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Research paper

Perylene and coronene derivatives binding to G-rich promoter oncogene sequences efficiently reduce their expression in cancer cells

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ABSTRACT

A novel approach to cancer therapeutics is emerging in the field of G-quadruplex (G4) ligands, small molecules designed to stabilize four-stranded structures that can form at telomeres as well as in other genomic sequences, including oncogene promoter sequences, 5′-UTR regions and introns. In this study, we investigated the binding activity of perylene and coronene derivatives PPL3C, CORON and EMICORON to G4 structures formed within the promoter regions of two important cancer-related genes, c-MYC and BCL-2, and their biochemical effects on gene and protein expression. In order to fully characterize the ability of the selected ligands to bind and stabilize the G4 structures originated by the c-MYC and BCL-2 promoter sequences, we performed electrospray ionization mass spectrometry (ESI-MS), Fluorescence Resonance Energy Transfer (FRET) measurements, Circular Dichroism (CD) spectra and polymerase stop assay. Altogether our results showed that the ligands had a high capacity in binding and stabilizing the G4 structures within the c-MYC and BCL-2 promoter sequences *in vitro*. Notably, when we evaluated by quantitative real-time PCR and western blotting analysis, the effects of treatment with the different G4 ligands on c-MYC and BCL2 expression in a human melanoma cell line, EMICORON appeared the most effective compound in reducing the mRNA and protein levels of both genes. These results encourage to consider EMICORON as a promising example of multimodal class of an antineoplastic drug, affecting different tumor crucial pathways simultaneously: telomere maintenance (as previously described), cell proliferation and apoptosis via down-regulation of both c-MYC and BCL-2 (this paper).

1. Introduction

G-quadruplexes are a family of very stable DNA secondary structures, derived by the folding of sequences containing four runs of 3–4 consecutive guanines (G) in a four-stranded helical structure, stabilized by Hoogsteen hydrogen bonds between four Gs (G tetrads) that stack on top of each other [1]. Recently, G-quadruplexes raised great interest in their possible biological role [2]. At first, G-quadruplex (G4) structures were proposed at telomeres, composed of G-rich repeated sequences, $(TTAGGG)_n$ in mammals. The observation that telomerase, the enzymatic complex that adds telomeric repeats at the end of chromosomes, is up-regulated in 80–90% of cancers [3], led to the study of G-quadruplex stabilizing ligands as possible telomerase inhibitors [4]. However, it emerged that the G4-interacting agents are more than simple telomerase inhibitors and that their direct target is rather telomere than telomerase [5,6]. More recently, it became evident that telomeres are not the only possible targets for G-quadruplex

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ligands. A genome-wide bioinformatics analysis identified more than 300.000 potential G-quadruplex forming sequences (PQS) in the human genome [7]. PQS are not randomly distributed: they are enriched in promoter regions, in 5′ UTR and introns. Interest in the more general therapeutic significance of G4 has expanded during the past decade to include G4 structures in the promoters of a wide range of genes important in cell signaling, recognized as hallmarks of cancer: c-MYC, c-KIT and KRAS (self-sufficiency), pRB (insensitivity), BCL-2 (evasion of apoptosis), VEGF-A, and VEGFR (angiogenesis), hTERT (limitless replication), and PDGF-A (metastasis) [7,8].

In the field of G-quadruplex interactive compounds [9], we have synthesized and studied many perylene [10,11] and coronene [12] derivatives over the years, focusing our attention mainly on their effects on telomeric sequences. More recently, we found that the presence of at least one piperidine ring on perylene bay-area greatly enhances G-quadruplex versus duplex binding selectivity of these compounds, leading to selective telomere damage and consequent antiproliferative effects on cancer cells, as in the case of PPL3C [13]. Lately, we showed that, combining this structural feature with a wider aromatic core, the “chimera” molecule EMICORON (half coronene and half bay-substituted perylene) was observed to be the most efficient G-quadruplex ligand and the most selective with respect to duplex DNA of all the previously synthesized compounds of these series [14]. For these reasons, we decided to extend the study of this compound together with previously reported telomeric ligands and telomerase inhibitors PPL3C and CORON (Fig. 1) to their possible effects on the G-rich sequences found in oncogene promoters.

Here, we focused on c-MYC and BCL-2, two of the more widely overexpressed proto-oncogenes in human tumors [15,16], and whose promoters contain well-characterized GC-rich elements capable of forming stable G-quadruplexes *in vitro* [17,18]. Aberrant high levels of c-MYC protein have been shown to cause tumorigenesis by promoting unrestrained cellular proliferation, blocking differentiation and by inducing genomic destabilization [19]. BCL-2 encodes a protein that inhibits both apoptosis and the autophagic-associated cell death and it is also involved in physiological DNA repair [20]. The up-regulation of BCL-2 was found to mediate the resistance of cancers to conventional chemotherapeutic agents and radiotherapy and was associated with poor prognosis [21]. Hence, finding novel approaches to directly target c-MYC and/or BCL-2 gene expression could be relevant from a therapeutic point of view in killing cancer cells resistant to traditional chemotherapeutics due to overexpression of both oncogenes [22].

2. Material and methods

2.1. Oligonucleotides and DNA

DNA oligonucleotides were purchased from MWG-Biotech (Ebersberg, Germany). The sequences of the oligonucleotides are shown in Table 1.

2.2. ESI-MS (electrospray ionization mass spectrometry)

Instrumentation: All the experiments were performed on a Q-TOF MICRO spectrometer (Micromass, now Waters, Manchester, UK) equipped with an ESI source, in the negative ionization mode. Full scan MS spectra were recorded in the *m/z* range between 800 and 2,500, with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Sample preparation protocol: The oligonucleotides bcl2-G4 and myc-G4 were dissolved in bi-distilled water to obtain the starting stock solutions and were annealed in 150 mM ammonium acetate buffer by heating at 90 °C for 10 min and then cooling to room temperature slowly. The final concentration of oligonucleotides stocks was 50 μM in quadruplex units. Samples were prepared by mixing appropriate volumes of 150 mM ammonium acetate buffer, 50 μM annealed oligonucleotide stock solution, ligands 100 μM stock solutions and methanol. The final concentration of DNA in each sample was 5 μM (in quadruplex unit) and the final volume of the sample was 50 μL. Drugs were added at 1:1 drug/DNA ratios, ranging between 0.5 and 4.

Data analysis: Binding constants (K_1 and K_2) and percentage of bound DNA were calculated according to previously reported formulae [23], considering drug-DNA complexes in 1:1 and 2:1 stoichiometry, which were proven to be the main species present in solution in all the experiments. The association constants K1 and K2 can be calculated directly from the relative intensities of the corresponding peaks found in the mass spectra, with the assumption that the response factors of the oligonucleotides alone and of the drug-DNA complexes are the same. The constants were determined at different drug/DNA ratios, depending on the intensity of

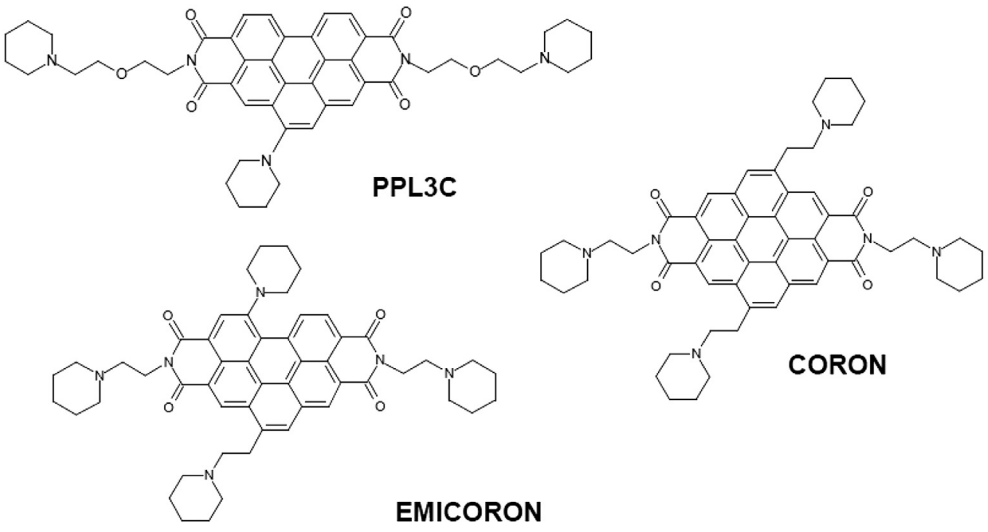

Fig. 1. Chemical structures of the studied perylene and coronene derivatives.