Interaction of cationic porphyrin-imidazophenazine conjugates with DNA quadruplex: FID assay and quantum-chemical modeling

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Aim. To study the efficiency of tricationic porphyrin–imidazo[4,5-b]phenazine conjugate and its Zn(II) and Mn(III) complexes as G-quadruplex (G4) DNA ligands. Methods. FID (Fluorescent Intercalator Displacement) assay was used to evaluate the affinity of compounds for a model duplex and Tel22 quadruplex DNA at various ionic strengths. Molecular modeling of the conjugate interaction with G4 DNA was performed using the Density Functional Theory (DFT) calculations with M06-2X functional and 6-31G(d) basis set. Guanine octet stabilized with K+ ion was used as a G4 model. Results. DC50 values and dissociation constants were determined for the complexes of three conjugates with duplex and quadruplex DNA. The structures and energetic parameters of G-octet complexes with Zn-metalated conjugate were obtained. Conclusions. All complexes have a strong affinity to the Tel22 quadruplex. The increase of ionic strength results in an increase in selectivity for quadruplex over duplex DNA and the decrease of binding affinity of the ligands. The structure of ligand–G4 complexes is determined by stacking interaction of porphyrin fragment with G-quartet, rather than an intercalative binging of the ligand.

Keywords: G-quadruplex, ligands, porphyrins, imidazophenazine, FID, DFT

Introduction

Quite recently established anticancer strategy is based on targeting the telomeres and telomerase by small molecules. Telomeres are guanine-rich DNA sequences located at the ends of the chromosomes. Telomeric DNA is able to fold into specific four-stranded structures called G-quadruplexes (G4) formed by the stacks of guanine quartets (G-quartets)
linked by non-canonical systems of hydrogen bonds and stabilized by monovalent metal cations located inside the channel formed by guanine residues [1, 2] (Fig. 1). Binding and stabilization of these structures by small molecules can result in the inhibition of telomerase, an enzyme responsible for telomere elongation upon cellular division [3, 4]. Telomerase is highly active in a majority (80–95 %) of tumor cells, in contrast to normal somatic cells [3, 5]. So telomerase system is directly associated with cancer, and both telomerase and telomeric DNA quadruplex structures are considered as targets for the development of novel anticancer drugs [6–9].

In the cell, G4 structures are also found in promoter regions of some genes, mainly proto-oncogenes [10]. Stable RNA quadruplexes have been discovered as well [11]. There is growing evidence that DNA and RNA quadruplexes play a crucial biological role as a class of regulatory elements involved in processes such as DNA replication, transcription and translation [12, 13].

The binding of a ligand to telomeric G-quadruplex can interfere with telomerase interaction with telomeric DNA blocking its elongation and thus inducing the death of cancer cells [2–4, 8]. Another mechanism of antitumor activity of G4 ligands has been shown to be quadruplex-mediated DNA damage [14, 15].

Usually specific G4 ligands contain large heteroaromatic systems allowing the efficient π-π-interaction with G-quartets [2, 7–9, 16]. Tetracationic porphyrin TMPyP 1 (Fig. 1) is a well established G4 ligand and telomerase inhibitor [17, 18]. Its tricationic analog tris(methylpyridinium)phenylporphyrin (TMP3) was used to attach porphyrin to a variety of other molecules [19–22]. We have previously synthesized TMP3 conjugate with imidazo[4,5-b]phenazine (TMP3-ImPhz) and their Zn(II) and Mn(III) complexes [23] (Fig. 2). ImPhz is an efficient intercalating agent stabilizing DNA duplexes [24–27], and its residue was expected to enhance ligand affinity for G4-DNA.

![Fig. 1. (A) The structure of guanine quartet; (B) side view of the stack of three G-quartets containing two metal cations (K⁺, Na⁺).](image-url)
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Conjugates 2a-c were shown to be efficient telomerase inhibitors in vitro active at 2–10 μM concentration [28]. They were also found to display antiproliferative activity in vitro (mouse Lewis lung carcinoma cell culture), with EC50 values for 2a, its Mn and Zn complex to be 21.8, 11.2 and 5.9 μM, respectively [23].

Absorption and fluorescence spectroscopy studies on the interaction of compounds 2a-c with monomolecular quadruplex Tel22 [29] and tetramolecular G4 formed by poly(G) [30, 31], as well as various double- and single-stranded nucleic acids [29–31], revealed a number of binding modes, including electrostatic interactions, external binding, aggregation and intercalation. However, binding parameters of these compounds to G4 and duplex DNA have not yet been determined.

In this work we have quantitatively evaluated the affinity and selectivity of porphyrin-imidazophenazine conjugates as quadruplex DNA ligands and their possible mode of binding to G4 structure.

Materials and Methods
Sodium cacodylate and Thiazole Orange (TO) were obtained from “Sigma” (USA). Conjugates of tricationic porphyrin TMP3 and its Zn(II) and Mn(III) complexes with imidazo[4,5-b]phenazine were synthesized as previously reported [23]. Oligonucleotides were from “Eurogentec” (Belgium).

DNA quadruplex was obtained by the folding of a model oligonucleotide Tel22 of the sequence 5’-AGGG(TTAGGG)3’-3’. Tel22 solution in a 10 mM sodium cacodylate buffer (pH 7.3) containing 100 mM KCl was heated at 95°C for 5 min and slowly cooled overnight to room temperature and then equilibrated for a day at 4°C. DNA duplex ds17 was prepared by standard procedure from complementary 17-mer oligonucleotides 5’-CCAGTTCGTAGTAACCC-3’ and 5’-GGGTTACTACGAACTGG-3’ [34, 35].

FID assay
Fluorescent Intercalator Displacement assay was used to study the conjugate binding to duplex [32, 33] and quadruplex [34–36] DNA with TO as a fluorescent dye.

FID titration experiments were performed in 96-well plastic microplates in semi-automatic mode with Synergy HT analyzer (BioTek, USA). For both quadruplex and duplex DNA, the mo-
lar ratio DNA/TO was 3:1. The reaction mixtures (total volume 50 μL) contained 100 or 200 mM KCl, 10 mM Na-cacodylate, 1 μM TO, 3 μM Tel22 or ds17, and various concentrations of tested compounds (solution in dimethyl sulfoxide). The fluorescence of samples was measured at room temperature with λ<sub>ex</sub> = 500 nm and λ<sub>em</sub> = 530 nm. The data were processed with Gen5 software. The percent of fluorophore displacement D was obtained for each concentration as follows: \( D = 100 - \left( \frac{F}{F_0} \times 100 \right) \), where \( F \) and \( F_0 \) — fluorescence of DNA/TO complex in the presence and absence of a ligand, respectively. The plots of \( D \) vs. ligand concentration were built, from which DC<sub>50</sub> values (ligand concentration required for 50% displacement of TO in dye-DNA complex) were obtained. Dissociation constants of complexes were determined using the Scatchard method from the plots of \( DF/\text{[free ligand]} \), where \( DF \) is a difference between the relative fluorescence of DNA/TO complex in the presence and absence of a ligand, and \([\text{free ligand}]\) is a concentration of unbound ligand in the probe [32, 33]. Three independent experiments were performed for each ligand.

**Molecular modeling**

Quantum-chemical modeling of the interaction of ligands with G4 structures was performed by DFT (Density Functional Theory) approach using M06-2X functional [37] and 6-31G(d) basic set. The structures of all porphyrin conjugates have been previously optimized by the same method [23]. Guanine octet stabilized by a potassium cation was used as a simple model of G-quadruplex [38].

Complexes of (Zn)TMP3-ImPhz with G-octet were investigated. Full geometry optimization was performed for the studied systems in water. The solvent effects were treated using the CPCM (Conductor-like Polarizable Continuum Model) method [39, 40]. Calculations were carried out with Gaussian 09 package (revision B.01) [41].

**Results and Discussion**

The synthesis of tricationic porphyrin-imidazophenazine conjugates containing a flexible linker (2a-c, Fig. 2) was based on the coupling of carboxyalkyl-modified porphyrin with aminoalkyl-functionalized phenazine derivative, as described in [23].

**Ligand affinity and selectivity**

Ligand interaction with two forms of DNA was studied using a Fluorescent Intercalator Displacement (FID) assay. FID is a simple and fast analytical method that allows to evaluate the DNA binding affinity and selectivity of low-molecular compounds. This assay proposed by Boger et al. in 2001 [32] is based on the loss of fluorescence of some dyes, usually Thiazole Orange (TO), upon their displacement from complexes with DNA by a ligand. TO is virtually non-fluorescent in a free state, whereas its complexes with DNA are highly fluorescent. Both duplex [32, 33, 42–44] and quadruplex [34–36, 44–46] DNA, as well as RNA [47], can be used as targets.

22-mer oligonucleotide Tel22 (sequence 5’-AGGG(TTAGGG)<sub>3</sub>-3’, a fragment of human telomeric DNA containing three telomeric repeats), was folded in the presence of K<sup>+</sup> cations to form G-quadruplex of parallel topology (PDB access code 1KF1). Model double-stranded DNA ds17 was formed by two
complementary non-quadruplex-forming 17-mer oligonucleotides.

To evaluate the effect of ionic strength on binding affinity and selectivity, FID experiments were performed in solutions containing 100 and 200 mM KCl.

DC$_{50}$ values were determined from displacement–concentration plots obtained in FID titration experiments (Fig. 3). DC$_{50}$ parameter, compound concentration resulting in 50 % fluorophore substitution in dye-DNA complex, is a measure of relative ligand affinity to DNA targets; the lower is DC$_{50}$, the higher is binding efficiency of the ligand.

Dissociation constants of ligand-DNA complexes ($K_d$) were determined from the titration data for duplex and G4 DNA using the classic Satchard analysis, according to [32, 33].

Ligand affinity data are presented in Table 1.

It should be noted that DC$_{50}$ values are directly obtained from the experiment and can be used for affinity ranking of a series of ligands in regard to a given DNA target. At the same time, $K_d$ is a calculated parameter to be taken with care, since FID method is an indirect technique involving the competitive DNA binding of two ligands with different affinities and binding modes. FID and the direct methods provide comparable results [32, 45], but

Fig. 3. FID titration plots obtained for non-metalated conjugate 2a with quadruplex (Tel22 G4) and duplex DNA (ds17) in 200 mM KCl buffer.

**Table 1. Binding parameters and selectivity indexes of TMP3-ImPhz and its metal complexes at various ionic strengths**

| Ligand | 100 mM KCl | | 200 mM KCl |
|-------|------------|------------|------------|
|       | DC$_{50}$, µM | $K_d$, $\times 10^{-6}$ M | Selectivity index ** |
|       | G4 | ds17 | G4 | ds17 | Sl$_{G4}$ | Sl$_{K4}$ | Sl$_{G4}$ | Sl$_{K4}$ | G4 | ds17 |
| 2a    | 17.4 | 5.0 | 4.6 | 3.7 | 0.29 | 0.81 |
| 2b    | 36.1 | 59.7 | 7.0 | 6.1 | 1.65 | 0.87 |
| 2c    | 14.4 | 14.2 | 1.6 | 2.9 | 0.99 | 1.81 |
|       | 43.8 | 79.9 | 20.1 | 89.5 | 1.82 | 4.45 |
| 2b    | 59.6 | 114 | 46.3 | 359 | 1.91 | 7.75 |
| 2c    | 28.6 | 72.3 | 3.8 | 42.0 | 2.53 | 11.1 |

* experimental errors are within 10 % (n=3)

** Sl$_{G4}$ = DC$_{50}$(ds)/DC$_{50}$(G4); Sl$_{K4}$ = $K_d$(ds)/$K_d$(G4)
some authors underline the difficulties in accurately determining DNA-ligand affinity constants, pointing out the need for comparing data obtained with various techniques [35].

All compounds demonstrate a strong binding to G4 DNA. Dissociation constants $K_d$ in 100 mM KCl are in the range $(1.6–7.0) \times 10^{-6}$ M. The most efficient G4 binder is Mn(III) complex 2c. At high ionic strength (200 mM KCl) binding affinity of all ligands to G4 substantially decreases, according to both DC$50$ and $K_d$ data, but remains high ($K_d = 3.8–46.3) \times 10^{-6}$ M).

High affinity of the conjugates for G4 is associated with a good ability to inhibit telomerase. The most efficient Zn(II) complex 2b was shown to completely inhibit it in vitro in TRAP assay [5] at 2.5 µM concentration being several times more active than non-conjugated porphyrin. Non-metalated conjugate 2a showed inhibition effect at 5 µM, whereas Mn(III) derivative 2c was less active [28].

Preferential quadruplex vs. duplex binding, along with high affinity to quadruplex DNA, is a key requirement for specific antitumor G4 ligands allowing to minimize their unfavorable biological side effects. Selectivity index (SI) is defined as the ratio between either DC$50$ values or dissociation constants of ligands for duplex DNA and G4. At low KCl concentration (100 mM) ligands 2a and 2b preferentially bind to duplex DNA, whereas Mn complex 2c has no (SI determined from DC$50$ values) or relatively low (SI based on $K_d$ is 1.81) preference for G4.

At 200 mM KCl all ligands bind to G4-DNA stronger than to duplex DNA, i.e. demonstrate a good selectivity for quadruplex over duplex DNA. For example, the selectivity index determined from $K_d$ values is 4.45 for TMP3-ImPhz and 7.75 and 11.1 for its Zn and Mn complexes, respectively. Thus, an increase in ionic strength leads to an increase of G4 selectivity of ligands.

Generally, both electrostatic and stacking interactions may contribute to DNA binding of the studied ligands. The electrostatic binding of positively charged porphyrin fragment to DNA phosphate backbone was experimentally shown to occur upon interaction of 2a-c with quadruplex structures [29–31]. Ionic strength increase suppresses strong, but non-specific electrostatic interactions, resulting in the increase of ligand selectivity for G4, however, this effect is obviously associated with a decrease of binding affinity (Table 1). These data are in line with a recent finding that lowering the total charge on TMPyP decreases the affinity, but increases the selectivity to G4 vs. dsDNA [48].

**DFT modeling of complexes**

Computer modeling of the interaction of ligands with their molecular target, telomeric G-quadruplex, is required to get insights into their mechanism of action and for further structure optimization to design more potent telomerase inhibitors.

The complexity and flexibility of quadruplex structures is still a challenge for theoretical modeling of G4 and their ligand complexes. In addition to the large size of the molecular system, one of the main problems is a broad structural polymorphism of quadruplex DNA [1, 2, 8, 49].

Usually molecular modeling of quadruplex nucleic acids and their complexes is carried out by molecular dynamics (MD) approaches [50, 51]. For example, a series of cationic porphy-
rin-anthraquinone hybrids have been investigated for their interaction with G4 DNA by employing docking and molecular dynamics methods [52]. MD simulations provide valuable information on conformation, dynamics and thermodynamic parameters of the systems studied. MD methods, however, have a number of limitations, e.g. force-field imperfections and often short simulation times resulting in insufficient accuracy of modeling. On the other hand, quantum-chemical (quantum-mechanical, QM) approaches are much more accurate, although require more computational resources, and have been successfully used in the field of quadruplex modeling [38, 53–55].

To find a possible mode of binding, we have performed a QM study of the interaction of metalated TMP3-ImPhz conjugate \(2b\) with a simple model of G-quadruplex, so called G-octet previously developed by us [38]. This system consists of two guanine quartets containing a central K\(^{+}\) cation. Molecular modeling was carried out to construct the geometries of complexes in aqueous solution. Calculations were performed by DFT approach using M06-2X functional [37] that was proposed for the correct description of non-covalent interactions, including the representation of geometry and energetic parameters of p-stacking complexes [56]. Taking into account the complexity of the studied system, we have used 6-31G(d) basic set.

A number of possible structures of the complexes have been identified (Fig. 4, Table 2). In all cases, the stacking interaction of the porphyrin ring with G-quartet is observed. ImPhz fragment can bind in several ways: it can freely move (structure A), interact with G-quartet (B) or porphyrin (C), or intercalate between the pair of quartets (D). The most energetically favorable structures are those where ImPhz residue interacts with G-quartet (B) or with a porphyrin (C), i.e. complexes in which both fragments of the conjugate participate in binding interactions. Of course, model structure B formed upon the conjugate binding to G-octet cannot be realized with a real Tel22 quadruplex containing 3 stacked G-quartets, due to the insufficient linker length. At the same time, the formation of intercalative complex D where imidazophenazine chromophore is inserted between two G-quartets is associated with strong deformation of G-octet that results in significant increase of system energy and its destabilization. So the intercalative mode of binding of ImPhz moiety is hardly possible. The same is true for the porphyrin fragment. It should be noted that metal cation located between G-quartets prevents ligands from efficient intercalation.

| Structure | A | B | C | D |
|-----------|---|---|---|---|
| Total     | -6523385.80 | -6523413.28 | -6523412.74 | -6523380.59 |
| Relative  | 27.48 | 0.0 | 0.54 | 32.59 |

Table 2. Energetic parameters of (Zn)TMP3-ImPhz conjugate complexes with G-octet obtained with M06-2X/6-31G(d)/CPCM method.
Importantly, both spectral-fluorescent data [29] and DFT calculations [23] suggest that free conjugate 2a and its Zn(II) complex 2b in water exist mainly in the form of rather stable intramolecular head-to-tail heterodimers. Their formation results from the strong electronic

**Fig. 4.** DFT calculated structures of porphyrin-imidazophenazine 2b complexes with G-octet (side view). N–Zn coordination bond in complex C is shown as a dashed line.
interactions between porphyrin and ImPhz chromophores, via either p-p-stacking (2a) or metal coordination (2b). Moreover, it was shown by fluorescence spectroscopy techniques including the fluorescence polarization data that these conjugates can bind to G4 structures in the form of internal heterodimers [29–31], just as in case of the calculated complex C.

Non-intercalative binding mode of the conjugates 2a-c to Tel22 quadruplex of antiparallel topology was proposed; non-metalated conjugate 2a was shown to bind to G4 via the interaction of porphyrin moiety with a terminal G-quartet, whereas the intercalative binding of phenazine moiety to Tel22 was not observed [29]. Phenazine intercalation between the guanine bases was observed only for the interaction of (Zn)TMP3-ImPhz conjugate 2b with a long tetramolecular quadruplex formed by poly(G) [31]. TMPyP itself was shown to bind to the parallel Tel22 via the external end-stacking rather than intercalation [18].

Thus, DFT modeling results are in good agreement with experimental data on the conjugate binding to Tel22 G4.

Conclusion

All studied compounds have a strong binding affinity for Tel22 quadruplex, with $K_d$ values in micromolar range. Both binding affinity and selectivity of ligands depend on ionic strength of the solution. Its increase results in the decrease of ligand affinity for both duplex and G4 DNA. Interestingly, this decrease is associated with a significant increase of selectivity of compounds for G4 resulting presumably from the suppression of non-specific electrostatic interactions. Theoretical modeling has revealed that stacking interaction of G-quartet with a porphyrin fragment, but not the intercalative binding of any conjugate component, determines the structure of ligand–G4 complexes.

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ногого интеркалятора (FID) использован для оценки аффинности соединений к модельной дуплексной ДНК и квадруплексу Tel22 при разных значениях ионной силы раствора. Молекулярное моделирование взаимодействия конъюгата с G4-ДНК проведено при помощи вычислений теории функционала плотности (DFT) с использованием функционала М06-2X и базисного набора 6-31G(d). В качестве модели G4 использован гуаниновый октет, стабилизированный ионом K⁺.

Результаты. Определены значения DC₅₀ и константы диссоциации комплексов трех конъюгатов с дуплексной и квадруплексной ДНК. Рассчитаны структуры и энергетические параметры комплексов G-октета с Zn-металлизированным конъюгатом. Выводы. Все соединения проявляют высокую аффинность к квадруплексу Tel22. Увеличение ионной силы приводит к возрастанию селективности к квадруплексной относительно дуплексной ДНК и снижению аффинности связывания лигандов. Структуру комплексов лиганда–G4 определяет прежде всего стекинговое взаимодействие порфиринового фрагмента с G-квартетом, а не интеркаляционное связывание лиганда.

Ключевые слова: G-квадруплекс, лиганды, порфирин, имидазофеназин, FID, DFT

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