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STUDY ON THE INTERACTION BETWEEN PENTAMERIC OLIGONUCLEOTIDES AND RECOMBINANT SIGNALING PROTEINS AND RECEPTORS

Aim. *The aim of this study was to synthesize, purify, and investigate the interactions between oligonucleotides (OLN) and recombinant signaling proteins, specifically interferon a2-b, insulin, their receptors, and somatotropin, using docking and fluorescence spectroscopy methods.* ***Methods.*** *To analyze these interactions, we employed the Stern-Volmer equation in its general and modified forms, as well as the Hill equation, which enabled us to determine the binding affinity and binding constants. We synthesized oligonucleotides using the solid-phase phosphoramidite method, which offers high efficiency and specificity. The resulting oligonucleotides were purified by solid-phase extraction, which removed by-products and impurities, as confirmed by spectral analysis.* ***Results.*** *Fluorometric titration revealed that homopolymeric oligonucleotides bind to proteins within the medium-affinity range, forming non-fluorescent complexes. The most significant interactions occurred with shorter oligonucleotides. We observed positive cooperative binding between insulin and the oligoribonucleotide Poly(rG)$_5$. Notably, all proteins selectively bound with the oligonucleotide Poly(dG)$_5$.* ***Conclusions.*** *Understanding the mechanisms of protein-oligonucleotide interactions may open new possibilities for developing antibiotics, antiviral drugs, and treatments for cancer and genetic diseases.*

Keywords: *signaling proteins, protein-ligand interaction, spectroscopy, oligoribonucleotide, oligodeoxynucleotide, docking.*

Abbreviations: *Insulin (INS), Interferon a2-b (INF), Insulin receptor (INSR), Interferon-a/β receptor 1 (INFR), Somatotropin (STP), Homopolymeric oligonucleotides (OLN) of different lengths and compositions (A20 (Poly(dA)20); G20 (Poly(dG)20); T20 (Poly(dT)20); C20 (Poly(dC)20); A5 (Poly(dA)5); G5 (Poly(dG)5); rA5 (Poly(rA)5); rG5 (Poly(rG)5).*

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Introduction

The study of interactions between oligonucleotides and recombinant signaling proteins and their receptors has drawn significant attention in molecular biology [1]. These interactions provide valuable insights into molecular recognition in signaling pathways, crucial for understanding various biological processes. Oligonucleotides that bind to proteins and their receptors play a key role in regulating genetic information [2], signaling [3], and other cellular functions. For instance, they can be used as diagnostic tools to detect diseases or as therapeutic agents that modulate biological processes at the molecular level.

Oligoribonucleotides have garnered particular attention in recent years due to their potential for developing new drugs against viruses, inflammation, and tumors [4]. They are promising candidates for creating innovative antiviral drugs, as they can interfere with viral replication processes and enhance antiviral defense in cells [5]. However, the development of a compound that inhibits or activates the protein function requires knowledge of its complex spatial conformation. While some protein classes, such as membrane receptors, enzymes, ion channels, or transport proteins, can be therapeutically affected by conventional protein targeting strategies, others, like transcription factors, scaffold proteins, or structural proteins, are less amenable to traditional methods [6].

Materials and Methods

Preparation of ligand molecules

Preparation of ligand molecules was carried out using the Avogadro program [7]. First of all, it is necessary to optimize the geometry of the molecules. This procedure is performed by the Avogadro program. The next step is to minimize the structure and prepare for docking in the UCSF Chimera 1.18 program. The PyRx program uses files with the PDBQT extension in its work, so it is necessary to save the file in this format after all manipulations [8].

Preparation of the receptor molecule

The spatial structures of proteins were obtained from the AlphaFold database [9]. The preparation of protein structures for docking was carried out in the UCSF Chimera 1.18 environment [8] at two stages. At the first stage, the structure was cleaned of excess water molecules, ions, and uncoordinated cofactors, and polar hydrogen atoms were added. At the second stage, AutoDock atomic types and Gasteiger charges were added to all receptor atoms, and water was removed from non-biological regions. After that, the receptor file in PDB format was converted to PDBQT format, which is necessary for docking. All preparatory procedures were performed using the AMBER14 force field.

Grid parameters (GridBox)

The docking zone was defined using a virtual grid (Grid Box) measuring 60×60×60 $Å^3$, the centre of which was set according to the coordinates of the active site, determined from crystallography data or the spatial arrangement of key residues in the protein model. This grid size ensures coverage of the main binding pocket and possible allosteric sites.

Analysis of results

The obtained protein-ligand complexes were visualised and analysed using the BIOVIA Discovery Studio Visualizer program [11]. The main types of intermolecular interactions were identified, in particular: hydrogen bonds, hydrophobic interactions, π-π stacking and other contacts that are important for stabilising the complex.

Phosphoramidite synthesis

Oligonucleotide synthesis was performed by solid-phase phosphoramidite synthesis using an H-8 RNA/DNA synthesizer (K&A Laborgeraete, Germany). This method allows obtaining highly specific and pure oligonucleotides, which are necessary for further biochemical studies. The synthesis was performed on standard columns, designed for

200 nmol and with a pore size of 1000 Å, from Biosearchtech (Great Britain):

- 5'-DMT-dA(Bz) Synthesis Column;
- 5'-DMT-dG(iBu) Synthesis Column.
- DMT-2`O-TBDMS-rA(bz)
- DMT-2`O-TBDMS-rG(ib)

After synthesis, the oligonucleotides remained on controlled porosity glass (CPG) columns. For their isolation, 1 ml of 25% ammonia solution (Supelco, USA) was used, which ensured effective washing of the oligonucleotides from the columns. Isolation was carried out in a VORTEMP thermostat (Uniequip, Germany) at a temperature of 55 °C for 16 hours, which ensured complete removal of the protective groups and obtaining pure oligonucleotides for further use.

Oligonucleotide purification

Oligonucleotides were purified by solid-phase extraction (SPE). SPE was performed using a P-8 device from K&A Laborgeraete (Germany), which provides a precise and automated purification process. The purification process used columns with polymerized dextran MicroPure II Column from Biosearchtech (UK).

After the SPE process was completed, the purity of the resulting oligonucleotide samples was verified by spectrophotometric analysis at 260 nm. A NanoDrop One C spectrophotometer from the company (Thermo Fisher Scientific, USA) was used.

Fluorescence spectroscopy

Fluorescence analysis was performed on a FluoroMax-4+ spectrofluorimeter (HORIBA Jobin Yvon SAS, USA). The excitation wavelength was 295 nm (Ex = 295 nm), and the emission range was 305—405 nm (Em = 305—405 nm) with a slit width of 5 nm. The experiment used a protein at a concentration of 50 pM, dissolved in Tris-HCl buffer (50 mM, pH = 7.4) in a volume of 200 µl, 25 °C. Fluorescent titration was performed by sequentially adding 20 µl aliquots of oligonucleotide solution (2 nmol/ml) to the protein solution to a final volume of 400 µl.

The fluorescence quenching value (Q) was determined as the relative decrease in the emission intensity of the protein in the presence of oligonucleotides (OLN) compared to the control emission intensity of the protein itself.

The fluorescence intensity was calculated using the formula:

$$F_{corr} = F_{obs} \times 10^{(A_{ex} + A_{em})/2}$$

where F_{obs} is the measured fluorescence, A_{ex} is the absorption at 295 nm, and A_{em} is the absorption at λ_{em}. The optical density of the samples at 295 nm was less than 0.05, so the contribution of the internal filtering effect was considered insignificant.

Analysis of the quenching mechanism

To further elucidate the mechanism of oligonucleotide-induced fluorescence quenching of recombinant proteins, fluorescence quenching data were analyzed using the Stern-Volmer equation [12]:

$$F_0/F = 1 + K_{SV} \times [L],$$

where F is the fluorescence intensity of the protein in the presence of OLN, F_0 is the fluorescence intensity of the protein itself under the same conditions, L is the ligand concentration, Ksv is the Stern-Volmer quenching constant, which is determined from the Stern-Volmer equation. K_{SV} was determined as the slope of the linear approximation of the dependence of F_0/F on ligand concentration. K_{SV} is the Stern-Volmer constant, and the quencher concentration required for 50% fluorescence quenching is calculated as $[L]/2 = 1/K_{SV}$.

In case the plot was not linear, the fluorescence data were further examined using the modified Stern-Volmer equation [13]:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a \times K_{SV}} \times \frac{1}{[L]} + \frac{1}{f_a},$$

where fa is the fraction of the initial fluorescence that is available for quenching $(F_0 - F_{min})/F_0$ and $\Delta F = F_0 - F$.

The graph of $F_0/\Delta F$ versus 1/[L] gives the interaction constants [15].

The scheme (Fig. 1) demonstrates alternative binding modes of pentameric oligonucleotides to recombinant proteins. It highlights positive cooperativity (enhanced binding affinity upon initial ligand attachment), negative cooperativity (reduced affinity of subsequent ligands), and non-cooperative binding (independent interaction without affinity change). These modes reflect the diversity of molecular recognition in protein-nucleic acid complexes. Adapted from [15].

From the Van't Hoff equation:

$$\Delta G = R \times T \times \ln(Kd) \text{ or } Kd = e^{\Delta G/(R \times T)},$$

where R is the universal gas constant, T is the temperature in Kelvin, and is the binding constant [16].

To determine the number of binding sites and the binding constant, experimental data were fitted into a modified equation:

$$F = F_0 + \Delta F \times \frac{([L] + n \times P + Kd) - \sqrt{(([L] + P + Kd)^2 - 4n \times P \times [L])}}{2},$$

n — number of binding sites, P — protein concentration, L — ligand concentration [17].

If there were two sites, we used a model dependent on [L] to fit the binding constants Kd1 and Kd2:

$$F = \frac{F_0 + \frac{[L]}{Kd1} \times Fbound1 + \frac{[L]}{Kd2} \times \times Fbound2 + \frac{[L]^2}{Kd1 \times Kd2} \times Fbound12}{1 + \frac{[L]}{Kd1} + \frac{[L]}{Kd2} + \frac{[L]^2}{Kd1 \times Kd2}}$$

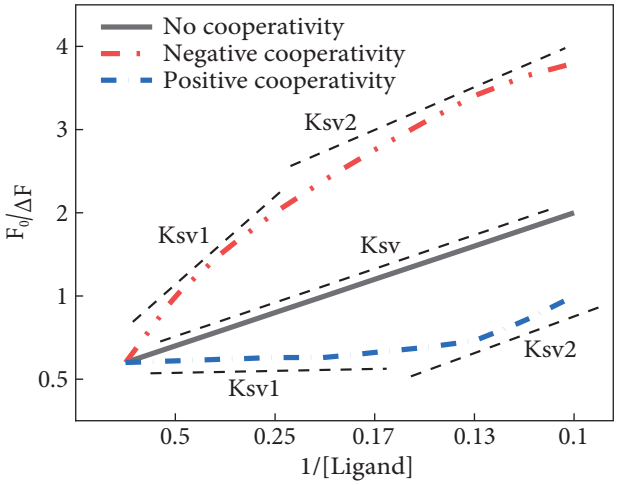

Fig. 1. Different variants of oligonucleotide interaction when binding to recombinant signaling proteins

This equation describes the dependence of fluorescence on ligand concentration [L] for a protein with two independent binding sites. Note that in this equation, we assumed that the binding constants Kd1 and Kd2 are microscopic constants for each site independently of the other, and we also assumed that the anisotropies for partially occupied states (Fbound1 and Fbound2) may be different, and also differ from the fully occupied state (Fbound12) [18, 19].

Results and Discussion

Using docking studies, we can estimate the binding affinity between a protein and an oligonucleotide (Table 1).

Table 1. **Scoring function values (ΔG_{bind} kcal/mol) and average values**

Protein/Ligand	dA5	dC5	dG5	dT5	rA5	rC5	rG5	rU5	Average
INF	–9.3	–8.5	–8.2	–8.5	–7	–7.4	–8.7	–6.6	–8.0
INFR	–7.8	–8.7	–8.5	–8.8	–6.7	–7.2	–8.7	–7.3	–8.0
INS	–6.9	–7.3	–8.1	–7.7	–6.8	–6.2	–7.1	–7	–7.1
INSR	–9.7	–9.6	–10.5	–9.4	–9.7	–8.8	–10	–9.1	–9.6
STP	–8	–8.4	–9.1	–9	–8.4	–6.8	–8.7	–7.6	–8.3
Average	–8.3	–8.5	–8.9	–8.7	–7.7	–7.3	–8.6	–7.5	

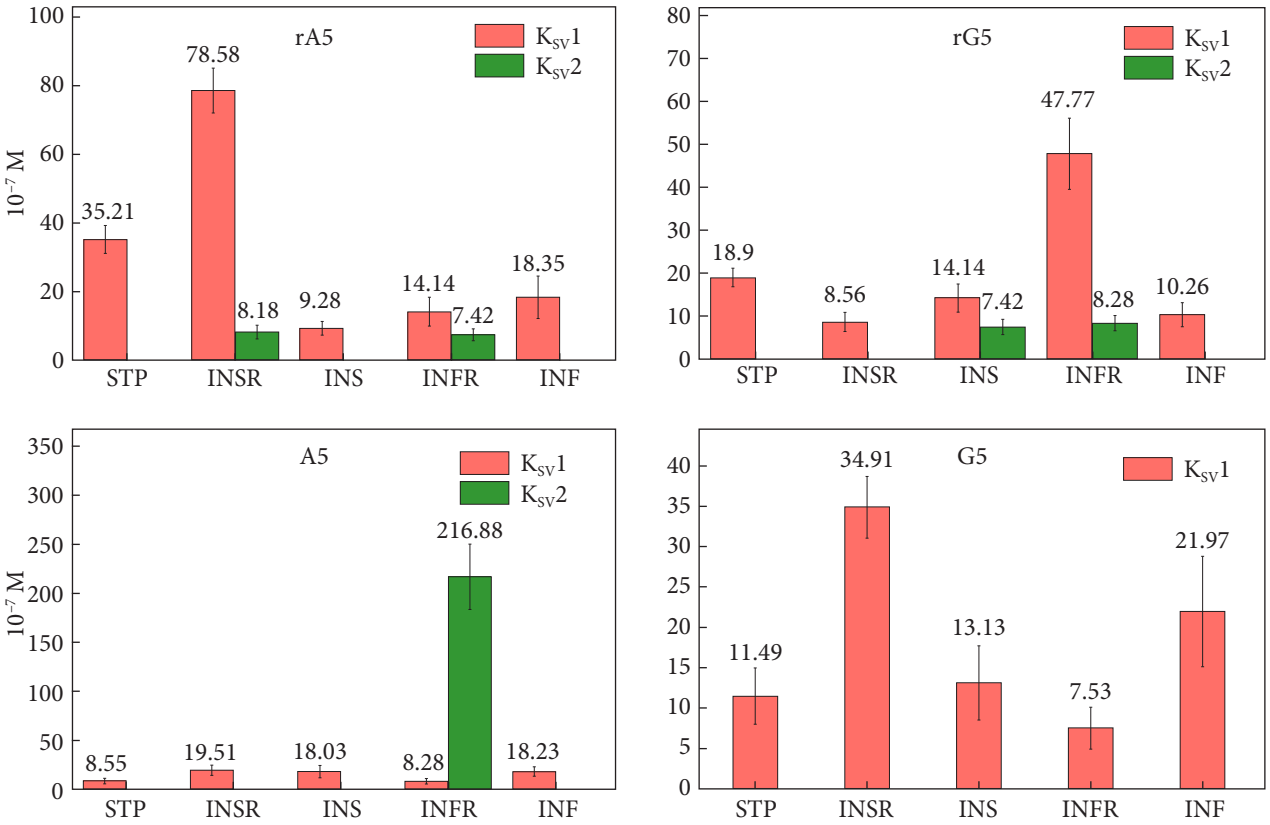

Fig. 2. Stern-Volmer constants of oligonucleotides in binding to recombinant signaling proteins. The bar chart presents Stern-Volmer quenching constants (Ksv) obtained for oligonucleotide-protein interactions. Variability of Ksv values indicates different quenching efficiencies, reflecting interaction strength and potential cooperative effects. Higher constants correspond to stronger quenching, suggesting tighter protein-ligand association

INF has a mean ΔGbind = –8.0 kcal/mol, indicating a rather moderate binding affinity. This may indicate that the protein does not have a very strong interaction with oligonucleotides. The binding affinity of INFR is similar to INF, indicating similar characteristics in the interaction with oligonucleotides. INS is noted to have a slightly weaker affinity than INF and INFR, with a mean ΔGbind = –7.1 kcal/mol, which may indicate a less efficient binding of this protein to oligonucleotides. INSR has the best affinity among all variants with a mean ΔGbind = –9.6 kcal/mol. This indicates that this protein binds very strongly to oligonucleotides. STP with an average ΔGbind = –8.3 kcal/mol has an average binding affinity slightly worse than that of INF and INFR, but still indicates a fairly strong interaction.

INSR has the best binding affinity to oligonucleotides, while INS has the lowest. INF, INFR, and STP have medium binding affinity to oligonucleotides, with similar results among themselves. dG5 has the highest average binding affinity, indicating its strong interaction with proteins. rC5 has the lowest affinity, followed by rA5 and rU5, indicating weaker interaction of these oligonucleotides with proteins (Fig. 2).

In cases where Ksv1 did not correspond to a linear dependence ($R^2 < 0.9$), second-order Stern-Volmer constants (Ksv2) were calculated based on spectroscopic results, which allowed combining computer modelling data and

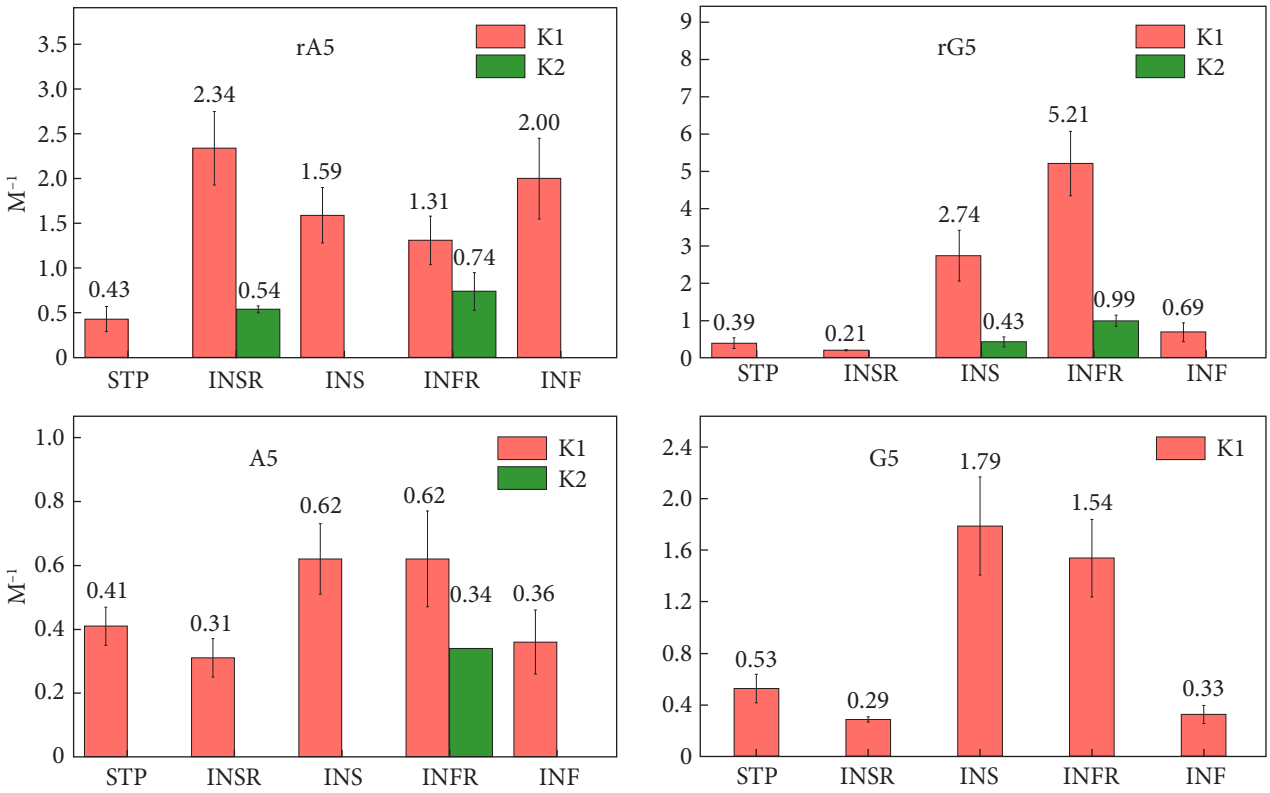

Fig. 3. Binding constants of oligonucleotides in binding to recombinant signaling proteins. The diagram compares binding constant (Kd) (as shown in Fig. 3) values for various oligonucleotide-protein pairs. Data illustrate differences in affinity between DNA and RNA pentamers, as well as distinct behavior of interferon, insulin, somatotropin, and their receptors. Clustering of proteins according to similar affinities highlights structural preferences in ligand recognition

experimental measurements. These constants reflect the effect of one or more OLN molecules on the fluorescence intensity during interaction with the protein. For example, when rA5 or rG5 was added to INFR, negative cooperative binding was observed. This means that binding of one OLN molecule may reduce the probability of binding of another OLN molecule or protein.

Analyzing the obtained data in transposed form with a focus on individual oligonucleotides, we found significant differences in their interaction activity. These differences are explained by the different nature and composition of the oligonucleotides, which determine their unique properties when binding to proteins.

In the diagrams presented, the binding constants are displayed as colored markers. Orange indicates the reaction in which one protein binds to one oligonucleotide molecule (A + B = AB), while green indicates the process of binding a protein to two oligonucleotide molecules (A + 2B = AB2). This demonstrates that a protein is capable of binding to several molecules simultaneously, and the nature and the efficiency of these interactions depends on the specific conditions.

The analysis of the binding constants, presented in transposed form, allowed us to assess the interaction of individual oligonucleotides with proteins and their receptors. When studying the binding to the rG5 oligonucleotide, two statistically similar groups were identified: the first in-

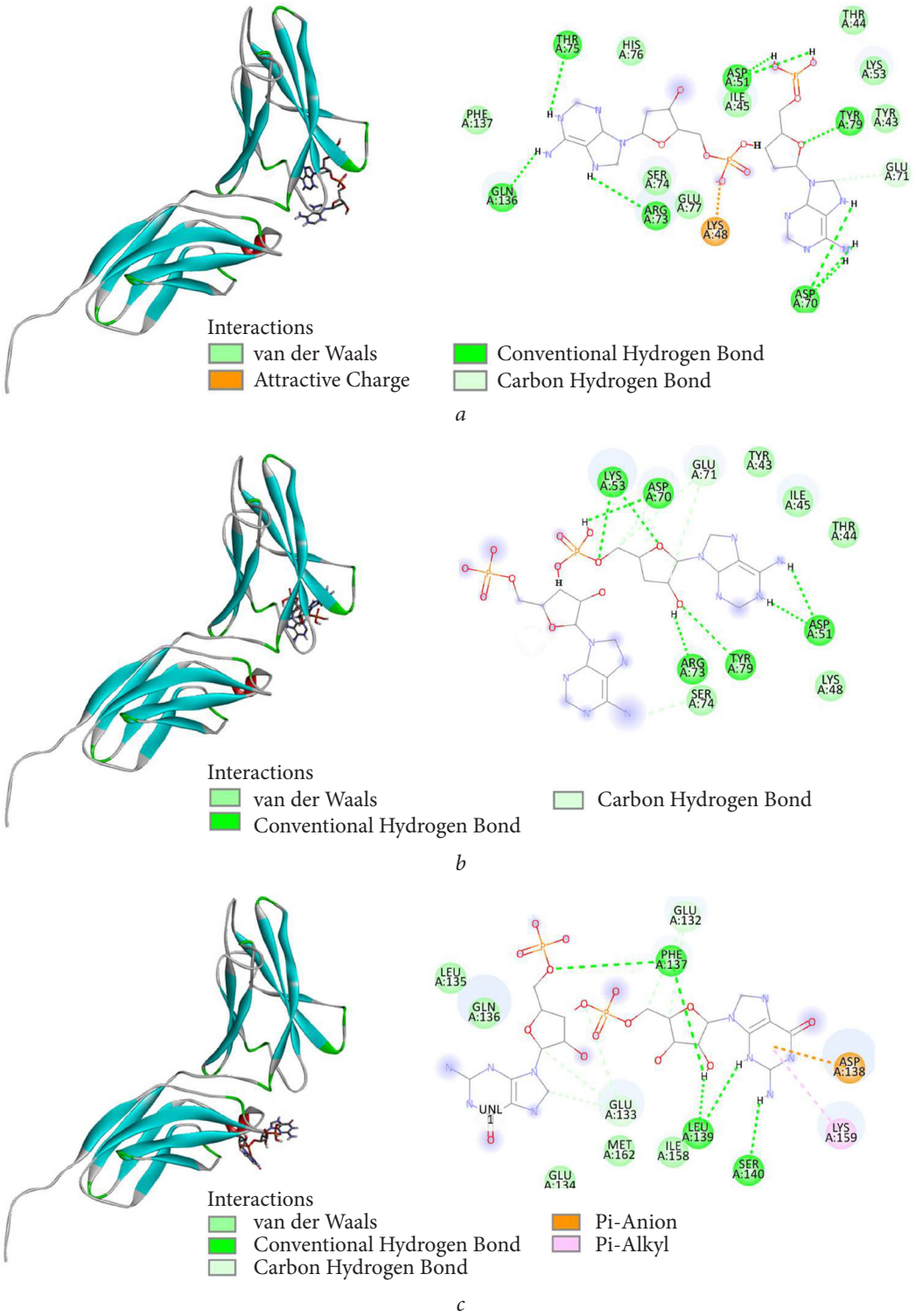

Fig. 4. *a* — INFR protein molecule and two A5 molecules. *b* — INFR protein molecule and two rA5 ligand molecules. *c* — INFR protein molecule and two rG5 ligand moleculesv

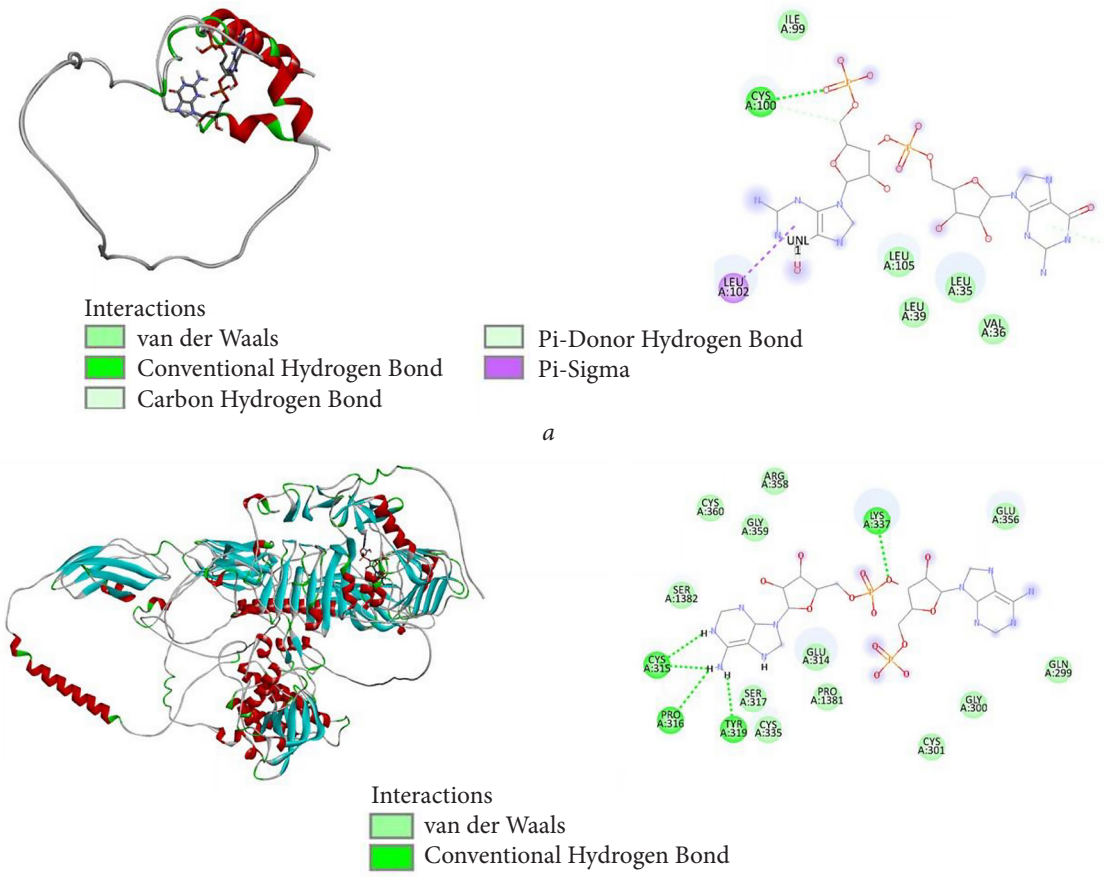

Fig. 5. *a* — INS protein molecule and two rG5 l nolecules. *b* — INSR protein molecule and two rA5 ligand molecules

cluded insulin and its receptor (INS and INFR), and the second — somatotropin, interferon (INF) and the insulin receptor. A similar division was observed for binding to oligonucleotides A5 and G5, where the INS and INFR groups were distinguished, as well as somatotropin with INF and the insulin receptor. When interacting with rA5, one statistically similar group was distinguished, which included insulin, interferon and their receptors.

rA5 (RNA) has higher Kd1 values than A5 (DNA) in all conditions except STP (where they are similar) (Depending on the number of Kd values Fig. 3). G5 (DNA) shows a higher affinity than rG5 (RNA) in INF, INFR, INS. However, in INSR and STP rG5 binds better (e.g., INSR: G5 = 0.29 mkM^{-1} vs rG5 = 0.21 mkM^{-1}). In INFR the lowest affinity is for rG5 (Kd1 = 5.21 mkM^{-1}), which is 3.5 times lower than for INS (2.74 mkM^{-1}). Two binding sites for rG5 are also visible (Kd1 = 5.21, Kd2 = 0.99 mkM^{-1}). In the case of the INSR titration, the maximum affinity is for rG5 (Kd1 = 0.21 mkM^{-1}), but the lowest is for rA5 (Kd1 = 2.34 mkM^{-1}). INS: High affinity for G5 (Kd1 = 1.79 mkM^{-1}) and rG5 (Kd1 = = 2.74 mkM^{-1}). STP: Generally low Kd values for all ligands (e.g. A5 = 0.41, rG5 = 0.39 mkM^{-1}).

Of particular interest is the positive cooperativity exhibited by rG5 in its interaction with insulin. A similar property was also found for the homopolymeric oligonucleotides rA5 and A5, which showed a strong interaction with the interferon receptor. These results confirm that rG5 in combination with insulin exhibits stable and significant

Table 2. **Protein-ligand binding energies (ΔG), binding constants, and scoring function values for protein and ligand groups**

Protein/ Ligand	$A5_1$	$A5_2$	G5	$rA5_1$	$rA5_2$	$rG5_1$	$rG5_2$
Binding constants, Kd 1(Kd 2) *mkM*							
INF	0.36	—	0.33	2.00	—	0.69	—
INFR	0.62	0.34	1.54	1.31	0.74	5.21	0.99
INS	0.62	—	1.79	1.59	—	2.74	0.43
INSR	0.31	—	0.29	2.34	0.54	0.21	—
STP	0.41	—	0.53	0.43	—	0.39	—
Binding energy, ΔG *kJ/mol*							
INF	0.595	—	0.645	–0.404	—	0.215	—
INFR	0.277	0.629	–0.251	–0.158	0.174	–0.961	0.005
INS	0.277	—	–0.339	–0.270	—	–0.588	0.492
INSR	0.681	—	0.722	–0.495	0.359	0.908	—
STP	0.519	—	0.370	0.492	—	0.547	—
The value of the scoring function, ΔG_{bind} *kcal/mol*							
INF	–9.3	—	–8.2	–7.0	—	–8.7	—
INFR	–7.8	–7.5	–8.5	–6.7	–7.6	–8.7	–7.8
INS	–6.9	—	–8.1	–6.8	—	–7.1	–7
INSR	–9.7	—	–10.5	–9.7	–9.2	–10	—
STP	–8.0	—	–9.1	-8.4	—	–8.7	—

positive cooperativity. The general conclusion is that all the oligonucleotides studied, in particular rA5, rG5 and A5, enhance protein binding, indicating their possible role in the regulation of biological processes.

Docking visualization shows the interferon receptor (INFR) bound with two A5 ligands (Fig. 4*a*). The first ligand forms stable hydrogen bonds with GLN136, THR75, ARG73, and an ionic interaction with LYS48, providing high binding specificity. The second ligand interacts via hydrogen bonds with ASP51, TYR79, ASP70, and GLU71, stabilizing the complex through multiple contacts. Together, both ligands demonstrate complementary stabilization of the receptor interface.

The Fig. 4*b* depicts INFR in complex with two rA5 ligands. The first ligand interacts through hydrogen bonds with ASP51, LYS53, ASP70, and GLU residues, enhancing selectivity. The second ligand establishes a single hydrogen bond with SER74, suggesting a weaker but potentially modulatory interaction. Such differences indicate diverse binding strengths of RNA versus DNA analogs at the receptor site.

Fig. 4*c*. INFR — protein molecule and two rG5 ligand molecules. Docking results for two rG5 molecules with INFR show extensive interaction networks. The first ligand forms multiple hydrogen bonds (with SER140, LEU139, GLU132, PHE137), as well as π-anionic and π-alkyl interactions with LYS159 and ASP138, ensuring high stability. The second ligand binds via GLU133 and PHE137,

overlapping with contacts of the first ligand, suggesting cooperative binding at shared sites.

The interactions described in Fig. 4 are part of a broader mechanism of receptor activation, where the first ligand induces stable molecular bonds to ensure high specificity and high efficiency of interaction with the receptor. The second ligand continues activation through interactions with specific amino acids that also interact with the first ligand, providing a cooperative effect.

The insulin protein (INS) is shown bound with two rG5 ligands (Fig. 5*a*). The first ligand forms a hydrogen bond with GLY32, providing specific recognition. The second ligand engages CYS100 and LEU102 via hydrogen, π-donor, and π-sigma interactions, indicating a more diverse but less specific binding. This combination highlights differences in ligand stabilization between primary and secondary binding sites.

The insulin receptor (INSR) complexed with two rA5 ligands.(Fig. 5*b*) The first ligand shows specific hydrogen bonding with CYS315, PRO136, TYR319, and ionic interaction with LYS337, ensuring stable and selective binding. The second ligand interacts mainly through van der Waals forces, providing weaker stabilization. Together, these interactions demonstrate a dual mechanism of receptor activation: one strong and specific, the other weaker and supportive.

Considering three indicators simultaneously (ΔG, Kd, molecular docking results) [20] in the study has significant value because they complement each other and provide multi-level analysis interactions ligand-protein (Table 2).

To evaluate the characteristics of the interaction of different ligands with nucleotide sequences, binding constants (Kd, mkM) were determined for ligand-oligomer complexes. The type of binding was assessed based on the number of sites identified: the presence of one site was interpreted as non-cooperative binding, and the presence of two sites was interpreted as the presence of multiple binding sites with different affinities. Such a situation may correspond to cooperative, anti-cooperative, or independent binding; additional analysis is required to establish cooperativity.

At the same time, binding was detected for individual ligand-sequence combinations, mostly with signs of positive cooperativity, when the binding of the second ligand occurs with higher affinity. The most pronounced cooperativity was recorded for INS on rG5 (Kd1/Kd2 ≈ 6.37).

This study analyzed the interactions between pentameric oligonucleotides and recombinant signaling proteins, including interferon α2-b, insulin, somatotropin, and their receptors. We used molecular docking methods and fluorescence spectroscopy to evaluate these interactions *in silico* and *in vitro*.

Conclusions

The study revealed a strong positive correlation between the binding constant and the scoring function when desoxyoligonucleotides interact with all studied proteins, and also when interferon interacts with all ligands. This consistency suggests that the applied methods accurately assess protein-oligonucleotide interactions.

The interactions between rG5 and insulin, as well as between homopolymeric oligonucleotides rA5 and A5 with the interferon receptor, demonstrated positive cooperativity, highlighting their role in stabilizing protein-nucleic acid complexes.

Analysis of the binding constant and energy revealed that the structural specificity of oligonucleotides, not just their pentose composition, determines how they bind to signaling proteins.

Molecular docking of proteins with two ligand molecules identified various interaction mechanisms, including hydrogen, ionic, pi-anionic, and pi-alkyl bonds, which provides deeper insight into protein-oligonucleotide interactions.

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ВИВЧЕННЯ ВЗАЄМОДІЇ МІЖ ПЕНТАМЕРНИМИ ОЛІГОНУКЛЕОТИДАМИ ТА РЕКОМБІНАНТНИМИ СИГНАЛЬНИМИ БІЛКАМИ ТА РЕЦЕПТОРАМИ

Мета. Метою цього дослідження було синтезувати, очистити та дослідити взаємодії між олігонуклеотидами (OLN) та рекомбінантними сигнальними білками, зокрема інтерфероном α2-b, інсуліном, їх рецепторами та соматропіном, за допомогою методів докінгу та флуоресцентної спектроскопії. ***Методи.*** Для аналізу цих взаємодій ми використовували рівняння Штерна-Фольмера в його загальній та модифікованій формах, а також рівняння Гілла, яке дозволило нам визначити параметри зв'язування та константи. Ми синтезували олігонуклеотиди за допомогою твердофазного фосфорамідитного методу, який забезпечує високу ефективність та специфічність. Отримані олігонуклеотиди потім очищали твердофазною екстракцією, яка видаляла побічні продукти та домішки, що підтверджено спектральним аналізом. ***Результати.*** Флуорометричне титрування показало, що гомополімерні олігонуклеотиди зв'язуються з білками в діапазоні середньої афінності, утворюючи нефлуоресцентні комплекси. Найбільш значні взаємодії відбувалися з коротшими олігонуклеотидами. Ми спостерігали позитивне кооперативне зв'язування між інсуліном та олігорибонуклеотидом Poly$(rG)_5$. Примітно, що всі білки вибірково зв'язувалися з олігонуклеотидом Poly$(dG)_5$. ***Висновки.*** Розуміння механізмів білок-олігонуклеотидної взаємодії може відкрити нові можливості для розробки антибіотиків, противірусних препаратів та методів лікування раку та генетичних захворювань.

***Ключові слова*:** сигнальні білки, взаємодія білок-ліганд, спектроскопія, олігорибонуклеотид, олігодезоксинуклеотид, докінг.