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REVIEW ARTICLE

Conjugates of Classical DNA/RNA Binder with Nucleobase: Chemical, Biochemical and Biomedical Applications

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Abstract: Among the most intensively studied classes of small molecules (molecular weight < 650) in biomedical research are small molecules that non-covalently bind to DNA/RNA, and another intensively studied class is nucleobase derivatives. Both classes have been intensively elaborated in many books and reviews. However, conjugates consisting of DNA/RNA binder covalently linked to nucleobase are much less studied and have not been reviewed in the last two decades. Therefore, this review summarized reports on the design of classical DNA/RNA binder – nucleobase conjugates, as well as data about their interactions with various DNA or RNA targets, and even in some cases protein targets are involved. According to these data, the most important structural aspects of selective or even specific recognition between small molecule and target are proposed, and where possible related biochemical and biomedical aspects were discussed. The general conclusion is that this, rather new class of molecules showed an amazing set of recognition tools for numerous DNA or RNA targets in the last two decades, as well as few intriguing *in vitro* and *in vivo* selectivities. Several lead research lines show promising advancements toward either novel, highly selective markers or bioactive, potentially druggable molecules.

Keywords: DNA, RNA recognition, aryl-nucleobase conjugate, fluorescence, circular dichroism, bioimaging, cytotoxicity.

1. INTRODUCTION

Formation of small molecule-DNA/RNA complex depends on several non-covalent binding modes, such as intercalation, minor or major groove binding and external electrostatic binding [1]. The interest for such small molecules is constantly present [2]. Very recently, even thoroughly studied molecules as classical DNA/RNA intercalator ethidium bromide had to be re-evaluated, since it became obvious that mechanisms of non-covalent interactions between small molecules and DNA/RNA have not been completely understood, and close analogues offered many intriguing biomedical implications [3]. Among the large number of small synthetic compounds that can bind to nucleic acids, those with selective binding to specific single-stranded regions (hairpins, bulges), abasic sites of DNA or single-stranded (ss-) RNA sequences are rather rare [1]. However, their implementations are numerous, like recognition of targeted ss-regions of DNA or RNA, with applications as fluorescent markers, as well as potential antitumor or antiviral agents. There are several approaches for ss-DNA/RNA recognition. The best known is antisense strategy based on oligonucleotides or eventually PNA analogues, well elaborated in many reviews and books and intensively studied for drug applications. However, such oligo-nucleobase constructs are mostly large molecules, which makes their application prone to many problems, like biological degradability *in vitro* and *in vivo*, cellular and tissue permeability, solubility, aggregation, and many others. Low molecular weight molecules (Mw > 800) [2] with biologically atypical bonds (thus resistant to the most of biodegradation mechanisms) often show better bio-administration performance; however, the smaller size of a molecule reduces the number of available recognition patterns for particular bio-target (ss-DNA, or ss-RNA). Thus, the design of such small molecular con-

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structs targeting ss-DNA/RNA became a very challenging task within the last 20 years.

One of the approaches took advantage of supramolecular bis-aromatic ligands (cyclo-bis-intercalands), which were able to bind small aromatic ligands, like nucleobases, with high affinity. Due to the steric restrictions, these cyclo-bis-intercalands showed higher affinities toward ss-DNA/RNA in respect to ds-DNA/RNA [1, 4, 5], and some also showed highly selective binding to viral RNA ss-bulges [1, 6].

However, many research groups decided to design hybrid, multifunctional small molecules constructed from several DNA/RNA binding motifs: a) one or more DNA/RNA intercalator or groove binder units with high affinity toward DNA/RNA; b) covalently attached nucleobase to provide Watson-Crick or non-canonical hydrogen bonding with DNA/RNA bases and c) linker between intercalator and nucleobase, which could control not only distance and flexibility of small molecule but also take active part in DNA/RNA recognition. This research and its biomedical potential were not summarized in a review or book within the last decades, thus we endeavored here to collect and discuss the available data on, as here dubbed, "DNA/RNA binder-nucleobase conjugates". Of course, these studies sometimes border with other research topics and targets, thus we had to make choice on more focused results, whereby some of the border-cases could be unintentionally omitted.

We would like to give an overview of such hybrid molecules and especially, to underline the importance of a fine interplay between molecule steric rigidity and spatially oriented interactions of substituents attached to a DNA-binder moiety with respect to achieved selectivity toward various DNA or RNA targets. Also, available biomedical implications will be discussed. Thus, the review is intended to provide valuable database for design and preparation of the new generations of such compounds, useful for the research community of organic and medicinal chemistry.

2. OVERVIEW ON DNA/RNA BINDER - NUCLEOBASE CONJUGATES AND DISCUSSION OF RESULTS

2.1. DNA/RNA Binder Linked to Nucleobase by Linker which Actively Contributes to Binding

Abasic (apurinic or apyrimidinic, AP) sites are probably the most common DNA mutagenic lesions; they are the result of N-glycoside bond breaking and appear as intermediates in the enzymatic repair of damaged DNA or by the exogenous factors, *e.g.* alkylating agents or ionizing radiation [7]. The abasic sites are potentially mutagenic and lethal lesions that can block DNA replication and transcription [8]. Specific binding of a ligand to the AP site masks this lesion for enzymatic repair. Thus the suppression of DNA repair can be a useful tool for potentiation of the cytotoxic effect of antitumor drugs [7, 9] (Fig. **1**).

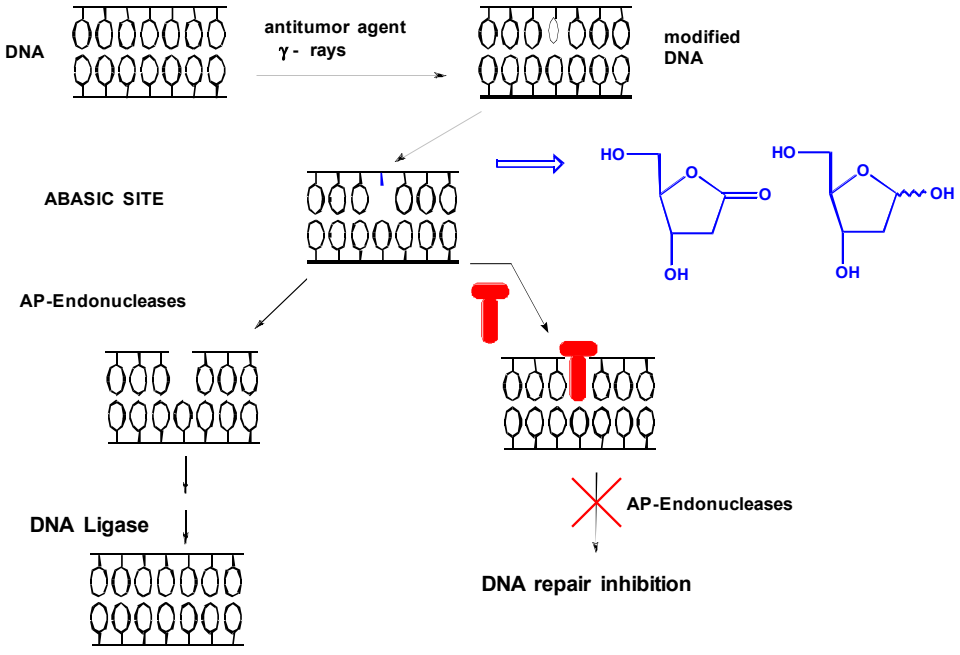

Fig. (1). Schematic representation of AP-site generation, repair and repair inhibition. Reproduced with permission from [7]. Copyright 1999 John Wiley and Sons.

Lhomme's group designed, synthesized and examined the group of various acridine-adenine conjugates, linked by different aliphatic-amine or guanidine linkers, for recognition and/or cleavage of abasic sites (Fig. **2**) [7, 9, 10-16]. The main idea of acridine-adenine conjugates design was intercalation of acridine between DNA bases and insertion of covalently linked adenine into the abasic pocket. The latter was accompanied by specific hydrogen bonding to the complementary base in the opposite strand, while the aliphatic positively charged linker interacted within the minor groove of the DNA backbone (Fig. **2**, down) [7].

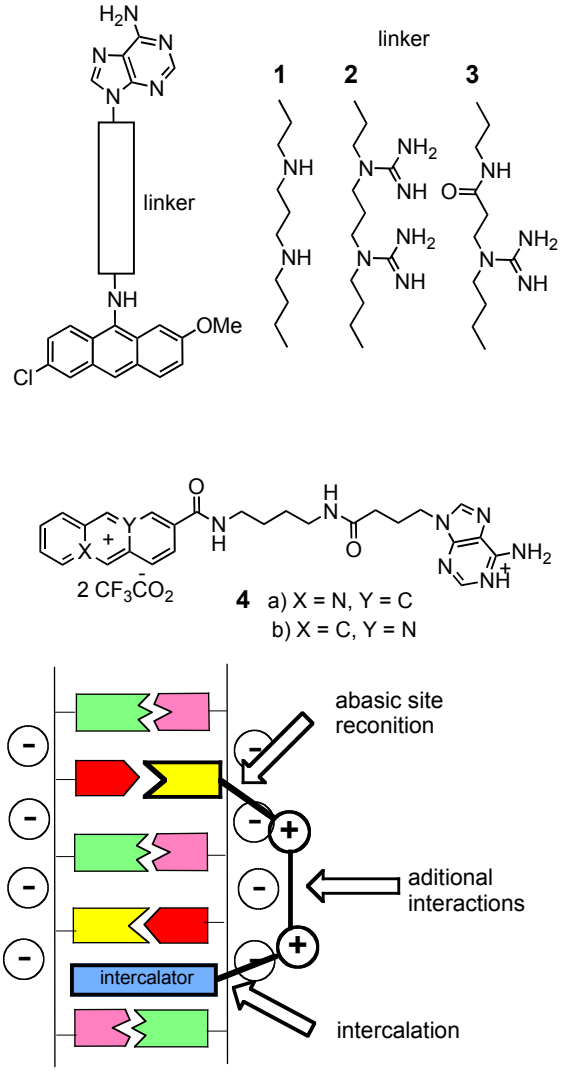

Fig. (2). Acridine-adenine conjugates (up); Schematic representation of AP-site recognition by acridine-adenine conjugates (down).

The linker between acridine and adenine unit also interacted considerably with DNA. Particularly, the aliphatic-amino linker of compound **1** [10-12] showed strong DNA cleavage activity at abasic sites, at variance to the aliphatic-guanidine linker of **2** [13-15] which strongly interacted with DNA backbone without cleavage involved. Compound **3** also interacted with the abasic sites in DNA and inhibited the major base excision repair enzyme in *Escherichia coli*, Exonuclease III [16]. However, **2** showed apparent synergistic effect with anticancer drug BCNU (bis-chloroethylnitrosourea) on murine leukaemia L1210 and human adenocarcinoma A549 cell lines. *In vivo*, the compound showed no antitumor activity in the murine leukaemia P388, but potentiated the action of BCNU [7]. On the other hand, application of more rigid amide linker in compound **4** instead of positively charged amino-linker caused decreased selectivity toward abasic site compared with **1**-**3** [17].

Bis-nucleobase-intercalator conjugates were also prepared using well-known threading intercalator naphthalene diimide (NDI) symmetrically substituted by two thymines over aliphatic amine linkers (**5**, Fig. **3**) [18]. Such NDI bis-thymine conjugate showed an increased binding affinity for complementary adenine sequences, with binding constants for ss-poly rA and poly rA-poly rU being an order of magnitude higher than those measured for NDI alone. The proposed explanation was additional stabilization of hydrogen bonding between thymine and RNA-adenines besides intercalation of NDI. While the structure of the complex in the case of ss-poly rA is easy foreseeable (intercalation of NDI and H-bonding of thymines with adjacent adenines), the structure for complex **5**/poly rA-poly rU remained unsolved. Namely, NDI is well-known threading intercalator [19], whereby side-chains are positioned in both minor and major groove, and there could form additional interactions. However, NDI can also partially intercalate by keeping both chains in the same groove – from experimental data for the complex **5**/poly rA-poly rU it is not clear which structure is dominant.

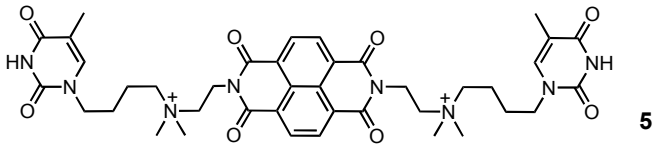

Fig. (3). Naphthalene diimide-bisthymine conjugate **5**.

To investigate whether the aromatic unit (intercalator, groove binder) was necessary for abasic-site recognition, Abe and co. [20, 21] prepared conjugates **6** of nucleobases (adenine, thymine, uracil, guanine, and cytosine) and an aliphatic oligoamine, thus omitting DNA intercalator or groove binder (Fig. **4**). They hypothesized that weak and non-specific interactions of an oligo-cation (aliphatic oligo-amine) with DNA

backbone would yield sufficient affinity to allow selective interaction of ligand's nucleobase with complementary base opposite to AP-side by forming Watson–Crick base pairs.

No stabilization effect was observed by UV-melting measurements to the full match duplexes lacking the AP site. On the contrary, high selectivity of A-, G-, C- and U- ligands (all except T-ligand) was observed for duplexes that contained complementary nucleobases opposite AP-site [20], indicating selective Watson-Crick base-pair formation (Fig. **4**). Further on, G-ligand promoted β-elimination of oligodeoxyribose unit of the AP-site that led to DNA strand cleavage without selectivity for the opposing base. Interestingly, A-ligand selectively cleaved the AP site with the opposing dT. In addition, products of β-elimination in DNA strand, namely 3'-phosphate end structures, were hydrolyzed by an AP endonuclease thus interfering base excision repair process [22]. This made A-ligand and G-ligand good candidates for the potentiation of the antitumor efficacy of the DNA drugs.

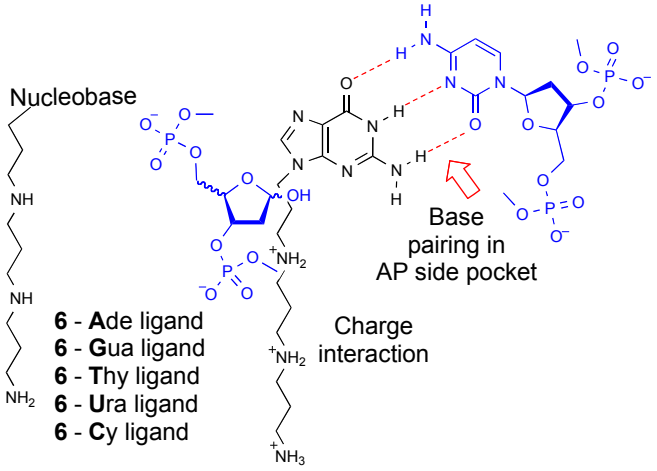

Fig. (4). Polyamine-nucleobase conjugates (left); Schematic representation of AP-site binding by the polyamine-guanosine conjugate (right) [20, 21].

In addition, other cyclic aliphatic amines, such as well-known drugs neamines, were also conjugated with nucleobases. Namely, among numerous antibiotics that bind to ribosomal RNA thus interfering protein synthesis, aminoglycosides were the best characterized. It was found that aminoglycosides that are positively charged at physiological pH [23], bound to a conserved sequence of rRNA that was near the site of codon-anticodon recognition in the aminoacyl-tRNA site (A site) of 30S subunits. This binding stabilized tRNA–mRNA interaction in the A site and consequently affected translation by decreasing tRNA dissociation rates, as well as misreading of the genetic code [24]. Aminoglycoside neamine, like many other aminoglycosides, bound in the major groove of the A-site RNA in a unique binding pocket formed by non-canonical base pairs and a bulged nucleotide [25-27].

To improve the selectivity of neamine, series of conjugates of neamine and nucleoside thymidine/uridine linked by a short aliphatic chain were designed and prepared (Fig. **5**) [28]. Computer simulation demonstrated groove binding of neamine moiety supported by specific binding of nucleoside unit into Escherichia coli rRNA A-site fragment (16S RNA). Surface plasmon resonance (SPR) screening showed that neamine-nucleoside conjugates exhibit somewhat higher affinity to 16S RNA in respect to the neamine-ribose (deoxyribose) reference. The most selective conjugate was **7** (Fig. **5**).

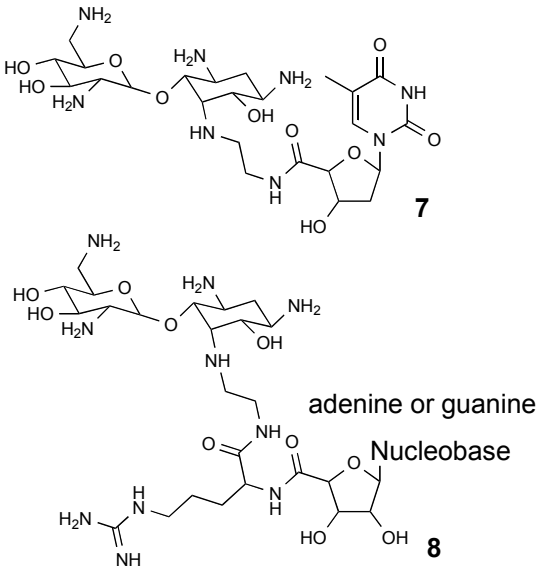

Fig. (5). Neamine-nucleoside conjugates.

The further design was based on neamine–arginine and neamine–lysine conjugates, which showed improved inhibition of HIV-1 TAR-Tat interaction compared to referent neamine [29]. Thus, the addition of arginine or lysine to linker between neamine and nucleoside, and the introduction of purine-bases instead of pyrimidine-bases significantly improved binding properties. For instance, neamine-nucleoside conjugates possessing ethylenediamine-lysine or ethylenediamine-arginine linker showed remarkably improved binding to A site of 16S RNA as well as to TAR RNA compared to the referent un-substituted neamine [30]. Molecular docking study of adenine and guanine conjugates **8** (Fig. **5**) indicated the electrostatic interactions as major binding force responsible for the accommodation of ligands **8** into A site of 16S RNA pocket.

Quinoxaline antibiotic triostin A, isolated from *Streptomyces aureus* [31], is an antitumor agent that inhibits RNA synthesis by DNA-bis-intercalation of quinoxalines, while its peptide scaffold binds to DNA by hydrogen bonding [32, 33]. Replacing of quinoxaline intercalating units by different nucleobases led to a wide family of rigid nucleobase-depsipeptide bicyclic conjugates **9** (Fig. **6**, up) [34-36]. Conjugates were investigated for their DNA-binding potential and for selectivity to the DNA-abasic site.

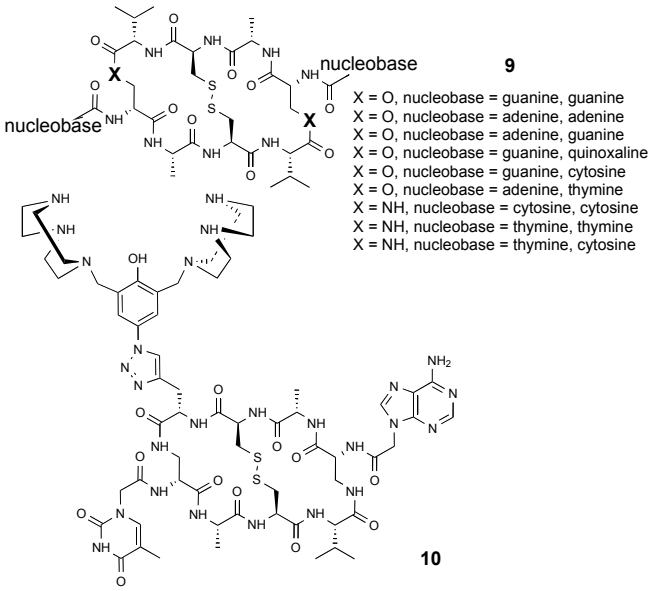

Fig. (6). Nucleobase analogues of triostin A (up); Triostine A derivative aza-TANDEM joined to two [1,4,7]triazacyclononane (TACN) moieties and adenine and thymine (down).

Although experiments indicated that mode of conjugate binding differed from that of triostin A bis-intercalation, none of the conjugates **9** showed favored recognition of abasic sites [36]. Another modification of triostin A comprised replacement of quinoxalines by adenine and thymine combined by additional functionalization with a metal binding moiety [37] (Fig. **6**, down, comp. **10**). The intention was to bring functional metal complex in proximity to the oligonucleotide backbone by use of DNA recognition unit. Indeed conjugate retained DNA binding properties and simultaneously could bind zinc.

Des-N-tetramethyl triostine A derivatives (TANDEM) were functionalized by the introduction of four nucleobases, ATTA and TTTT to give derivative **11** [38] (Fig. **7**). The TTTT self-stacking and self-aggregation differed from ATTA derivative. While TTTT lacked any DNA binding, interestingly, ATTA aza-TANDEM derivative showed recognition of ds-DNA complementary sequence. The tetra-nucleobase conjugates showed low cytotoxicity, thus having the potential for development as DNA-selective probes *in vitro*.

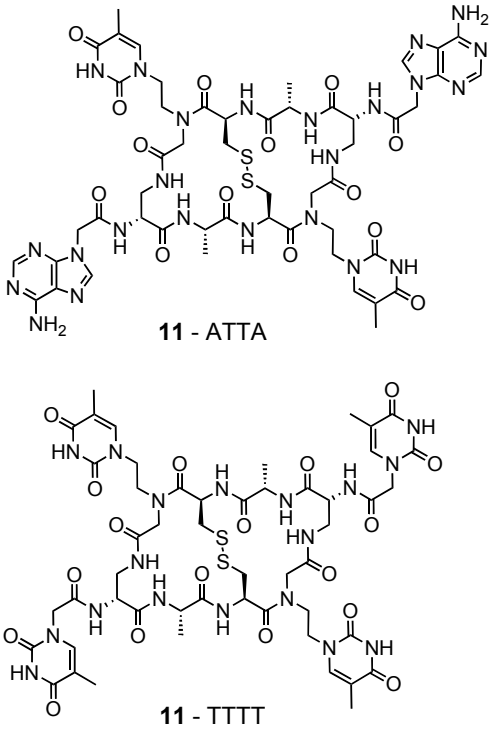

Fig. (7). ATTA and TTTT derivatives of TANDEM [38].

Above mentioned DNA-intercalation ability of quinoxaline [39] was also combined with a triazole-thymidine capacity for human thymine kinases inhibition [40]. Namely, thymidine kinases (TKs) had elevated expressions in cancer cells, thus TKs had been considered as a target for anticancer therapeutic. Thus, 3'-deoxythymidine phenylquinoxaline conjugate was prepared (dT-QX, **12**, Fig. **8**) [41] and *in vitro* study revealed selective cytotoxicity of conjugate dT-QX toward various cancer cells, while remaining low cytotoxicity for normal hepatocytes. Further, *in vivo* study on mice model also showed significant inhibition of tumor growth. dT-QX cytotoxicity in tumor cells was attributed to selective inhibition of DNA synthesis, yielding an extensive mitochondrial superoxide stress.

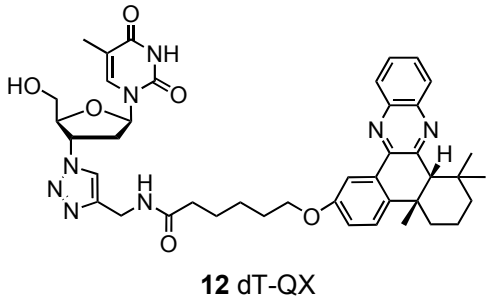

Fig. (8). 3'-Deoxythymidine phenylquinoxaline conjugate.

2.2. DNA/RNA Binder Linked to Nucleobase by Inert Linker

So far, linkers between two or more nucleobases or between DNA binder (intercalator) and nucleobase were also DNA-active, either due to positive charges (oligo amines, guanines, Figs. **2-4**) or due to H-bonding pattern within RNA groove (Fig. **5**) or DNA grooves (cyclic peptides, Figs. **6-7**).

The question arose whether recognition could be achieved also by application of DNA-inert linker; like for instance simple aliphatic chain. Actually, among the very first intercalator-nucleobase conjugates were ethidium-thymine conjugate **13** and ellipticine-thymine conjugate **14** [42, 43]; both containing short, inert aliphatic linker. One of them (ethidium analogue) showed distinct recognition of single base A-bulge in TAR sequence *(trans-activating responsive region)* of RNA HIV-1 virus (Fig. **9**). Since the three-base bulge in TAR region is of great importance as a biological target because Tat (*trans-activating transduction protein*) binds to this sequence, which influences gene expression in HIV-1 [44, 45], thus, already one of the first series of nucleobase-intercalator conjugates showed potential for anti-viral drugs.

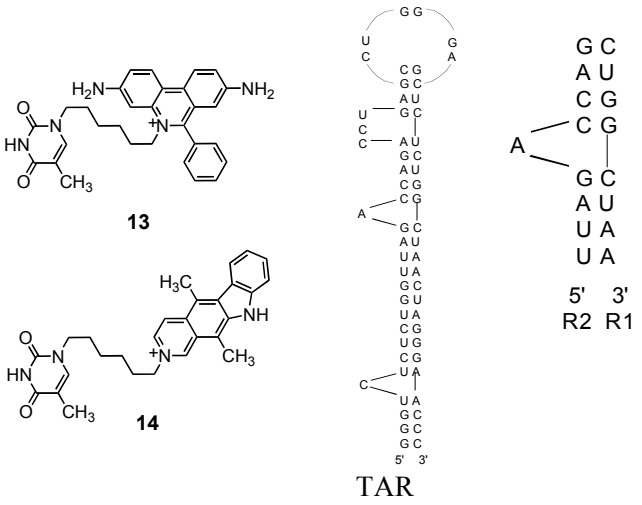

Fig. (9). Ethidium-thymine conjugate **13**, ellipticine-thymine conjugate **14** and their biological targets: TAR RNA sequence HIV-1 and RNA model system R2R3.

Ellipticine-thymine conjugate **14** didn't show improved stabilization of model system R2R3 compared to ellipticine. On the other hand, ethidium-thymine conjugate **13** showed significantly better stabilization than ethidium bromide and thymine, indicating that conjugates are able to interact specifically with bulged-base structures in control regions of RNA viruses. Intriguingly, ellipticine is of similar size and charge as ethidium, but only latter conjugate (**13**) showed recognition, thus suggesting that fine interplay of bonding and steric interactions is essential for the recognition.

Inspired by the above-mentioned efficiency of ethidium (phenanthridinium) derivative **13**, numerous phenanthridine-nucleobase conjugates were prepared to study in more detail the impact of steric properties of inert linkers on various DNA/RNA sequences recognition. Systematic variations were introduced regarding length and rigidity of the linker between phenanthridine and nucleobase, number and type of nucleobases (adenine or uracil), as well as number of phenanthridine units (one or two) [46]. In addition, reversible protonation of phenanthridine nitrogen was used to selectively switch-on (at pH 5) and in some cases switch-off (pH 7) the binding of the conjugate to DNA/RNA. This pH regulation of the DNA/RNA affinity is particularly interesting for tumor tissue and cell uptake, since extracellular pH of many solid tumors was shown to be significantly lower than its intracellular environment [47]. Likewise, several antitumor drugs owed their preferential accumulation in tumor tissue to this weakly acidic *pKa* value [48].

Further on, very simple phenanthridine-adenine conjugates **15** exhibited two orders of magnitude higher affinity toward complementary ss-RNA (poly rU) in comparison to analogous uracil conjugates [49]. It should be stressed that **15** with longer linker (n=5) could in theory bis-intercalate into poly rU, but short linker analogue (**15**, n=3) cannot, thus strong selectivity can only be attributed to synergistic effect of intercalation and adenine-uracil H-bonding.

Moreover, bis-nucleobase phenanthridines (Fig. **10**, **16**) were prepared with the idea of increasing selectivity by two base-pairing events adjacent to phenanthridine intercalation.

However, bis-adenine analogue did not show any selectivity toward poly rU, probably due to excessive intra- and/or intermolecular stacking, which competed to RNA binding [50]. Nevertheless, due to a smaller aromatic surface of uracil, bis-uracil conjugate **16** did not exhibit such strong self-folding. Accordingly, **16** showed a significant selective recognition of consecutive poly-adenine sequences (*i.e.* poly dA–poly dT, poly rAH^+–poly rAH^+ and poly rA-poly rU), owing to the specific interactions of uracils in **16** with adenine in the polynucleotide. Namely, both tethered uracils were located in the same groove and one of them was involved in additional hydrogen bonding interactions with adjacent adenine, while phenanthridinium was partially intercalated Fig. **10**, down) [50].

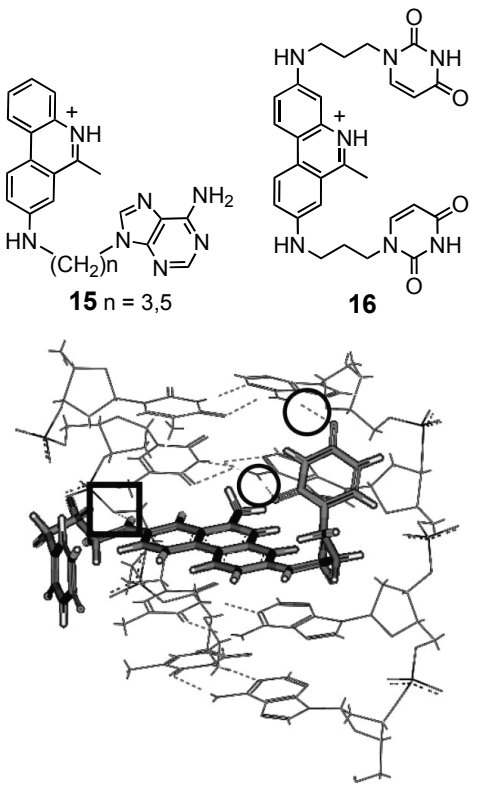

Fig. (10). Phenanthridine-nucleobase conjugates (up); the optimized structure of poly dA-poly dT – **16** intercalative complex. Hydrogen bonds between uracil 2,4-carbonyl groups and amino groups of consecutive adenines are indicated by circles, while interaction of the phenanthridinium-8-amino group with the sugar 5'-OH oxygen of the DNA backbone is indicated by a square (down) [50] - Reproduced by permission of The Royal Society of Chemistry.

Pyrene was used as large, planar intercalating building-block for new conjugates; its fluorescence was sensitive to the environment that made it a good candidate for fluorescent DNA probes [51]. Adenine or thymine was linked to pyrene by a short linker while alteration of hydroxyl or carbonyl functionalities was used to control flexibility of conjugates **17** and **18** (Fig. **11**) [52]. Both adenine conjugates showed selectivity for binding to the complementary oligonucleotide T_{10}, where more flexible **17b** with sp^3-hybridized C-atom vicinal to pyrene moiety showed more efficient binding. **17b** also bound efficiently to the double-stranded $(dA\text{-}T)_{10}$ template. Further, both adenine and thymine conjugates with hydroxyl linker (**17b** and **18b**, respectively) produced granular staining pattern revealed by confocal microscopy and accumulated in mitochondria of HeLa cells, whereas adenine compound **17b** was also localized in nucleoli.

Next, bis-phenanthridine – nucleobase conjugates were prepared, inspired by high DNA or RNA affinity of previously studied bis-intercalands [4, 5], with the idea that attached nucleobase would add selectivity toward complementary DNA or RNA sequence. Uracil-conjugate **22a** surprisingly showed high affinity toward ss-RNA poly rU, which cannot be attributed to uracil-uracil H-bonding but rather more likely to the bis-intercalation of phenanthridine-subunits into poly rU. Also, **22a** showed selectivity for poly rAH^+–poly rAH^+, which was attributed to the significantly less structural change of ds poly rAH^+–poly rAH^+ upon binding of rigid conjugate **22a** in comparison to the impact of flexible conjugate **22b** (Fig. **12**), and confirmed by molecular dynamics (MD) simulations [53].

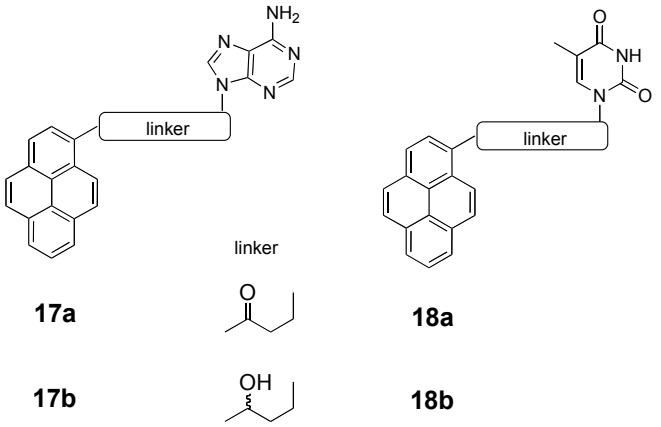

Fig. (11). Pyrene-adenine and pyrene-thymine conjugates.

Finally, bis-phenanthridine-bis-nucleobase derivatives were prepared (*e.g.* **21b**, Fig. **12**) and compared with mono-nucleobase analogues (*e.g.* **21a**). The bis-phenanthridinium–adenine conjugate **21a** revealed a fluorescent recognition of alternating AT-DNA with respect to other ds-DNA/RNA and ss-RNA; while bis-phenanthridine-bis-adenine **21b** didn't show such selective fluorescence response on various DNA/RNA, but gave highly selective induced (I)CD spectra pattern strongly dependent on polynucleotide secondary structure [54].

So far, preparation of various series of aryl-nucleobase conjugates included very laborious synthetic procedures, whereby the structural modification required several synthetic steps. Thus, to simplify the design and preparation of novel generations, the peptide linkers were introduced instead of aliphatic ones. For instance, peptide-bridged phenanthridine-thymine conjugates (Fig. **12**) [55] were prepared as analogues of previously reported aliphatic derivatives (Fig. **10**, Fig. **12**, **22a,b**). However, peptide-derivatives did not show recognition pattern of aliphatic analogues. Instead, peptide-bridged phenanthridine-thymine conjugates showed similar spectroscopic results as phenanthridine

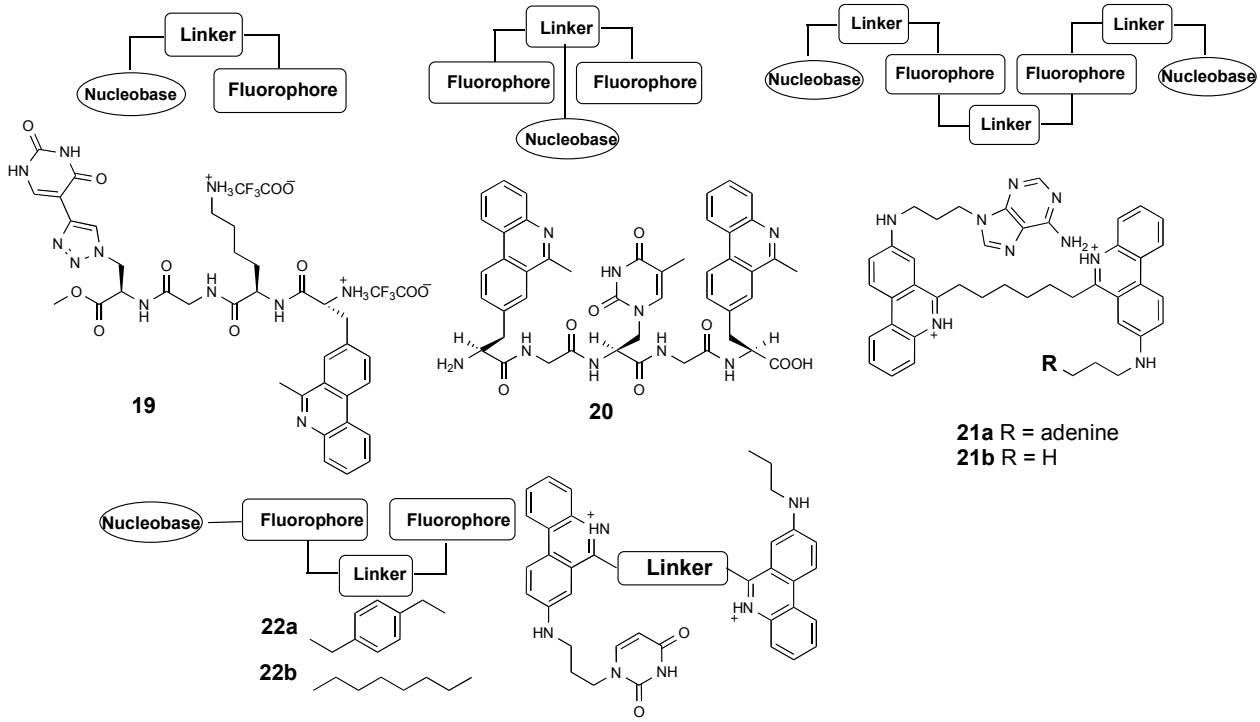

Fig. (12). Complex nucleobase-bis-fluorophore systems (and related mono-fluorophore): Peptide-linked phenanthridine-pyrimidine conjugates [55-57] (**19, 20**); Bisphenathridinium-adenine conjugates [53] (**21a,b**); Bisphenathridinium-uracil conjugates [53] (**22a,b**).

amino-acids [56], thus suggesting that intercalation into ds-DNA (dominant binding mode) was not significantly influenced by attached thymine and the peptide backbone.

The stacked phenanthridine-thymine-phenanthridine model **20** (Fig. **12**) revealed characteristic phenanthridine-excimer fluorescence emission and very specific CD spectrum: a novel property, not previously observed in bis-phenanthridine-nucleobase analogues (**21a**, Fig. **12**). Obviously, due to intrinsic chirality, peptide backbone of **20** (which **21a** does not have) oriented aryl-units attached to amino-acid side chains significantly better, thus yielding chiral aromatic stacking system with the efficient formation of fluorescent excimer.

At variance to the most of phenanthridine derivatives, peptidic phenanthridine-thymine conjugates exhibited negligible antiproliferative activity on human cell lines and thus, present potential selective blockers of the repair of abasic DNA sites (Fig. **1**), caused by antitumor therapy.

Recently, peptidomimetic **19** that comprised phenanthridinyl-L-alanine, triazolyluracilyl-L-alanine, lysine and glycine demonstrated binding to poly rA-poly rU with micromolar affinity and selective fluorescence response (Fig. **12**) [57]. Again, parallelly prepared and tested phenanthridine-aliphatic linker-uracil analogues showed inferior interactions with DNA/RNA.

Till now, aryl-moieties in aryl-nucleobase conjugates were built of 2-3 condensed aromatic units. Intended increase in aryl-binding to DNA/RNA was achieved by preparation of bis-aryl analogues (Fig. **12**: bis-phenanthridines, Fig. **6**: bis-quinoxalines). However, the surface of aryl-moiety itself can be doubled. With this aim, different porphyrin – nucleobase/nucleoside derivatives were designed and prepared [58-60] (Fig. **13** up). The intention was to employ large DNA-binding affinity and selectivity of porphyrin (intercalation in the GC-rich regions and external binding in AT-rich regions). In addition, porphyrin was able to act as telomerase inhibitor upon binding to telomeric DNA and consequently influenced the lifetime of tumor cells [61, 62].

Also, an increase of selectivity by attaching nucleobases to porphyrin was expected. However, the porphyrin-adenine or -thymine conjugates (Fig. **13**) did not show stronger binding to complementary single-stranded polynucleotides in respect to non-complementary. The reason for such behavior is attributed to the dominant affinity of large porphyrin. However, porphyrin-adenine **23** did show different spectroscopic response upon binding to complementary poly

rU, which was attributed to the influence of specific interactions of adenine and uracil on a positioning of the chromophore (porphyrin) within the complex **23**/poly rU [63]. Among nucleobase-porphyrin conjugates bridged by the short linker, guanine-conjugate (Fig. **13**, compound **24**) showed stronger intercalating ability as well as stronger cleavage efficiency than other conjugates or porphyrin analogue without nucleobase [64].

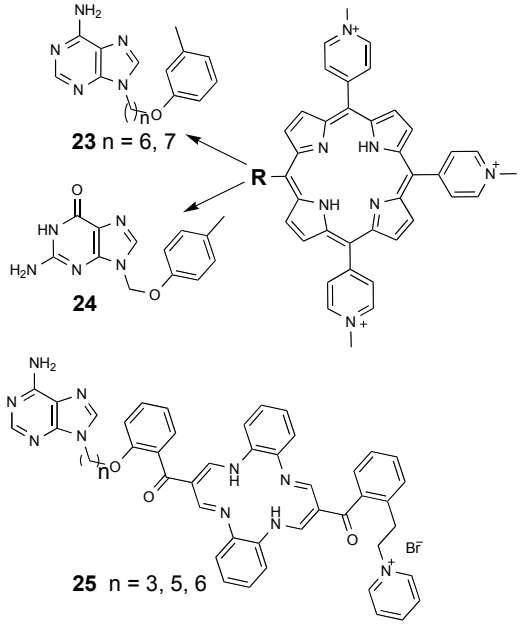

Fig. (13). Porphyrine-nucleobase conjugates **23, 24** [63,64] and DBTAA–adenine conjugates **25** [66].

Another large aromatic moiety, the dibenzotetraaza[14]annulene (DBTAA, Fig. **13** down) is known DNA/RNA ligand structurally related to porphyrin. The efficiency of the anti-proliferative effect of DBTAA derivatives on human cell lines was proportional to their ds-DNA/RNA affinities [65], which made DBTAA attractive building block for new bioactive molecules. The DBTAA-adenine conjugate with the shortest flexible propyl bridge allowing self-stacking of aromatic units showed significant selectivity upon binding to complementary polynucleotides. Very intriguingly, DBTAA–propyl–adenine conjugate showed specific recognition of the consecutive oligo dT sequence, both by increased affinity and specific induced chiro-optical signal, in comparison to other homogenous ss RNA and DNA [66]. This unique selectivity is preserved even in the presence of large excess of oligo rU and explained by intramolecular self-stacking of conjugate's aromatic sub-units, hydrogen bonding between adenine (conjugate) and thymine (oligo dT) and additional interaction of pyridyl (conjugate) and thymine methyl (oligo dT). The complete inactivity of close analogues (Fig. **13**; n=5 or n=6) successfully demonstrated the importance of the linker on recognition process. Moreover, DBTAA–propyl–adenine conjugate showed specific recognition of homogeneous AT-DNA sequence by an ICD band pattern, which was not observed for other ds-DNA/RNA [67].

2.3. Nucleobase Conjugates with Atypical DNA/RNA Binding Systems

Antibiotic pleuromutilin bound to ribosomal RNA by hydrogen-bonding network and hydrophobic contacts [68, 69] in the peptidyltransferase center (PTC), thereby inhibiting peptide bond formation by hindering a correct location of the tRNA. Pleuromutilin was conjugated by a click chemistry protocol to nucleobases adenine, uracil or thymine and nucleoside uridine by different linkers to improve selectivity and stacking properties (14) [70, 71]. Molecular modelling and chemical footprinting of nucleotide U2506 were used to examine the binding of conjugates to Escherichia coli ribosome, where docking results were in good agreement with the footprinting results. Docking analysis indicated stacking interaction between triazoles and nucleotide U2506. Further on, footprinting of nucleotide U2585 showed that all examined conjugates interact with above-mentioned position, which was otherwise unaffected by pleuromutilin itself. Although variation in affinity was relatively low, pleuromutilin-adenine conjugates (*e.g.* conjugate **26**, Fig. **14**showed higher binding affinity than thymine conjugates, and both groups were superior to a simple phenyl group. Conformational restriction of the linker contributed to conjugate binding affinity. Furthermore, compounds showed antibacterial activity with three different bacterial strains, namely drug-hypersensitive *E. coli* AS19 (Gram-negative), *Bacillus subtilis* 168 (Gram-positive), and *Listeria innocua* (Gram-positive).

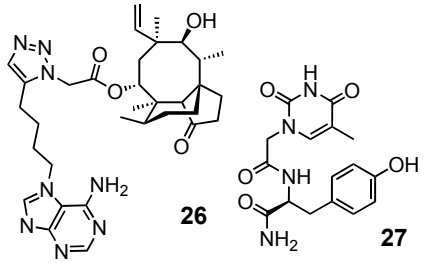

Fig. (14). Pleuromutilin-adenine conjugate **26** and thymine-tyrosine conjugate **27** [72].

Naturally occurring aromatic amino acids are not considered typical DNA/RNA intercalators but in some specific cases, they can at least partially intercalate.

Conjugate of amino acid tyrosine and nucleobase thymine (TyrT **27**, Fig. **14**) indeed interacted with ss-poly rA [72]. CD measurement showed no binding of TyrT to ds-DNA, while CD spectra of poly rA changed upon complexation of TyrT, whereby molecular modelling studies of poly rA-TyrT complex suggested intercalation of TyrT thymine between two adenines of poly rA. Further, scanning electron microscopy (SEM) enabled visualization of TyrT samples morphology in the presence of poly rA. Such highly selective binding to a poly-adenine sequence of RNA, which has distinct biological and biochemical implications, encouraged further studies of TyrT **27** and its close analogues.

2.4. Metal Complexing Ligands as DNA/RNA Binders Conjugated with Nucleobase

Kimura and his group prepared cyclen–Zn(II) complexes, as well as acridine or anthraquinone-cyclen–Zn(II) complexes that bound thymine and uracil, and also analogues having imide group [73-75]. Conjugates of adenine and cyclen-Zn(II) complex were synthesized in order to combine cyclen and adenine properties that resulted in high selectivity and binding affinity for uracil and uridine [76]. Next modification of cyclen-Zn(II) complex was the introduction of uracil linked by a phenylene-dimethylene bridge (compound **28**, Fig. **15**). The ability of conjugate-Zn(II) complex to act as artificial nuclease was examined and it was found that conjugates dramatically accelerate the plasmid DNA cleavage. That was explained by the generation of the stable intra- and intermolecular complex formed by binding of the metal ion to the nitrogen atom of uracil moiety [77]. Cyclen unit was also used for the design of uracil-cyclen-dipeptide conjugate **29**, where uracil could recognize adenine combined with cyclen-DNA, with high binding affinity. Mononuclear Cu(II) complex **29b** showed high catalytic activity in the DNA cleavage process that occurred through hydrolytic pathway [78].

The biological applications of ferrocene benefited from two unique properties associated with this complex: its sandwich structure [79-82] and reversible one-electron oxidation [83]. The redox activity of ferrocenes had two major implications in biology. The first referred to the design of electrochemical biosensors [84] and the second pertained to its redox-related mechanisms for the anticancer activity of ferrocenyl derivatives. The ferrocenyl-nucleobase systems prepared in the 1990s (*e.g.* **32,** Fig. **16**) interacted with DNA in solution by electrostatic interactions, while in the case of immobilization of DNA onto an electrode surface, both the electrostatic and non-electrostatic contributions were observed [85]. The recent increase in interest for the synthesis of ferrocene-nucleobase conjugates was stimulated by the numerous biological applications of this class of organometallic nucleobase derivatives [86]. Ferrocenyl-nucleobases of general formula **30** (Fig. **16**) revealed great potential as antioxidants [87], electrochemical DNA sensors [88], or as drugs for various targets. For example, artificial nucleoside **31** in which the iron-containing ferrocenyl moiety mimicked the ribose fragment of the natural nucleoside, showed significant *in vivo* antitumor activity and also synergistic effect with an anticancer drug cyclophosphamide [89].

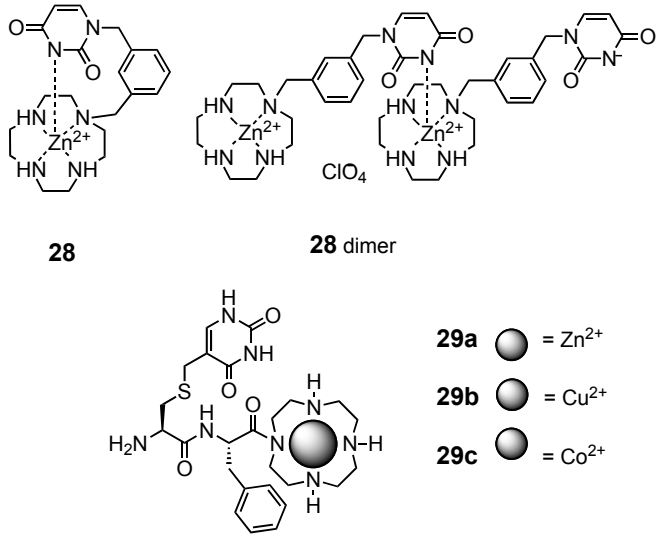

Fig. (15). Cyclen-nucleobase conjugates [76-78].

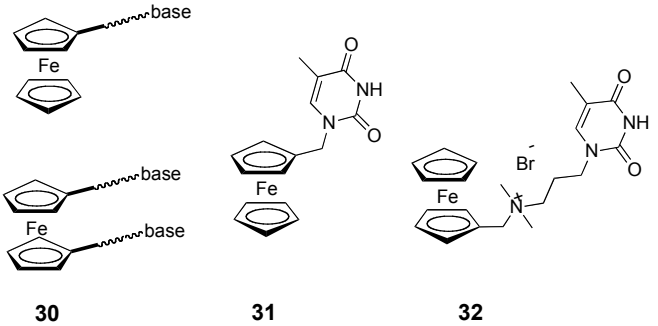

Fig. (16). The general formula of ferrocenyl-nucleobase derivatives and examples of organometallic nucleobases.

Some recent biological studies demonstrated promising anti-trypanosomal and cytotoxic activity of half-sandwich cymantrene and cyrhetrene-nucleobase compounds as closely related ferrocenyl-nucleobase congeners. Moreover, the compounds synthesized in this study were amongst the most potent anti-trypanosomal organometallics described to date [90]. Concerning the latter, the combination of the redox-active, unnatural

ferrocenyl group with biogenic nucleobases could facilitate the development of the novel therapeutic agents, xeno nucleic acids, electrochemical sensors and self-assembled molecular materials.

3. BIOLOGICAL AND BIOMEDICAL ASPECTS AND CONCLUSIONS

Nucleobases are very attractive building blocks for the design of new hybrid, multifunctional ligands for DNA/RNA binding and selective recognition. Inspired by nature, such multifunctional ligands can provide selective recognition of specific regions, secondary structures and structural motifs by Watson-Crick and non-canonical hydrogen bonding and even more, can simultaneously target DNA- or RNA- related proteins.

Results presented herein, based on a particular design of a small molecule or DNA/RNA binder-nucleobase conjugate, showed a number of different approaches for the recognition of various DNA or RNA sequences. Huge variety of structures and ligands connected to nucleobases enables different functionalities and therapeutic potential of this group of hybrid compounds.

Some of the results presented here elucidated in detail the mechanism of selective or specific recognition of particular DNA or RNA by small molecule but did not follow-up into *in vitro* or *in vivo* experiments. However, even on this level, presented results addressed DNA/RNA sequences of high interest.

For instance, the recognition of poly rA sequence [18, 50, 53, 72] could have considerable biological or biochemical importance: *e.g.* most eukaryotic mRNAs had poly rA sequence at their 3′-termini. This region was of great significance for nuclear export, translation, and stability of mRNA, by protecting it from enzymatic degradation in the cytoplasm. [91,92] Consequently, poly rA region was recognized as a potential target for anticancer biomedical approaches [93]. Therefore small molecules that were able to bind either to ss- poly(A) tail or the double helical poly rAH^+-poly rAH^+ could strongly influence the RNA function. Moreover, single base A-bulge in TAR sequence *(trans-activating responsive region)* of RNA HIV-1 virus (Fig. **9**) is also a target of the highest biomedical interest.

Moreover, thymine sequences, which have been widely explored as a part of diverse research lines, are addressed here mostly as thymine-related DNA-abasic (AP) sites (Fig. **2**) and through related biochemical or biomedical applications for detection and blocking of AP. However, to these issues is also related the highly selective recognition of longer oligo-thymine sequences (**25**, Fig. **13**). Corresponding single stranded uracil – sequences are part of important RNA-loops or bulges (*e.g.* in TAR sequence of RNA HIV-1 virus uracil is present both in a bulge and hairpin loop), thus recognition by small molecule offers prospects of both, spectrophotometric detection and affinity blocking of these positions [49,63,76].

Aside simple recognition, some of conjugates induced significant and selective damage in DNA or RNA structure (*e.g.* DNA/RNA cleavage; [13-15,21,77,78], thus acting as artificial nucleases, which makes them perspective agents for selective biochemical degradation and maybe applicable to biomedical implications. For instance, the development of agents capable of cleaving RNA and DNA has attracted considerable attention of researchers in the last few years, because of their application in biotechnology and pharmacology. Artificial nucleases are designed to imitate the active centers of natural enzymes by simple structures possessing minimal sets of the most important characteristics that are essential for catalysis. Being less efficient and specific than natural enzymes, the primitive mimics are smaller, more robust and able to function in a broad range of conditions [94].

Even more interesting, for some ligands (Fig. **2**) DNA/RNA damage could be controlled by outer stimuli – pH, offering intriguing applications in antitumor therapy related to druggable higher acidity of tumor tissues in respect to normal ones [47, 48]. Namely, the microenvironment within solid tumors is slightly acidic, and manipulation of this extracellular acidity to cause intracellular acidification might be used to increase selective antitumor effects of some anticancer drugs. Potential mechanisms include inhibition of repair of DNA damage and inhibition of repopulation of tumor cells between successive courses of chemotherapy. For instance, decrease of intracellular pH enhanced activity of well-known anticancer drug against human cancer cells [95].

Furthermore, pH-dependence of here presented compounds (Figs. **2, 10, 12**) have even more pronounced potential for either druggability or *in vitro*/*in vivo* sensing of particular targets, since absorption, distribution, metabolism, excretion and toxicity (ADMET) are profoundly affected by the charge state of compounds under varying pH conditions [96].

Several groups of conjugates also showed applicable *in vitro* activity. Not surprisingly, classical intercalators like acridine of phenylquinoxaline showed pronounced cytotoxicity, but due to the nucleobase at-

tachment the conjugates were significantly more toxic to tumor cells in comparison to normal human cells [16,41] and in some cases also had synergistic effect if applied with established anticancer drugs [16] namely: anticancer drug BCNU (bis-chloroethylnitrosourea) an on murine leukaemia L1210 and human adenocarcinoma A549 cell lines. The most active group of compounds are metalo-complex-nucleobase conjugates, which exhibited anticancer and antibacterial action due to high cytotoxicity [97] and antibacterial activity against Gram-positive strains of *Staphylococcus aureus*, including methicillin-resistant and vancomycin-resistant strains [102]. Moreover, they have also shown anti-trypanosomal activity comparable to the currently applied drugs [90].

The *in vivo* activity of here presented conjugates was, as usual, much less studied. Some of promising cytotoxic agents lost their activity in living systems [16]. However, some classical intercalator-nucleobase conjugates extended their *in vitro* activity to *in vivo* conditions on animals [41]. Aside, intercalator-conjugates, also ferrocenyl-nucleoside showed significant *in vivo* antitumor activity and also synergistic effect with an anticancer drug cyclophosphamide [89]. Nevertheless, at this point it is not clear whether *in vivo* effect on solid tumors was result of only intercalator action or attached nucleobase has some positive contribution; thus more comparative *in vivo* experiments are needed.

Prospects: Taking into account the showed bioactivity and also the derivatization advantages, two groups of here presented conjugates are the post promising leads in the future research. One group, metalo-complex-nucleobase conjugates, already showed even *in vivo* intriguing activity; can be easily modified by changing vast number of different ligands around coordinated metal, as well as broad choice of bioactive metal cations, thus allowing easy preparation of large libraries. Another group are peptide-based conjugates, whereby peptide-part actively contributes to bioactivity – in this aspect only few bioactive peptides coupled with nucleobase already showed promising results, thus supporting further systematic research, which due to easy and fast peptide synthesis can also cover large structural diversity.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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