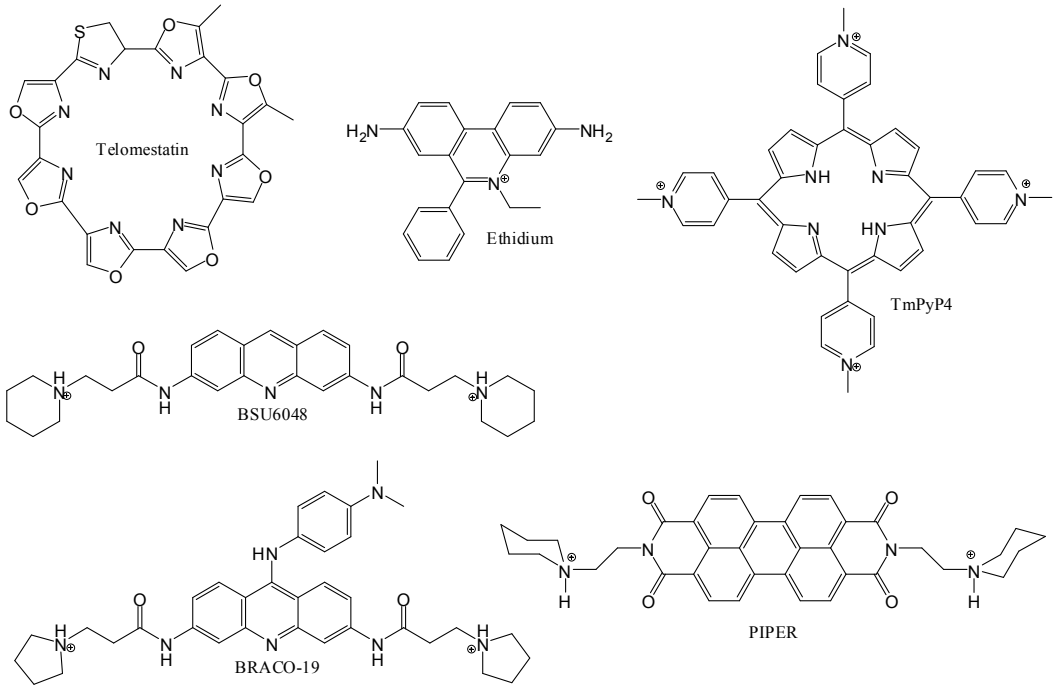
"Synthesis of new carbazole ligands, potential inhibitors of telomerase in antitumor therapy"

Completion of Human Genome Project has brought about great interest in the ligands entering into specific interactions with DNA, both in the bioanalytical aspect (detection and diagnostics of DNA sequences) and biomedical aspect (new formulations and strategies for treatment of cancer, viral infections or genetic diseases). The majority of classical DNA ligands have been addressed to the applications with the double stranded DNA. In recent years intense study has been undertaken on selective ligands capable of intercalation to the triple stranded DNA helix (e.g. BePI, naphthylquinolines, phthylquinolines, anthraqinones) [1,2], and capable of stabilization of guanine quadruplexes (G-quadruplexes) – the folded structures made of oligonucleotides rich in guanine residues [1,3-8] (Scheme 1).

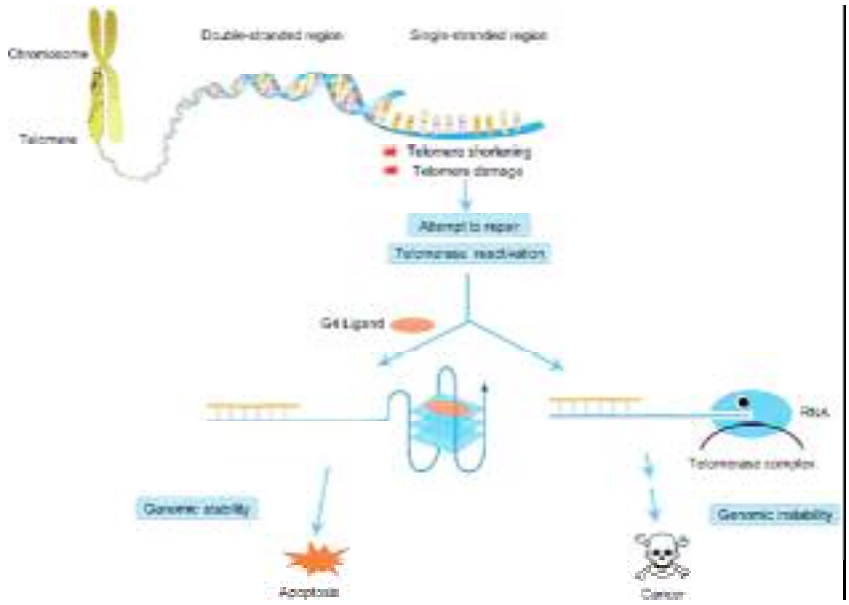


Scheme 1. Structures of exemplary G-quadruplex ligands.

The therapeutic effectiveness of such ligands in treatment of neoplasmic diseases is attributed to inhibition or poisoning of some DNA enzymes, in particular topoisomerases and telomerase [4,5,7-13]. Although no reliable evidence has been reported yet on a close correlation between the ligands ability to stabilize DNA triplexes or quadruplexes and their anticancer properties, such a correlation is generally assumed to exist [4,5,7-10,12-14].

The ligands stabilizing the DNA triplex structure show inhibition or poisoning of topoisomerases, which leads to the DNA strand cut by the enzyme. In other words, the ligand changes the normal enzyme into a cell toxin by stabilizing the cleavable complex of DNA-topoisomerase [14]. The ligands stabilizing quadruplex structures were shown

by the in vitro study to be able to inhibit telomerase [4,5,7,8,10]. Telomerase shows elevated activity in cancer cells, while in the majority of somatic cells it is inactive [15]. The activity of telomerase is associated with the linear ends of chromosomes (telomeric DNA), with a characteristic sequence rich in guanines [16]. In a normal cell telomeres get shortened upon each cell division, which acts as special biological clock and restricts the number of possible replications. In cancer cells telomerase is active and maintains a constant length of telomeric DNA, which guarantees unlimited number of replications of the cell making it practically immortal. However, for telomerase to get active it must first make a complex with single-stranded fragment of telomeric DNA (3'-overhang) (Scheme 2).



Scheme 2. Structure and biological roles of telomeres (Ou, T.; Lu, Y.; Tan, J.; Huang, Z.; Wong, K; Gu, L. *Chem. Med. Chem*. **2008**, *3*, 690-713).

At this point the question of quadruplex structures formation appears. It has been proved that the single-stranded DNA of the repeatable sequence (TTAGGG)n is *in vitro* capable of formation of the four-stranded structure (G-quadruplex) [17,18]. Figure 1 gives a schematic organization of the guanine quartet (a) and the quadruplex structure (b). The molecular structure formed in this way on the single-stranded telomere end acts as a plug, preventing the complex formation with telomerase and hence telomere extension. Consequently, the cancer cell should die after a certain number of divisions and cancer development should stop. G-quadruplex structures are particularly favored in the presence of certain metal cations (e.g. K^+) and can be stabilized by specific ligands [3-7,9,10,17,18].

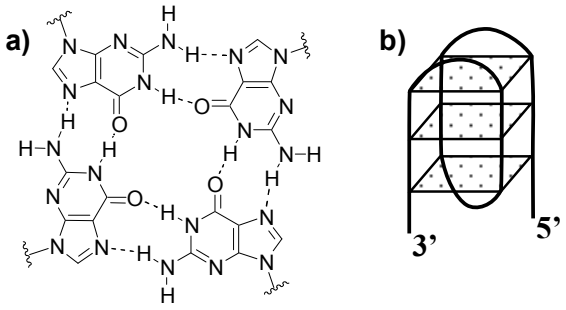


Figure 1. A scheme of guanine quartet (a) and a quadruplex structure (b).

The project proposed is a fragment of a wider program of studies on synthesis of new ligands interacting with nucleic acids for biotechnological and bioanalytical purposes and is consistent with PO IG priority theme of "new medical products and techniques".

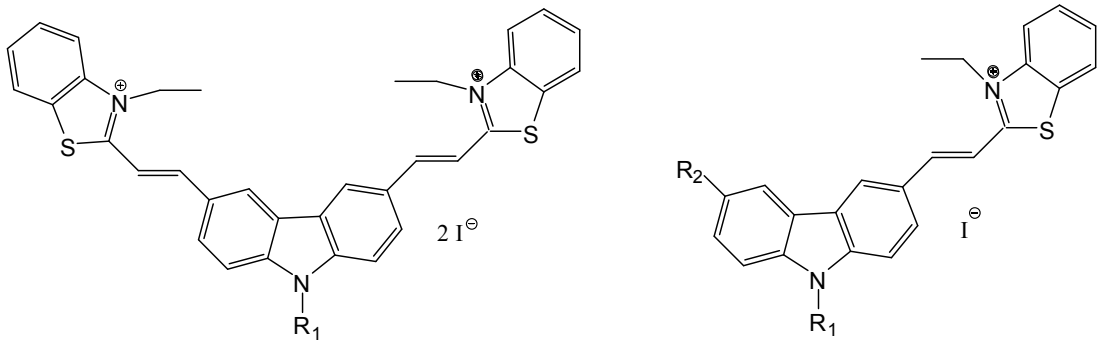
Investigation of the processes of interaction of new ligands in the presence of double-, triple- and tetra– stranded DNA, determination of mechanisms of the above processes should provide interesting experimental material for design of effective DNA ligands potentially active in anticancer therapy and useful as fluorescence probes for structural studies of DNA or detection of trace amounts of DNA. In our opinion research work on the border of analytical chemistry and biotechnology aimed at development of modern tools in biomedical diagnostics and/ or new formulations for treatment of cancer diseases can and should be performed also in Poland. The results may have stimulating effect on economic development of the country.

The ability of ligands to make complexes with triple-stranded and tetra-stranded DNA is the first indication of potential anticancer properties of the ligands studied. The ligands active towards DNA are obtained by biotechnological methods or from substances of natural origin (e.g. antibiotics: anthracycline or daunorubicin; alkaloids such as berberine or coralline), many are synthesized (porphyrins, anthraquinones, benzacridines). They share the following features.

- Planar rigid structure containing 3 or more condensed aromatic rings – the ligands should show ability to interact with DNA;
- The presence of heteroatoms often endowed with positive charge; the presence of a cationic group enhances the ligands solubility in aqueous environment and allows electrostatic interactions with DNA;
- Various functional substituents.

After a through survey of literature, we decided to study the ligands with carbazole skeleton as central element and containing heterocyclic benzothiazolium group (or another one possessing positive charge) and additional substituents (functional groups, C=C bond), as all these structural elements are associated with interesting biological and photophysical properties (Scheme 3):

- Carbazole ring is a planar aromatic system and shows ability to bind with different forms of DNA,
- Opportunistic infections – carbazole dications make an unusual class of DNA binding agents active against opportunistic infections,
- Alzheimer's disease - carbazoles are recognized as potential prevention agents for Alzheimer's disease,
- Some derivative of carbazole:
  - inhibit telomerase ($IC_{50} = 5x10^{-8}M$),
  - bind to G-quadruplexes,
  - inhibit topoisomerase I and II,
- Carbazole ring and benzothiazolium group exhibit interesting fluorescent characteristics,
- Ability for c*is-trans* photoisomerization is expected due to the presence of C=C bond.



R_1 = H, C_2H_5, $CH_2CH_2CH_2CH_2NH_3^+$, $CH_2CH_2CH_2CH_2(C_2H_2N_3)$, $CH_2CH_2CH_2CH_2(C_3H_3N_2)$

R_2 = H, CHO

Scheme 3. Structures of carbazole ligands.

The carbazole dications make an unusual class of DNA binding agents that are active against opportunistic infections. Such infections caused by pathogens (bacterial, viral, fungal or protozoan) that usually do not cause disease in a healthy host, are hazardous for the persons with immunological system weakened by HIV virus. Some derivatives of carbazole with antibacterial activity, behave essentially as DNA minor groove binders although they contain a planar chromophore, which is generally a characteristic of DNA intercalators [19-22]. Alzheimer's disease is characterized by the presence of β-amyloid fibril formation. The inhibition of this peptide accumulation may be a prevention method for Alzheimer's disease. Several classes of drugs have been reported to inhibit β-amyloid fibril formation and among them carbazoles are recognized. One such derivative was synthesized and examined for interactions with DNA samples in *in vitro* experiments by the applicant of grant. On the other hand, carbazole ligands are able to cross membrane and to penetrate rapidly inside the cells, where they do not accumulate inside the mitochondria but rapidly bind to DNA and are then slowly reduced by intracellular reducing agents [23]. Some derivatives of carbazole recognize specific quadruplex structures, particularly the quadruplex of human telomeric sequence $d(T_2AG_3)_4$ and inhibit telomerase ($IC_{50} = 5x10^{-8}M$) [7, 24-31]. The compounds with carbazole structure also inhibit topoisomerase I and II [22,32,33]. The benzothiazolium group is known as a structural element in highly fluorescent cyanine dyes (TOTO, BOBO) used for detection and dying of DNA [34]. The presence of nitrogen atom bearing a cationic charge will increase the ligand solubility in water environments and permit electrostatic interactions with DNA. Finally, the presence of the C=C double bond offers a possibility of controlling the ligand structure by light (*cis-trans* photoisomerization).

Expected practical outcomes:

- New compounds can extend the range of anticancer drugs,
  - As inhibitors of telomerase,
  - As poisoning agents of topoisomerases I and II (recognition and cleavage of DNA),
- The ligands can be used as fluorescence probes in structural investigation of DNA or for detection of trace amounts of DNA,

- The method of equilibrium dialysis will be recommended as a fast method for investigation of ligand – DNA interaction for fast testing of compounds expected to show mutagenic or carcinogenic properties,
- New strategies for detection of selected gene sequences or their mutation analogues can be developed using synthesized ligands,
- Ligands can be exploited as fluorescent probes in modern technologies of genetic diagnostics (DNA biochips, DNA microarrays, molecular beacons),

Expected scientific outputs
- Synthesis of new carbazole compounds,
- Determination of correlation between the ligand structure and affinity to particular structural DNA forms,
- Investigation of affinity of new ligands (drugs, DNA probes) to certain DNA sequences (stability, stoichiometry) will add new results to worldwide database,
- Analysis of the possibility of use carbazole derivatives in anticancer therapy

Methodology of research
- Synthesis of new ligands,
- Physicochemical and spectral investigation of the new compounds (UV-Vis spectrometry, spectrofluorimetry, circular dichroism),
- Investigation of ligands interaction with DNA by the method of equilibrium dialysis using the procedure to be proposed,
- Experiments leading to examine quadruplex structure and dynamics and interactions of G-4 DNA with new ligands, using NMR spectroscopy,
- Biochemical experiments - biological activity of the new compounds will be examined, including cytotoxicity tests, telomerase activity assays (TRAP - telomeric repeat amplification protocol) and many others.

The first stage of the project is devoted to synthesis of new ligands containing a carbazole ring, heterocyclic benzothiazolium ring or other charged ring and functional groups (ethylene bond, polar substituents). In synthesis of new systems the applicant has successfully been using a method based on condensation of formyl derivatives of aromatic compounds with 3-ethyl-2-ethyl-benzothiazolium iodide and piperidine in methanol. Final products will be separated and purified using conventional methods including semi-preparative TLC and HPLC. All new compounds will be characterized as to their physicochemical and spectral properties (UV-Vis absorption spectra, fluorescence spectra). Interaction of ligands with DNA will be studied by the method of equilibrium dialysis using the procedure to be proposed by us [35]. We intend to use 14 different forms of nucleic acids. Single-stranded DNA and RNA are represented by Poly[dA], Poly[rA] and Poly[rU]. Duplex DNA is represented by Poly [dA]*Poly[dT], Poly[dG]*Poly[dC], Poly[dA-dT]*Poly[dA-dT], Poly[dG-dC]*Poly[dG-dC], 5'-CAATCGGATCGAATTCGATCCGATTG-3' and 24mer GA – natural samples and synthetic polydeoxynucleotides of defined sequence. Triple-stranded structures are represented by Poly[dT]*Poly[dA]*Poly[dT] and synthetic polydeoxynucleotides of defined sequence. The quadruplex are represented by oligonucleotide which has human C-MYC oncogene sequence-5'-AGGGTGGGGAGGGTGGGG-3', oligonucleotide

which has human telomere sequence-5'-AGGGTTAGGGTTAGGGTTAGGG-3' (both can form intramolecular G-quadruplex structures by folding of a single oligonucleotide molecule) and structure 5'-TTGGGGGGGGGGGGGGGGGGGGTT-3' of nucleic acid which can form tetramolecular parallel G-quadruplex ($T_2G_{20}T_2)_4$). The data will be supplemented with spectrophotometric titration and spectrofluorometric measurements. Identification of the mode of interaction of the ligands with DNA (intercalation vs. external electrostatic interactions or minor groove complexes) is planned to be performed by circular dichroism spectroscopy and results of Scatchard analysis of titration data. Experiments aimed at determination of quadruplex structure, dynamics and interactions of G-4 DNA with new ligands will be performed using NMR spectroscopy (essential tool in the study of quadruplex nucleic acids). This research task will be carry out in cooperation with the group of doc. Zofia Gdaniec from the Institute of Bioorganic Chemistry PAS in Poznan. The biological activity of new compounds will be examined in cooperative studies with research group of Prof. Maria Rybczynska from the University of Medical Sciences. Biochemical experiments will be carried out on cancer cells including cytotoxicity tests, telomerase activity assays (TRAP - telomeric repeat amplification protocol) and many others.

Our hitherto experience permit us to expect that the group of the ligands to be obtained will contain the derivatives that would be useful for bioanalytical purposes (e.g. selective fluorescence DNA probes) and biomedical purposes (e.g. inhibitors of telomerase in cancer treatment. Results obtained in realization of the project will be used for preparation of habilitation dissertation.

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