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Identification of new GATA4-small molecule inhibitors by structure-based virtual screening

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ABSTRACT

Members of the GATA family of transcription factors are zinc finger proteins that were shown to play evolutionary conserved roles in cell differentiation and proliferation in different organisms. We hypothesized that by finding new molecules that inhibit their function to be crucial in future therapeutical interventions for various diseases.

By virtual high throughput screening using a version of GLIDE (Schrodinger®) program with both crystal and NMR structure of GATA C-terminal zinc finger, we identified new small molecular weight chemicals with lead-like properties. We used in vitro cell-based assays to show that these molecules selectively and efficiently inhibit GATA4 activity by inhibiting its interaction with the DNA. In addition we showed that these molecules can block the activation of downstream target genes by GATA4. Moreover these compounds can moderately enhanced a mouse model of myoblast differentiation into myotubes. This might be partially due to decreased GATA4/DNA interaction as shown by gel retardation assays.

Further investigation is needed to reach selectivity and efficacy. Our study however do show that in silico screening combined with in vitro studies are efficient tools to unravel new molecules that interact with zinc finger proteins such as GATA4.

1. Introduction

GATA proteins are zinc finger transcription factors evolutionary conserved in terms of structure and function.[1] In vertebrates the family is composed of six members (named GATA1 to 6) divided into two subgroups according to sequence homology and tissue distribution. GATA1/2/3 are preferentially expressed in hematopoietic cells, whereas GATA4/5/6 are highly expressed in the heart, gonads, and derivatives of the digestive system.[2] The most prominent features of GATA proteins at the structural level are two highly conserved type IV zinc fingers referred to as N-terminal zinc finger (N-ZF) and C-terminal zinc finger (C-ZF) that mediate specific binding to the A/TGATAA/G conserved sequence on the DNA.[1] It has been shown that only the C-terminal zinc finger and the adjacent basic domain are sufficient and necessary for specific DNA binding in vitro, while the N-ZF is involved in stabilizing the protein–DNA interaction.[3–5]

Binding motifs for GATA-factors have been identified within the promoters of many genes and functional studies have implicated all six GATA members in embryonic and postnatal development.[2,6] More importantly they play essential roles in cell fate specification, cell proliferation and differentiation. The role of GATA factors in human diseases was abundantly studied in the past decade. Those studies linked both *GATA4* and *GATA6* genes to congenital heart disease, *GATA1* to thrombocytopenia, *GATA2* to chronic myeloid leukemia (CML), and *GATA3* to hypoparathyroidism, deafness, and renal disease (HDR).[7–10] In addition to the role of GATA2 in CM, the expression of all other GATA proteins was shown to be altered in different types of cancer.[11–16]

GATA4 known to be one of the master proteins controlling cardiac cell differentiation and morphogenesis, and has been shown to be implicated also in cell cycle regulation and apoptosis.[17,18] In fact GATA4 is a key regulator of physiological and pathological cardiac hypertrophy: its overexpression is enough to induce cell growth depicted in terminally differentiated cardiomyocytes as an increase in cell size and re-expression of fetal genes.[19] In addition it was recently shown to be an upstream regulator of the Bcl family of apoptosis gene regulators and to activate the cyclin/cyclin dependent kinase (cdk) pathway early on during the proliferation of cardiac precursor cells in the anterior heart field.[20,21] Therefore, it was challenging to identify inhibitors that can disturb the activity of GATA4 by targeting its binding to DNA.

Transcription factors are however usually considered unlikely to be 'drug-targeted' since they have no enzymatic activities. But recently it was shown that many of these proteins could be selectively targeted by interfering with their DNA binding activity. The best example is the S3I-201 compound, a new inhibitor of STAT3 DNA binding activity which was discovered by a study that applied virtual high throughput screening of chemical databases.[22] The

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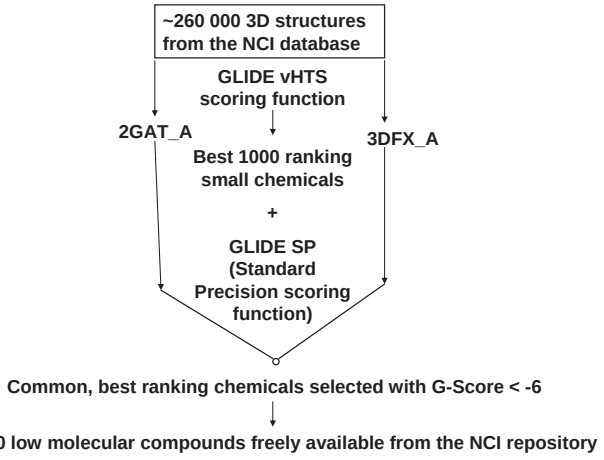


Figure 1. Docking protocol and scoring strategy used in this study.

study was a success because of the availability of the three-dimensional structure of phosphorylated STAT3 and the well-defined SH2 domain. Similar studies were done on NF-kB and Jak3 that prompted us to follow the same strategy for GATA4.[23,24] Unfortunately, the full three-dimensional structure is still not yet determined, but the 3D structure of the C-terminal zinc-finger domain was resolved as an NMR structure (1993) and X-ray structure (2008).[25,26]

Based on these facts, we hypothesized that it is possible to define a structural binding site at the interface, where the zinc finger core interacts with the major groove of the (A/T)GATA(A/G) site, and the start of the basic loop that interacts with DNA residues in the minor groove, without neglecting the cysteine residues that coordinate the zinc ion. Furthermore, it was interesting to test the feasibility of the virtual screening technique against such a hard target where no reference ligand was available. We expected that chemicals with metal chelating moieties or those with nucleotide like properties will be ranked at the top of the final results. Among the 40 compounds obtained from the National Cancer Institute (NCI) four were included in this study because the rest were either water insoluble and/or showed little effect on GATA4 DNA binding activity. The results of the four compounds show specific inhibition of the DNA binding activity of GATA4. More importantly the compounds have no cytotoxic effects on three different cell lines and their addition to the cells do inhibit GATA4 activity through diminishing binding to the DNA as assessed by electrophoretic mobility shift assay (EMSA).

2. Results and discussion

2.1. Virtual screening simulations

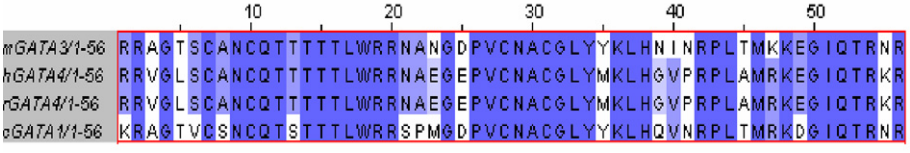
Docking results of the virtual high throughput screening yielded few chemicals that performed well with both crystallographic and NMR structures. We used the 56 amino acids region of the rat GATA4 sequence which is highly similar to the corresponding region of the NMR structure of the chicken GATA1 and the crystal structure of the murine GATA3 (Fig. 2). Because no reference ligand was available, it was challenging to correlate between structural predicted interactions and real biological activity. It is unfeasible to determine whether the studied chemicals interact directly with the DNA binding region constituted of basic amino acids independently of the zinc coordinating sphere or if they would chelate the zinc ion by approaching its coordinating residues. Unfortunately, no data is available at this time to support a direct interaction with GATA zinc fingers.

Forty low molecular weight chemicals were purchased for experimental assessment, but only few compounds showed slight to moderate impact on DNA binding activity as assessed by gel shift analysis using GATA4 overexpressed protein in Hek293 cells (data not shown). Furthermore, many displayed low solubility in pure water and few lead-like properties. The results of four chemicals with lead-like properties will be analyzed in this study (10% success rate). Those four compounds are: **NSC126866** (2-hydroxy-6-methyl-3,4-pyridinedicarboxylic acid) which is a dipicolonic acid derivative; **NSC140905** 2-(1,3-benzodioxol-5-ylmethyl) which is succinic acid; **NSC274937** (ICN 3847) or ribavirin monophosphate (RMP); and **NSC85661** 5-(((2-hydroxyethyl) amino) sulfonyl) isophthalic acid (Fig. 3). Docking scores and ranking for each compound to either the NMR or crystal structure were comparable (Table 1).

2.2. Predicted interactions for the best poses

Although the real interaction profile is not known for the studied compounds, it is interesting to discuss the predicted interactions for the best poses (Table 1).

For the NMR structure (Fig. 4A) of 2GAT (chain A), **NSC126866** occupied a region formed by the following residues: Thr14, Thr16, Cys28, Ala30, Lys48, Gly50, Ile51, Gln52, the carboxylate group engages hydrogen bonds with Thr16 and Ala30; the hydroxyl group interact with Gln52. **NSC140905** is surrounded by Thr16, Cys28, Ala30, Arg42, Met46, Ile51, Gln52. The carboxylate functions make hydrogen bonds with Thr16, Ala30, Gln52 and



SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score(%identity)
1	rGATA4	56	2	hGATA4	56	100
1	rGATA4	56	3	mGATA3	56	80
1	rGATA4	56	4	cGATA1	56	71
2	hGATA4	56	3	mGATA3	56	80
2	hGATA4	56	4	cGATA1	56	71
3	mGATA3	56	4	cGATA1	56	80

Figure 2. Percentage identity of amino acids residues between the sequences of GATA factors from different species (rat, mouse, chicken and human) cited in our study. The 56 amino acids region that include the C-terminal zinc core domain and adjacent basic region is highly conserved.

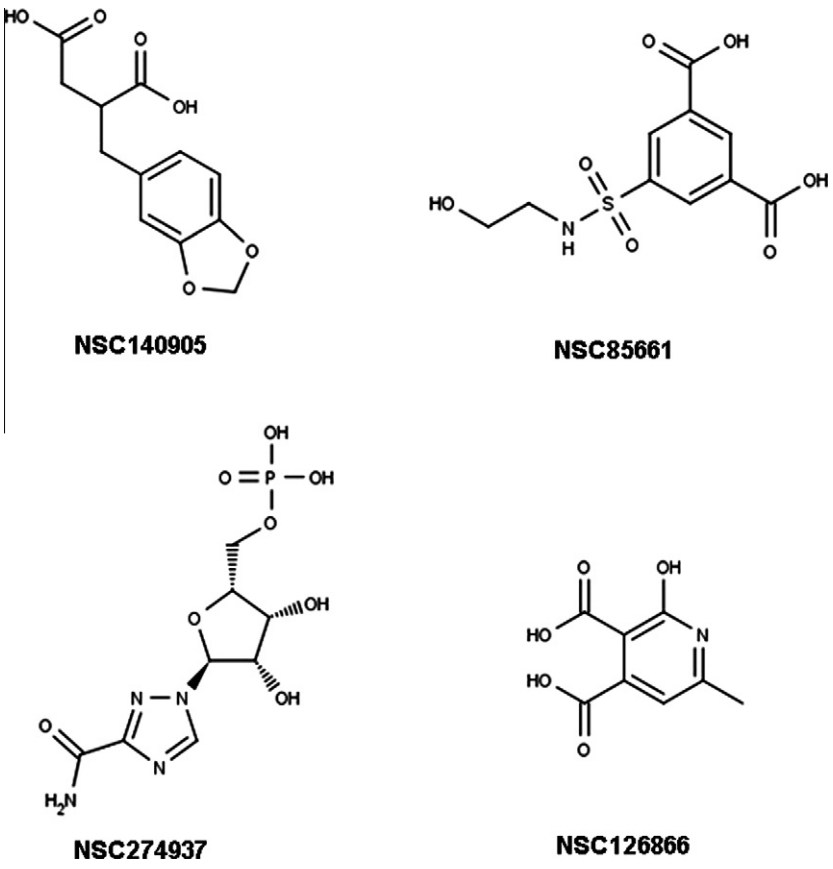


Figure 3. Chemical structures of the predicted GATA4-DNA binding inhibitors.

Table 1
Ranking of the poses of predicted inhibitory molecules

Cpd.	GlideScore
2GAT (*GATA1 C-terminal zinc finger NMR regularized mean structure*)	
NSC126866	−9.29
NSC140905	−9.02
NSC85661	−7.31
NSC274937	−7.26
3DFX (*GATA3 C-terminal zinc finger X-ray resolution 2.70 Å*)	
NSC140905	−8.61
NSC85661	−8.39
NSC126866	−8.18
NSC274937	−7.29

the benzodioxol group interacts with Arg42. **NSC274937** is docked closer to the zinc ion with the phosphate group oriented in the direction of the metal ligation sphere. It occupies a region containing the zinc ion, Thr16, Cys28, Cys31, Ala30, Arg42, Met46, Lys48, Gln52. The diol groups and the carbonyl group engage hydrogen bonds with Gln52 and Thr16, respectively. It is also possible that the triazole group would interact with the zinc ion. **NSC85661** occupies a region shaped by Thr14, Ala30, Met46, Gln52. the terminal OH group makes hydrogen bonds with Lys48. The NH group engages with Thr14 and the carboxyl group stabilizes the interaction by an additional hydrogen bond with Gln52 (Fig. 4A).

All four compounds contain zinc chelating moieties (carboxyl, sulfonyl, hydroxyl, triazole, and phosphate) and these interactions must be validated however by co-crystallization experiments.

For the crystal structure (Fig. 4B) of 3DFX (chain A), **NSC140905** occupies a pocket including the residues: Thr326, Asn339, Ala340, Arg 352, Met356, Lys358. The benzoxazole group engages hydrogen bonds with Thr326 and Asn339. The carboxylate group forms a salt bridge with side chains of Arg352 and Lys358. **NSC126866** occupies a region formed by Cys338, Ala340, Arg352, Lys358, and Ile361. Carboxylate group stabilizes the interaction by creating a salt bridge with the side chains of Lys358 and Arg352 as for NSC140905. **NSC274937** is surrounded by the following residues: Thr326, Cys338, Arg352, Lys358, Ile361 and Gln362. The terminal amino group makes a hydrogen bond with Gln362, and the phosphate group engages hydrogen bonds with Lys358 and Arg352. **NSC85661** occupies a cleft formed by Thr325, Thr326, Cys338, Arg352, Lys358, Glu359, and Ile361. The amino sulfonyl group makes different hydrogen bonds with Lys358 and Arg352 and the terminal hydroxyl group makes a hydrogen bond with Glu359. Moreover, the carboxyl group makes hydrogen bonds with Thr325 and Thr326.

All other hydrophobic amino acids in the pockets defined above made weak interactions with the studied ligands. Interactions with the zinc ion could not be accurately assessed because of the coordinating nature of the interaction and the lack of terms for the specific geometry of metal–ligand complexes. On the other hand, the coordinating sphere is highly buried in the finger core and protected by polar amino acids which create a barrier for docking feasibility.

Our findings suggest that the docked compounds interact with crucial residues involved in the DNA binding activity since Thr326, Arg330, Asn339, Ala340; Lys346 residues from the core zinc module make contact with bases and sugar phosphate backbone of the DNA molecule. In addition to Arg352, Met356, Lys358, and Ile361 from the first half of the C-terminal basic tail that also contribute to the major groove binding.

The residues mentioned above are highly conserved between chicken GATA1 CZF and GATA3 CZF and thus these compounds are predicted to bind similarly to the other five GATA C-terminal

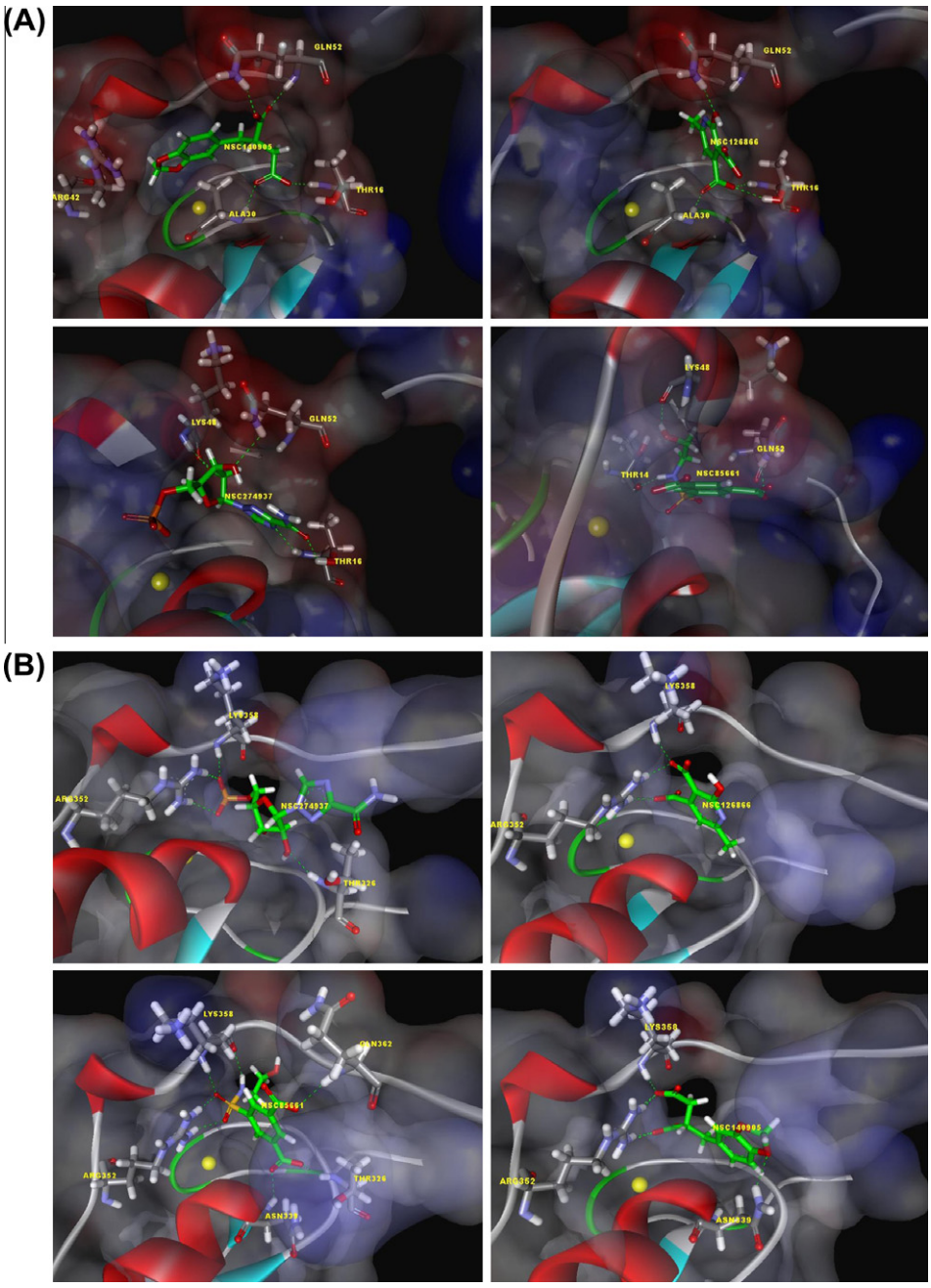


Figure 4. Binding modes of the selected molecules in the DNA binding region of (A) 2GAT_A (Chicken GATA1 C-terminal zinc finger) and (B) 3DFX_A (Mouse GATA3 C-terminal zinc finger). Residues involved in the interaction with the ligand are highlighted in stick representation. The yellow sphere is the zinc ion. Zinc fingers are rendered according to their secondary structure showing helices in red and sheets in cyan. The molecular surface of the protein is also defined using electrostatic potentials in Discovery studio visualizer (Accelrys, Inc., San Diego, USA).

zinc fingers but maybe with different affinities. These molecules may thus interact directly to the region formed by the hydrophobic alpha helix and the first amino acids of the basic tail that are essential to the binding of GATA4 to its target sequence. This kind of binding can modify the electrostatic potential by including one or more carboxylic group that is negatively charged at physiological pH. Only ribavirin monophosphate resembles natural nucleosides and can mimic the interaction to DNA components.[27] However, this hypothesis cannot be confirmed without crystallographic studies. On the other hand it is clear from the list of compounds obtained through GLIDE that the top ranked molecules are in fact chemicals with metal chelating properties which can disrupt the coordinating sphere and destabilize the whole structure. For instance **NSC126866** and **NSC140905** are derivatives of known compounds with zinc chelating properties (picolinic acid and succinic acid). **NSC85661** contains sulfonamide, isophtalate and an alcohol group all known to interact with zinc ion. **NSC274937** possess a charged phosphate group that has an affinity to cationic ions. This particular compound might however bind directly to a pocket at the DNA binding interface or it might be modifying another structural region close to the zinc fingers. Further experimental approaches should be made to confirm our hypothesis.

2.3. In vitro effects on GATA4/DNA interaction

It is well known that if the structure of the C-terminal zinc finger is destabilized, GATA4 transcription factor will lose its binding affinity because this finger is sufficient and essential for

binding to the GATA consensus sequence. Accordingly, we performed EMSA in the presence of increasing amounts of the four compounds (50, 100, and 150 μM). The results clearly demonstrate their ability to reduce the interaction of GATA4 with DNA target in a dose-dependent manner (Fig. 5A). All compounds were able to abrogate the protein/DNA binding by 70–96% at the highest tested dose (150 μM). However, **NSC140905** was the most effective in reducing the binding by 60% even at the lowest tested dose (50 μM). This suggests either high affinity and/or specificity of the compound to GATA4, and thus **NSC140905** could be used even at sub-micromolar doses. The same compounds were used on other transcription factors, namely Tbx5 and NFATc1, showing no effect and thus confirming the selectivity of these compounds to zinc finger proteins (data not shown).

2.4. In vivo transcription activity and biological significance

In order to show that the abrogation of GATA4/DNA binding activity affects the transcriptional activity of GATA4, HeLa cells were co-transfected with a constant amount of GATA4 expression vector and a gata reporter gene fused to luciferase. Hundred microliters of each of the four compounds tested were added to the cells 24 h after transfection and luciferase assays was assessed and compared to untreated group overexpressing GATA4.

All compounds except **NSC85661** slightly reduces the GATA4 mediated transcriptional activity of the reporter gene proving that they can enter the cell membrane and specifically preventing GATA4 binding to its DNA target (Fig. 5B). The transcriptional activation of GATA4 was reduced by 35% upon the addition of

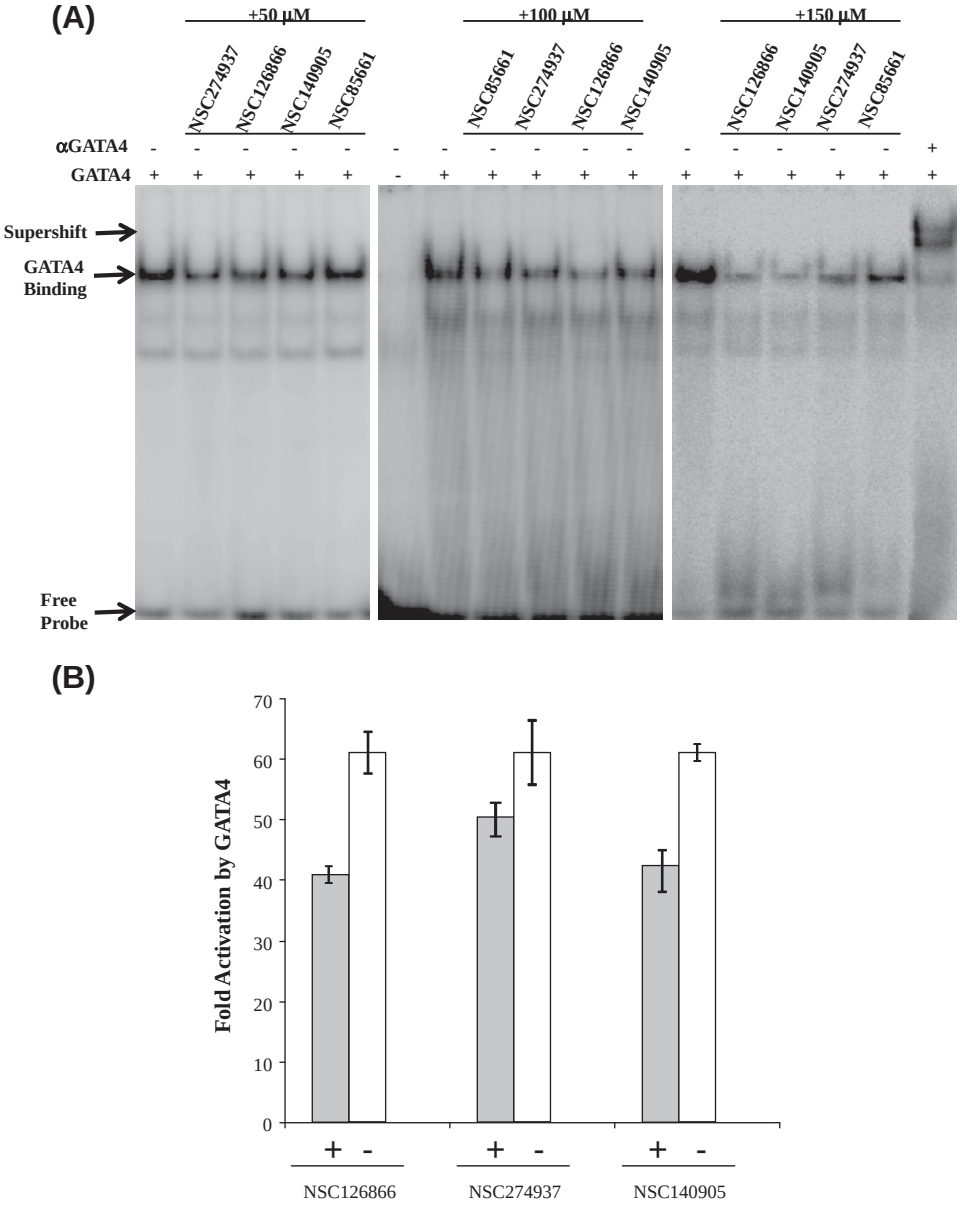


Figure 5. DNA binding and transcriptional activity of GATA4 in the presence of the studies compounds. (A) EMSA analysis with nuclear extracts from HEK293 cells overexpressing GATA4 incubated for 15 min in the presence of increasing amounts of compounds (50, 100, 150 μM). The results show a dose-dependent decrease in the specific GATA4 binding as identified by supershift analysis using a specific anti-GATA4 antibody. The most drastic effect is with NSC140905 whish shows a 60% inhibition of GATA4 binding at 50 μM and 96% inhibition at 150 μM. (B) GATA4 transactivation of the minimal BNP promoter carrying only the two proximal GATA binding sites is partially inhibited (25–30%) by three out of the four compounds. Transfected Hela cells were incubated with 100 μM of each compound for 24 h before luciferase assays. The results represent the mean of two independent experiments done in duplicates.

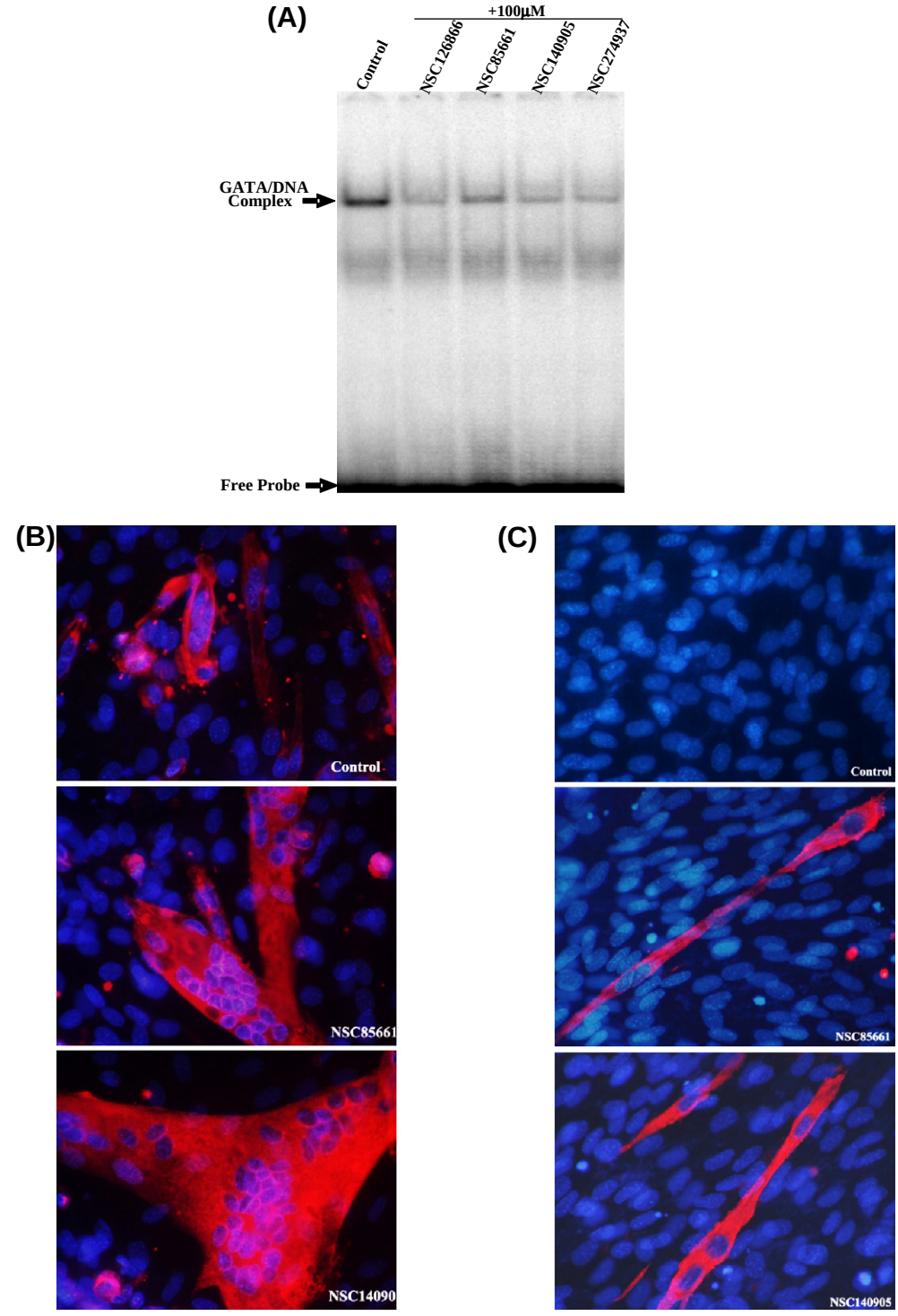


Figure 6. Abrogation of GATA4 endogenous activity and cell differentiation in C2C12 cells. (A) C2C12 cells were treated for 24 h with the selected compounds. EMSA was performed on nuclear extracts as described previously. The results show a diminished binding activity of GATA4 as assessed by the decreased intensity of the bound GATA4 complex. (B) C21C2 cells treated with 100 μM of selected compounds in a serum depleted medium (0.5% FBS) for 48 h. The cells were fixed and analyzed by immunofluorscence with anti-myosin (red signal) antibody to assess levels of differentiation. Nuclei are stained with Hoechst (blue signal), Magnification is ×20. More myotubes are formed in cells treated with the selected compounds as compared to the untreated cells. (C) C21C2 cells treated with 100 μM of selected compounds in normal serum conditions (20% FBS) for 48 h. The cells were fixed and analyzed by immunofluorscence with anti-myosin (red signal) antibody to assess levels of differentiation. Nuclei are stained with Hoechst (blue signal), Magnification is ×20. No myotubes were formed under these conditions in untreated cells whereas some myotubes are formed in cells treated with the selected compounds.

NSC126866 and **NSC140905** whereas this reduction was slightly lower (~16%) when **NSC274937** was used. It is important to indicate that all the studied compounds have low passive absorption rate and low bioavailability because of the acidic groups (two carboxylate groups, log P <0). This slight reduction is thus very significant in terms of its specificity, and ultimately

opens the way for the development of optimized leads that will increase physico-chemical properties and show improved activity at sub-micromolar range on cells and tissues.

The results also point out for the first time for a role of derivatives of ribavirin in targeting GATA4 activity. We think that RMP (**NSC274937**) is transported into cells via an active transport mediated by nucleosides transporters. It is well known that ribavirin monophosphate is a metabolite of ribavirin, an antiviral drug that and can inhibit different pathways; it is interesting to test ribavirin against GATA4 to find out whether the binding is mediated by the phosphate group or the other functional groups.[27]

2.5. In vivo effects on muscle differentiation

Our results on the transcriptional activity of GATA4 prompted us to look at the effect of the compounds on the in vivo GATA4 binding activity in cultured cells. Our unpublished results on the expression and function of GATA4 in the C2C12 myoblasts cells together with recent evidence of the expression of GATA4 in these cells prompted us to look at the modulation of GATA4 in these undifferentiated myoblasts.[28,29] The results did show a diminished binding activity of GATA4 at the same dose used for transcriptional activation in Hela cells (Fig. 6A). In addition there was no effect on cell proliferation nor cell death in any of the cell lines used namely Hela, Hek293, and C2C12 even at the highest dose 150 μM. Since C2C12 is a valid model to study muscle differentiation, we tested the effect of the four compounds on myotubes formation by assessing the formation of multi-nucleated cells and the expression of myosin. Differentiation of C2C12 cells was assessed, in the presence or the absence of differentiating media (20% or 0.5% FBS, treated with 100 μM of compounds for 24 h after inducing differentiation). Only, **NSC140905** and **NSC85661** groups showed significant numbers of myotubes compared to the untreated control in differentiating conditions (Fig. 6B). Interestingly, even in normal growth conditions (20% FBS), we could see formation of few myotubes in the cells treated with those two compounds as compared to the untreated cells where no myotubes were formed (Fig. 6C). It is possible that these compounds may be activators of membrane receptors that induce differentiation through a cascade of pathways not related to our study, or through a direct effect on GATA4.

3. Conclusion

In our study, we identified for the first time, new lead-like compounds using a virtual screening approach defined by GLIDE docking engine from Schrodinger that demonstrated accurate docking results through literature. Although, the use of GATA C-terminal zinc finger region as a target for docking studies and virtual screening is not feasible in the absence of co-crystallized ligands, we proposed a different strategy that combined different structures (NMR and X-ray) to find compounds that have significant biological activities.

Four low molecular weight chemicals were selected to validate the approach, biochemical analysis based on EMSA were performed and biological activity represented by the reduction of GATA4/DNA binding activity correlates with docking performance. It is noteworthy to mention that only the **NSC140905** compound shows consistency in reducing GATA4 physical and functional properties in vitro and in vivo. This suggests that the compound is relatively highly selective and stable and can be used directly on cells. The other three compounds show variable results although all of them do decrease GATA4 activity. This might be due to lower binding affinities since at higher doses they were able to match the effect of NSC140905 in gel shift assays.

Our study represents a preliminary assessment of virtual high throughput screening against GATA transcription factors represented in this study by GATA4. Further experiments should be carried on to assess the specificity towards GATA4 and/or to define the affinities of these compounds to the other GATA proteins. Our data do however suggest that GATA transcription factors ought to be considered an interesting target in drug design strategies in the future.

4. Materials and methods

4.1. Protein preparation and structure selection

At the beginning of the study, there was no crystal structure of the complex GATA/DNA. Two NMR structures were available from the protein data bank (PDB code: 1GAT and 2GAT). These structures correspond to the chicken GATA1 C-finger/DNA complex. The NMR structure (PDB code: 2GAT) was refined by residual dipolar couplings. Later on, crystal structures of the mouse GATA3 C-finger/DNA complex were submitted to the protein data bank (PDB code: 3DFX and 3DFV).[26] Since GATA C-finger domain is highly conserved among the six GATA vertebrate proteins, we selected to use this structure as a template for our study on GATA4 (Fig. 2), in addition to the 2GAT template because there was a good structural correlation between these two complexes (RMSD of 0.8 Å for 25 Cα atoms).

DNA macromolecule was removed from the structure. The protein chains were refined using the standard settings applied in the protein preparation wizard available with Maestro interface and the zinc ion was assigned a charge of +2, cysteines were deprotonated and tetrahedrally coordinated to the ion (zinc has a structural role in the model and not involved in a catalytic activity).

4.2. Ligand database and virtual screening simulations

The 3D configurations of 260,000 chemical compounds from NCI where obtained from the ZINC 8 database.[30] The three dimensional configurations of problematic structures were prepared using the default settings in LigPrep.

Grids were prepared starting from residues involved in DNA/GATA C-terminal zinc finger recognition interface and including zinc coordination region. Virtual high throughput screening experiments were conducted using GLIDE (Grid-Based Ligand Docking with Energetics) software from Schrodinger with vHTS (virtual high throughput screening) scoring settings (http://www.schrodinger.com). The first 1000 best ranking structures for each target were retained and re-docked using the SP (Standard precision) scoring function in GLIDE. High scoring chemicals that were common to the two sets (GlideScore <-6) were kept for further investigation. Forty non redundant low molecular compounds were requested from the NCI repository for experimental testing (Fig. 1).

4.3. Cell culture

The C2C12 myoblast cell line is a generous gift from Dr. Mona Nemer. Cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 20% fetal bovine serum, 1% penicillin–streptomycin. The cells were plated at a density of 50×10^3 cells/well in 12 well culture plates. The medium was renewed or changed to DMEM supplemented with 0.5% FBS (differentiation promoting medium, DM) when the cells reached 90% confluence. All cultures were incubated at 37 °C under 95% humidity and 5% CO2. Cells were treated with 100 μM of NCI compounds for 24 h after inducing differentiation.

Hela cells (confluency 70%) were plated in 12 well culture plates with 60×10^6 cells/well in DMEM medium supplemented with 10% fetal bovine serum. Hek 293 cells were plated in 100 mm^2 dishes and transfected using the calcium phosphate precipitation method as previously described with the plasmid encoding the rat GATA4 cDNA under the control of the CMV promoter.

4.4. Luciferase assay

To assay for the transcriptional regulation of the GATA site promoter by GATA4, HeLa cells were transiently co-transfected with the minimal BNP promoter carrying two GATA sites upstream of the luciferase reporter gene (kind gift from Dr, Mona Nemer), and a pcDNA3-GATA4 overexpressing vector using polyethyleneimine (PEI) from Sigma as a transfecting reagent. Twelve hours post-transfection, the medium was changed and the cells were treated in binaries with 100 μM of each tested chemical compound; after 24 h the cells were harvested in a lysis buffer (100 mM Tris HCl PH 7.9, 0.5% NP-40, 1 mM DTT) for 20 min at room temperature.

Luciferin (Promega) was prepared according to the manufacturer's protocol. The lysed cells were transferred to a 96 well plate to which luciferin was added and the signal was read immediately using the Ascent Fluoroscan at the Molecular Core Facility at the American University of Beirut. Luminescence values were normalized for total protein concentration.

4.5. Nuclear extract preparation

Cells were first washed with 2 ml PBS, and then they were scraped in PBS. Following centrifugation, the pelleted cells were homogenized in the hypotonic solution buffer A (10 mM Tris pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT and 0.5 mM protease inhibitors); the homogenate was incubated for 15 min on ice after which 50 μl of the detergent NP-40 was added. The supernatant was discarded and the pelleted nuclei were resuspended in the hypertonic solution buffer C (20 mM Tris pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 0.5 mM DTT and 0.5 mM protease inhibitors) and vortexed for 15 min at 4 °C. After this incubation the samples were centrifuged for 3 min at 13,000 rpm; the supernatant was aliquoted and stored at −80 °C.

Protein concentrations were determined using the BioRad DC Protein Assay (BioRad, Cat # 500-0114) according to the manufacturer's protocol.

4.6. Gel mobility shift assay

Primers corresponding to the GATA (Rat BNP promoter) consensus DNA binding site were selected. Forward: TGTGTCTGATAAAT CAGA reverse: TCTGATTTATCAGACACA. The single stranded primers were annealed and labeled with T4 Kinase. The bands corresponding to a double stranded probe were resolved on a 12% polyacrylamide gel, and then purified using Costar Spin-X columns (Costar) according to the manufacturer's protocol. The probe was then used in gel shift assay experiments. The nuclear extracts were run on a 6% non-denaturing polyacrylamide gel (Acrylamide/Bis (29:1), 1.6%APS, TEMED, water and 0.25× TBE) in 0.25× TBE buffer at 200 volts. The reaction consisted of 3 μg of extracts, 4 μl binding buffer (20 mM Tris pH 7.9, 120 mM KCl, 2 mM EDTA, 25 mM $MgCl_2$ and 25% glycerol), 1 μl poly dI/dC (Amersham) and 1 μl of the probe (50,000 cpm). The reaction was completed to 20 μl with water. Protein extracts were pre-incubated with the studied compounds for 10 min at room temperature and then incubated with γ-[^{32}P] ATP-labeled consensus binding sequence for 20 min.

After incubation, the samples were loaded and the gel was run for 2.5 h. For supershift experiments, the samples were incubated with GATA4 polyclonal antibody (Santa Cruz, sc1237) for 10 min prior to the addition of the probe. The gel was then dried using the BioRad gel dryer (Model 583) for 2 h at 80 °C; this was followed by exposing the gel to a phosphor imager screen. The screen was then scanned using the STORM scanner (Molecular Dynamics, Amersham), and the bands were quantified using the ImageJ software (http://rsbweb.nih.gov/ij/).

4.7. Immunofluorescence

Immunofluorescence was performed on C2C12 cells. The cells were fixed with 4% *p*-formaldehyde for 15 min; after washing with PBS the cells were blocked with 3% BSA/PBT for 45 min. The primary antibody (MF-20, anti-myosin heavy chain) was diluted in BSA/PBT and added to the cells with an overnight incubation at 4 °C. The cells were then washed in PBT and the secondary antibody was added to the cells for 1 h at room temperature. After washing with PBT, cells were incubated with Streptavidin-Texas Red (GE catalogue nb RPN1233) for 1 h. Hoechst staining for the nucleus was also performed by applying Hoechst to the cells for 10 min. The cells were then washed and mounted on a rectangular slide containing an anti-fading agent (DABKO). The slides were examined using an Olympus BH-2 microscope.

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