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Benzo(*h*)quinoline derivatives as G-quadruplex binding agents

Hanumantharao Paritala, Steven M. Firestine *

Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI, USA

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ABSTRACT

G-quadruplexes are unusual structures formed from guanine-rich sequences of nucleic acids. G-quadruplexes have been postulated to play important roles in a number of biological systems including gene regulation and the inhibition of enzyme function. Recently, our laboratory reported on the synthesis and evaluation of a triaza-cyclopentaphenanthrene compound which bound to G-quadruplexes with good affinity and selectivity. This compound contains a 4-pyridone group which has not been previously utilized in other quadruplex binding agents. In this Letter, we describe the synthesis and evaluation of 4-pyridone containing 2- and 3-carboxy-benzoquinolines as G-quadruplex binding agents. We find that these compounds are capable of binding G-quadruplexes with a K_a in the range of $3 \times 10^5\ M^{-1}$ and with a 10-fold selectivity for quadruplex over duplex DNA.

G-quadruplexes are four stranded nucleic acid structures formed from guanine-rich sequences in which sets of four guanine bases interact with each other via hydrogen bonds.[1,2] Quadruplexes can be found in either DNA and RNA and can be formed both inter- and intramolecularly.[1] One of the earliest known examples of G-quadruplexes are the telomere sequences where quadruplexes are thought to play a role in maintenance of the chromosomal ends.[3] Since this time, putative quadruplex forming sequences have been found in well over 300,000 different places in the human genome[4] and biochemical studies have shown that quadruplexes can form from sequences derived from telomers,[5,6] the immunoglobulin switch regions,[7] the insulin linked polymorphic region,[8,9] and the c-myc oncogene.[10]

Small molecules that bind to quadruplexes have been extensively studied as potential anticancer agents and gene regulators.[11] Numerous chemical classes have been shown to possess quadruplex affinity with some examples being acridines,[12,13] cationic porphyrins,[14] ethidium bromide derivatives,[15] anthraquinones,[16] perylenes,[17] macrocyclic compounds,[18] and steroids.[19] Recently, our laboratory discovered that compound **1** (Fig. 1) was a selective G-quadruplex binding agent.[20] The compound possessed a K_{app} of $2.7 \times 10^4\ M^{-1}$ and bound approximately 20-fold better to quadruplex DNA than to dsDNA.[20] Compound **1** contains a 4-pyridone group which has not been previously utilized in G-quadruplex binding agents and thus we chose to investigate whether simple 4-pyridone derivatives would be selective quadruplex DNA binding agents. To examine this, we have prepared a number of 4-pyridone compounds and determined their ability to bind to G-quadruplex DNA.

To create simple analogs of **1**, we eliminated the *N*-methylpyrrole region of the molecule and converted the nitroquinoline into a naphthalene. After the eliminations described above, a benzo(*h*)quinolinone core remained (Fig. 2). To enhance quadruplex binding affinity, we anticipated that we would need to add either a cationic group, which could form favorable electrostatic interactions with the phosphate groups of the quadruplex, or aromatic groups which could enhance binding by forming additional π-stacking interactions with the guanine bases. To determine where these substituents should be added, molecule modeling was conducted on both the 2-carboxy and 3 carboxy derivatives

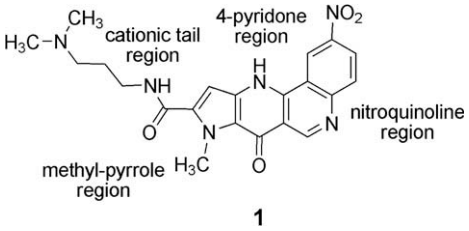

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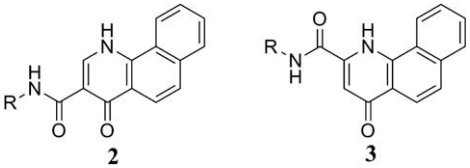
Figure 1. Triaza-cyclopenta(*b*)phenanthrene based quadruplex binding agent.



2 **3**

Figure 2. Targeted molecules: 3-carboxy-4-hydroxybenzo(*h*)quinoline derivatives (**2**) and 2-Carboxy-4-hydroxybenzo(*h*)quinoline derivatives (**3**).

* Corresponding author. Tel.: +1 313 577 0455; fax: +1 313 577 2033.
E-mail address: sfirestine@wayne.edu (S.M. Firestine).

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of the core as shown in Figure 2. These models (not shown) suggested that in both analogs the ring system could interact with the tetrad while placing the cationic tail into one of the grooves of the quadruplex. This binding orientation allowed for favorable hydrophobic interactions as well as good electrostatic interactions with the negatively charged phosphate groups located in the grooves.

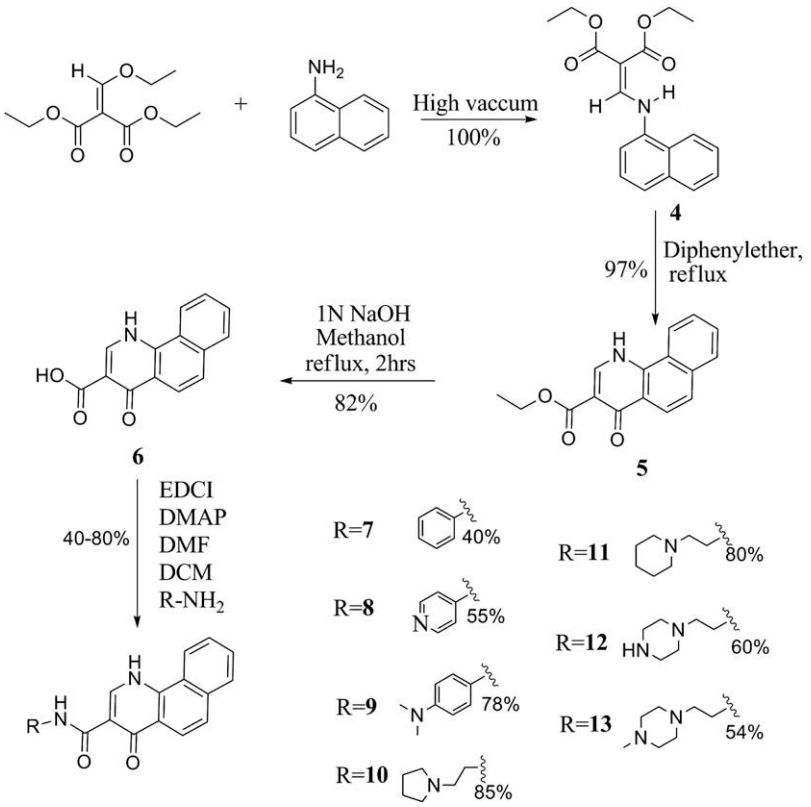
Synthesis of derivatives of **2** and **3** were accomplished according to the methodology outlined in Schemes 1 and 2. Synthesis of compounds **7–13** was done by amide bond formation from the common carboxylic acid, **6** (Scheme 1). Synthesis of **6** was accomplished from 1-napthylamine by slight modification of the reported method.[21] Reaction of 1-napthylamine with diethyl ethoxymalonate under vacuum resulted in the formation of **4**. We found that stirring this reaction under vacuum resulted in substantially higher yields in the reaction due to the efficient removal of ethanol. Cyclization of **4** to yield **5** was done by refluxing (240–250 °C) the starting material in diphenyl ether. Upon cooling, the desired material precipitated as a creamy solid which was collected and washed with petroleum ether. Hydrolysis of **5** in the presence of refluxing sodium hydroxide generated the acid in excellent yield. Synthesis of the derivatives was done using standard peptide coupling conditions under dry reaction conditions. Unreacted starting material was removed by a simple aqueous extraction and the desired product was obtained via silica gel chromatography. Overall, **7–13** were obtained in a 40–80% yield from **6**.

Synthesis of compounds **17–21** was accomplished using the common intermediate **16.**[22] Compound **14** was prepared by reaction of diethyl acetylenedicarboxylate with 1-napthylamine and then heated to reflux in diphenyl ether to afford cyclization to give **15**. Compound **15** was then treated with NaOH to provide **16** in good yields. Synthesis of compounds **17–21** was done using the same peptide coupling procedure described for **7–13**.

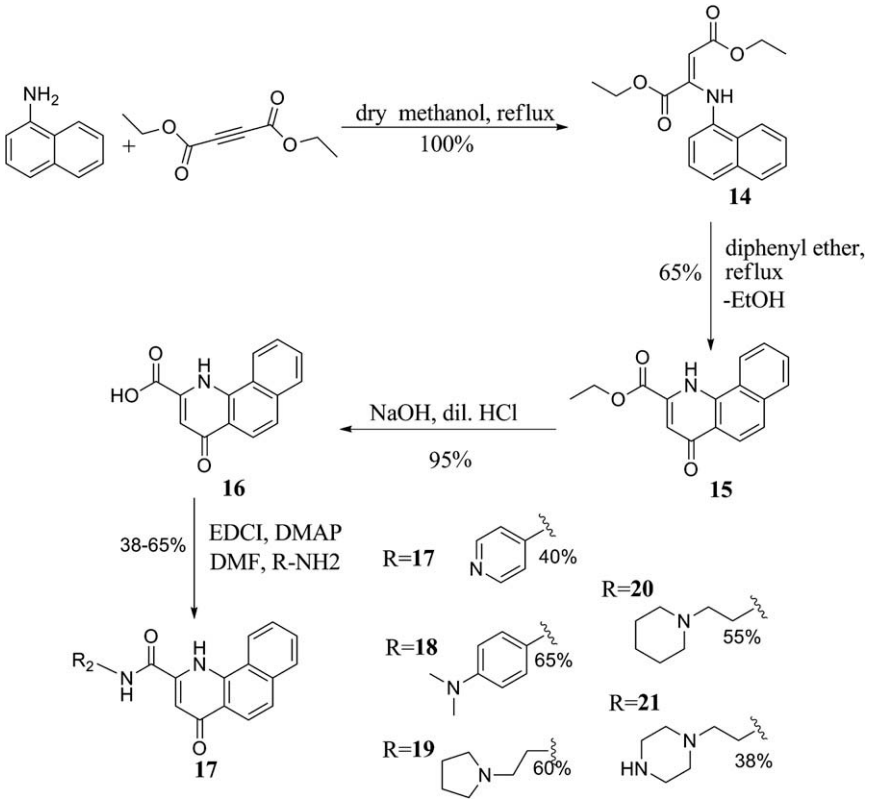
The synthesized compounds were evaluated for binding and selectivity to quadruplex DNA using a combination of fluorescence and SPR methods. We first conducted fluorescence melting studies using a commercially available G-quadruplex oligonucleotide (G-Quartet FRET Substrate, Calbiochem) containing a human telomeric repeat sequence.[23,24] The 5′ and 3′ ends of the oligonucleotide are labeled with a fluorescein–rhodamine FRET pair. Formation of a quadruplex results in energy transfer from the fluorescein to the rhodamine and upon heating, the quadruplex melts leading to a decrease in the FRET signal. If an agent binds to and stabilizes the quadruplex, an increase in melting temperature is observed.

FRET melting studies were conducted for all compounds at a concentration of 0.5 μM in 10 mM cacodylate, 100 mM NaCl buffer (pH 7.4). The concentration of GQ-FRET substrate was half that of the compound. Melting curves were determined by slowly heating each sample from 20 to 80 °C at a rate of 1 °C per minute while measuring the fluorescence at 518 nm at each temperature interval. During the assay, a two minute incubation time at each temperature interval was done prior to fluorescence measurement to ensure temperature equilibrium. The melting temperature (T_m) was determined using the first derivative of the obtained melting curves. As shown in Table 1, several of the compounds showed an increase in the melting temperature when compared to the oligonucleotide alone indicating binding to the quadruplex. Compounds **8**, **10**, **11** and **13** displayed the greatest changes in the T_m, although these were modest, ranging from 3.7 to 5.3 °C. All of these compounds are 3-substituted derivatives of the core hydroxybenzo(*h*)quinoline.

To gain additional information on quadruplex binding as well as to assess the quadruplex selectivity of these compounds, we utilized a fluorescence displacement assay.[25–27] This assay has been extensively used to measure binding of small molecules to DNA.[25–30] In this assay, the binding of the test compound is mon-



Scheme 1.



Scheme 2.

Table 1
FRET melting and fluorescence displacement studies to examine quadruplex binding by compounds **7–21**

Compd no.	Melting studies (°C) T_m		Thiazole orange displacement studies		
	T_m	Change in temperature (ΔT_m)	Quadruplex DNA binding constants 22G (10^5 M^{-1})	Duplex DNA dinding constants 17 bp (10^5 M^{-1})	Ratio 22G/17 bp
7	52.28	1.05	1.4	6.0	0.22
8	56.52	5.29	1.4	2.5	0.51
9	51.42	0.19	0.53	0.74	0.71
10	55.99	4.36	0.53	0.21	2.5
11	56.40	5.17	2.5	0.24	10.4
12	52.42	1.19	0.3	0.75	0.4
13	54.92	3.69	0.19	0.5	0.37
17	51.92	0.69	0.42	0.5	0.84
18	51.64	0.41	3.9	0.53	7.4
19	52.58	1.35	0.23	0.41	0.55
20	52.20	1.0	0.05	1.1	0.05
21	52.50	1.27	0.53	2.5	0.21

itored by a change in fluorescence due to the displacement of a bound fluorescent dye. Recently, it has been shown that thiazole orange is an excellent dye for these studies since it is safer than ethidium bromide and binds equally well to both quadruplex and double stranded DNA.[28,29]

Thiazole orange (TO) displacement studies were conducted using oligo 22G (5′-AG_3T_2A-$G_3T_2AG_3T_2AG_3$-3′), and oligo 17 bp (5′-C_2AGT_2C-GTAGTA_2C_3-3′;5′-$G_2TCA_2GCATCAT_2G_3$-3′).[29,30] Previous researchers have shown that 22G folds into a G-quadruplex while 17 bp adopts a duplex DNA structure. Binding to each oligonucleotide was determined by titrating the test compound into a solution containing the oligonucleotide (0.25 μM) and TO (0.25 μM) in 10 mM cacodylate buffer (pH 7.4) supplemented with 100 mM KCl. After each addition, the fluorescence spectra were taken and the area of the peak was determined using the instrument software. The area was plotted as a function of compound concentration and the concentration of compounds that reduced the area by 50% was taken as the IC_{50} value. Binding constants were calculated assuming a simple competitive binding model.[25,26] Binding constants and the ratio of quadruplex to duplex affinity is shown in Table 1.

An examination of the data presented in Table 1 indicate that there is little correlation between the compounds identified as quadruplex binders by melting versus fluorescence displacement studies. For example, compounds **10** and **13** display changes in melting of between 3 and 4 °C but have modest affinity as measured by the fluorescence displacement assay. Conversely, some compounds display good affinities as measured by fluorescence displacement (i.e., **18**) but not by the FRET melting assay. Similar observations have been made in the literature and these differences have been attributed to distinct binding modes or sites of the compounds being tested.[29,30] For **10** and **13**, it is likely that these compounds bind to a site distinct from that of thiazole orange and induce displacement by indirect mechanisms (i.e., chang-

ing conformation of the thiazole orange binding site). In contrast, for molecules like **18**, there is a direct competition with thiazole orange for binding to the terminal tetrad.[29,30] Given this, we predict that **11** and **18** likely bind to the terminal tetrad of the quadruplex whereas compounds **8**, **10** and **13** probably intercalate or bind to the grooves of the quadruplex.

The fluorescence displacement assay also provided us with a method to determine the selectivity of our agents (Table 1).[29,30] An examination of the selectivity ratio of these compounds indicates that most agents display a preference for duplex DNA (22G/17 bp ratio below 1). However, two compounds, **11** and **18** show marked selectivity for quadruplex DNA. Compound **11** binds to quadruplex DNA approximately 10-times better than duplex DNA, while **18** binds 7-times better.

We next conducted surface plasmon resonance (SPR) studies to examine binding affinities and selectivity for our agents.[31,32] These experiments were done on a Biacore instrument using biotin-labeled oligonucleotides immobilized onto a streptavidin-coated chip. In these experiments we examined binding of our agents to the human telomeric sequence, which is known to adopt a G-quadruplex structure, and to duplex DNA. Steady-state analysis was performed with multiple injections at various drug concentrations. The relative response of the instrument was then used to determine the binding constants of the compounds.[31–33] Most compounds displayed either weak affinity or non-specific interactions with the surface which precluded a determination of binding affinity. Compounds **8** and **11** were among the agents that displayed non-specific binding to the chip. However, we found that **10**, **13** and **18** bound to quadruplex DNA with equilibrium binding constants of 0.4, 0.5, and $0.3 \times 10^5\,M^{-1}$, respectively. These values are roughly equivalent to the affinity calculated by the fluorescence displacement assay for compounds **10** and **13**, but approximately 10-fold weaker for **18**. The difference in affinity for **18** could be the result of surface effects or covalent attachment of the oligonucleotide to the chip. Incubation of **10**, **13**, and **18** to dsDNA-containing chips resulted in weak signals that could not be accurately fitted to generate binding constants. While this indicates that **10**, **13** and **18** possess some measure of selectivity for quadruplex DNA, the magnitude of the selectivity could not be determined by SPR.

In conclusion, we have described the synthesis and evaluation of a series of 2- and 3- substituted benzo(*h*)quinolinones as quadruplex binding agents. These agents display binding affinities in the range of $0.05–4 \times 10^5\,M^{-1}$ for G-quadruplex sequences and two agents possess a 7–10-fold selectivity for quadruplex over duplex DNA. Competition with the well-known fluorescent dye, thiazole orange, suggests that these agents probably bind to the terminal tetrad. Both regioisomers appear to possess affinity for quadruplex DNA; however, the three-substituted isomer generally binds with greater affinity to the G-quadruplex. Given the ease with which these compounds can be synthesized, we believe that these agents may be of benefit for the future preparation of G-quadruplex binding agents.

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