Synthesis, Antitumor Activity, Molecular Modeling, and DNA Binding Properties of a New Series of Imidazonaphthalimides

Miguel F. Braña,*,† Mónica Cacho,† Mario A. García,† Beatriz de Pascual-Teresa,† Ana Ramos,*,† Nuria Acero,‡ Francisco Llinares,‡ Dolores Muñoz-Mingarro,§ Cristina Abradelo,§ María Fernanda Rey-Stolle,§ and Mercedes Yuste§

Departamentos de Química Orgánica y Farmacéutica, Biología y Ciencias Básicas, Facultad de Ciencias Experimentales y de la Salud, Universidad San Pablo CEU, Urbanización Montepríncipe, 28668-Boadilla del Monte, Madrid, Spain

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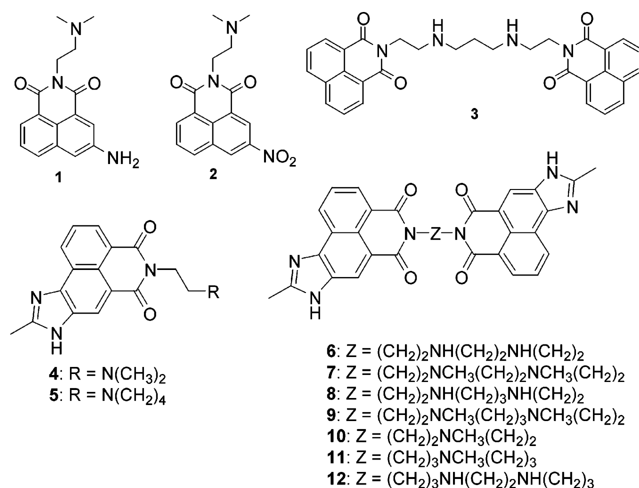
A series of mono and bisintercalators based on the 5,8-dihydrobenz[*de*]imidazo[4,5-*g*]isoquinoline-4,6-dione system were synthesized and evaluated for growth inhibitory properties in several human cell lines. All target compounds showed activity in the micromolar range. Representative compounds were evaluated using UV−vis spectroscopy and viscosimetric determinations, showing that they behave as DNA intercalators. Molecular modeling techniques were used in order to rationalize the moderate activity observed for bisnaphthalimides.

Introduction

Naphthalimides have been shown to be interesting chromophores in the design of mono- and bisintercalators with high antitumor activity.[1] Two of the most active mononaphthalimides, amonafide **1** and mitonafide **2**, were selected for phase II clinical trails.[2,3]



Bisintercalators obtained by dimerization of these naphthalimides exhibit higher activity than the monomeric compounds. Thus, elinafide **3** shows a potent cellular citotoxicity and an excellent in vivo antitumor activity[4,5] against several tumor xenograft models and has been selected for clinical trials. Footprinting experiments[6] and, more recently, NMR studies[7] have indicated that elinafide bisintercalates into the DNA helix via the major groove. The dinitrobisnaphthalimide DMP 840 shows a spectrum of activity similar to that of elinafide and has undergone clinical trials.[8]

To broaden the scope of this kind of highly active compound, we have designed a new series of mono- and bisnaphthalimides, where an imidazole ring fused to the naphthalene moiety has been introduced. The presence of a larger aromatic system can lead to a higher affinity for the DNA molecule and consequently to a greater growth inhibitory potency, as has been shown for azonafide.[9] Moreover, the presence of the heterocycle must have an effect on the electrostatic properties of the chromophore, which could improve the affinity and sequence selectivity of this kind of intercalator. Investigators from Du Pont Merck Pharmaceuticals have described the synthesis of bisnaphthalimides with this chromophore linked by a spermidine unit, finding a moderate activity against murine leukemia L1210 cells.[10] The antitumor activity of some mono-[11] and unsymmetrical bisnaphthalimides[12] characterized by the presence of an azaphenanthrene has been also reported.

The synthesis, biological activity, DNA binding properties, and molecular modeling of several mono- and symmetrical bisimidazonaphthalimides (**4**−**12**) are reported herein.

Results and Discussion

Chemistry. The synthesis of 2-methyl-3*H*-naphtho[1,2-*d*]imidazole-5,6-dicarboxylic anhydride **15**, from which the naphthalimides were prepared, was described previously starting from 4,5-diacetylaminoacenaphthene.[13] We used a similar method but starting from 3-acetylaminonaphthalene-1,8-dicarboxylic anhydride as outlined in Scheme 1. Mono- and bisnaphthalimides **4**−**12** were readily prepared by reaction of anhydride **15** with the corresponding polyamine. *N,N*-Bis(2-aminoethyl)-*N,N*-dimethyl-1,2-ethanediamine used in the synthesis of **7** was obtained following the procedure described by Alcock.[14] The synthesis of *N,N*-bis(2-aminoethyl)-*N,N*-dimethyl-1,3-propanediamine **18** was carried out starting from *N,N*-dimethyl-1,3-propanediamine as outlined in Scheme 2 (available as Supporting Information). The rest of amines were commercially available.

* To whom correspondence should be addressed. For M.F.B.: phone 34913724771; fax 34913724009; e-mail mfbrana@ceu.es. For A.R.: phone 34913724782; fax 34913510496; e-mail aramgon@ceu.es.
† Departamento de Química Orgánica y Farmacéutica.
‡ Departamento de Biología.
§ Departamento de Ciencias Básicas.

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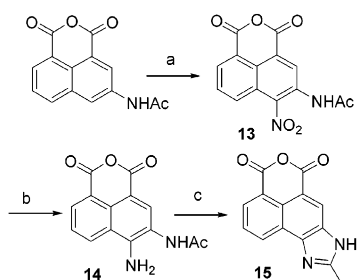
Scheme 1[a]



[a] Reagents: (a) HNO_3(f)/H_2SO_4, −8 °C; (b) H_2, 10% Pd/C, DMF; (c) AcOH refluxed.

Table 1. Growth Inhibitory Properties for Mono- and Bisnaphthalimides **4**−**12**

compd	IC_{50} [a]		
	HT-29	HeLa	PC-3
4	0.94	2.35	10.4
5	3.11	6.09	4.22
6	1.08	3.49	>100
7	3.83	8.62	>100
8	8.5	8.26	>100
9	0.32	0.80	3.22
10	17.5	4.45	>100
11	>100	>100	36.28
12	15	8.75	744
amonafide	4.67	2.73	6.38
elinafide	0.017	0.07	0.32

[a] IC_{50}: concentration of drug (μM) to reduce cell number to 50% of control cultures.

Biological Activity. The imidazonaphthalimides were evaluated for in vitro cytotoxicity against several cell lines. These include human colon carcinoma (HT-29), human cervical carcinoma (HeLa), and human prostate carcinoma (PC-3). The results are summarized in Table 1 and compared with the activity of amonafide and elinafide. Mononaphthalimides **4** and **5** showed improved cytotoxic activity over amonafide against the HT-29 cell line. This result is in agreement with our hypothesis that the introduction of a heterocycle fused to the naphthalene moiety should increase the affinity of the chromophore for the DNA molecule. To enhance the potency of this compound, we synthesized bisintercalators **6**−**12** by linking two chromophores with polyamine bridges. With the possible exceptions of **10** and **11**, all the compounds should have N−N distances compatible with a possible bisintercalative mode of binding.[15] Compound **10**, with the shortest linking chain showed an activity similar to that of amonafide against HT-29 and HeLa cell lines. This compound probably behaves as a monointercalator (see later). The addition of two additional methylene units brought about complete loss of activity, as shown for the closely related analogue **11**. This unexpected result could be explained assuming that the linker chain is long enough to allow bisintercalation, but it is still too short to form a stable complex with the DNA molecule. Compounds **6** and **8** with two nitrogen atoms in the bridge showed increased activity, but they remained less active than the parental monomer **4** and were considerably less active than the leader compound, elinafide. Compound **9**, with a $(CH_2)_2$-$NCH_3(CH_2)_3NCH_3(CH_2)_2$ linker, was chosen because methylation of the NH groups in related bis(indeno[1,2-*b*]-6-carboxamides)[16] and bis(9-methylphenazine-1-carboxamides)[15] led to an increase in potency. We observed a similar behavior for compound **9**, which was 26-fold more active than its unmethylated counterpart **8** against HT-29 and 10-fold more active against HeLa. This effect had not been observed previously in related bisnaphthalimides such as elinafide.[5]

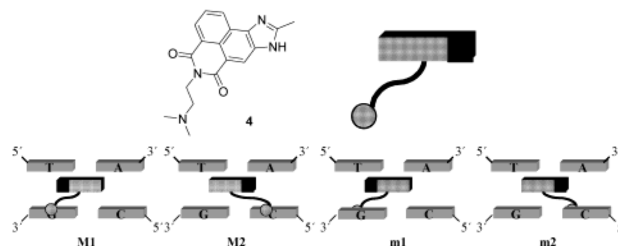


Figure 1. Schematic view from the major groove of the four complexes between compound **4** and $d(TG)_2$. The black square in the chromophore represents the orientation of the imidazole ring, and the sphere represents the protonated dymethylamino group from the side chain. Left: complexes with the linker in the major groove and the imidazole ring stacked between TG (**M1**) or between AC (**M2**). Right: complexes with the linker in the minor groove and the imidazole ring stacked between TG (**m1**) or between AC (**m2**).

In the case of compound **7**, with a shorter linking chain, the addition of methyl groups to the amine nitrogen atoms resulted in a loss of activity. This effect has been also observed for other bisnaphthalimides containing a $(CH_2)_2NCH_3(CH_2)_2NCH_3(CH_2)_2$ linker.[5]

Finally, the synthesis and biological evaluation of compound **12** were carried out on the basis of a molecular modeling study, the details of which are covered below.

Molecular Modeling. To rationalize the results of biological activity obtained for this new series of imidazonaphthalimides, we have carried out a molecular modeling study of mononaphthalimide **4** and bisnaphthalimides **8**, **9**, and **12**. The molecular modeling study was carried out making use of molecular mechanics and dynamics techniques. In the first approach, we studied a complex between a model system containing compound **4** and a $d(TG)_2$ dinucleotide (Figure 1). The sequence was selected according to the general pyr−pur preference exhibited by simple monointercalators[17] and to the binding preference demonstrated for this type of compounds in a recent NMR study.[7] To carry out this theoretical work, it was necessary to determine if, at physiological pH, the imidazole ring was protonated. UV−vis spectra of compound **4** registered at different pH values showed that, as expected, at physiological pH only the aliphatic nitrogen was protonated. We also had to take into consideration the two tautomeric forms of the imidazole ring. An RHF/6-31G*[18] calculation in a model compound revealed that the tautomeric form, where the hydrogen is located in the outer position, was 1.6 kcal/mol more stable than the inner tautomeric form. Two different model complexes were then constructed with compound **4**, by inserting the chromophore into the $d(TG)_2$ dinucleotide in the four possible orientations relative to the base pairs, denoted **M1**, **M2**, **m1**, and **m2**, as shown in Figure 1. The initial complexes were refined and submitted to 50 ps of molecular dynamics simulations. The root-mean-square (rms) deviation of the dimer complexes with respect to the initial structures was evaluated for all four complexes. This value was more stable for **M1**. In this complex, the side chain of **4**

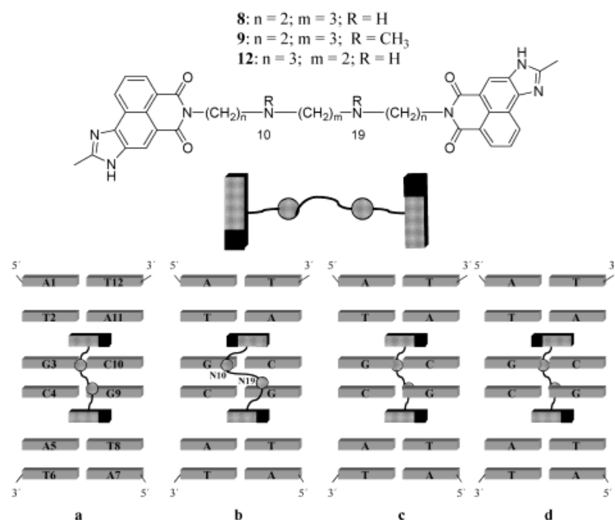


Figure 2. Schematic view from the major groove of the four complexes between compounds **8**, **9**, or **12** and d(ATGCAT)$_2$. Left: complexes with the ligand bound through the major groove and the chromophores in relative a parallel orientation (a) or antiparallel orientation (b). Right: complexes with the ligand bound through the minor groove and the chromophores in relative a parallel orientation (c) or antiparallel orientation (d).

is located in the major grove of the DNA dimer, locating the charged amino group between N7 and O6 of the guanine base. In fact, the distance between N7 and the nitrogen proton was monitored during the simulation time, giving an average value of (2.106 Å ± 0.246), indicating the presence of a hydrogen bond that remains stable during the entire simulation. The progression of these two parameters indicates that complex **M1** does not experience large conformational changes during the sampling time and thus can be considered to be in a state near equilibrium. In the remaining three complexes, the imidazole hydrogen is able to form a hydrogen bond with different positions of the sugar phosphate backbone, dominating the evolution of the molecular dynamics simulations. This leads to higher rms values. These results suggested a clear preference of compound **4** to bind the DNA dimer, leaving the charged side chain lying in the major groove of the dinucleotide and the imidazole ring stacked between the thymine and guanine bases.

Compounds **8** and **9** were then submitted to similar molecular dynamics simulations. Again, we considered the four possible orientations that compounds **8** and **9** could adopt when binding to d(ATGCAT)$_2$ (Figure 2). The sequence was selected according to the binding preference demonstrated for elinafide.[7] The root-mean-square deviation of these complexes with respect to the initial structures was evaluated during the simulation time, leading in all cases to average values over 4 Å. A detailed visualization of the dynamics trajectory showed relevant conformational changes over the entire simulation time. Average minimized structures of the last 10 ps of the molecular dynamics simulation for complexes **8b** and **9b** are shown in Figure 3 (available as Supporting Information) and compared to the initial refined structures. As shown in this figure, the DNA hexamer undergoes significant conformational changes by bending toward the major groove of the hexanucleotide. These results prompted us to propose that the linkers were too short for the dimerization of this type of chromophore. Compound **12** was then constructed and modeled following a similar protocol. The four complexes (Figure 2) were also evaluated, and the root-mean-square deviations were monitored during the simulation time. In this case, complexes **12a**, **12c**, and **12d** led to average rms values over 3.5 Å whereas complex **12b** gave an rms average value of 2.788 ± 0.290 Å. The initial minimized structure and the average minimized structure of the last 10 ps of the molecular dynamics simulation for **12b** are shown in Figure 3. As shown in this figure, this complex remains stable after the simulation time and the DNA does not undergo significant conformational changes with respect to the initial structure, prompting us to propose compound **12** as the next candidate to be synthesized. Disappointingly, compound **12** did not show increased biological activity. A detailed analysis of the time evolution of this complex showed the presence of a hydrogen bond between N19 of the protonated linking chain (Figure 2) and N7 and O6 of residue G9, whereas N10 is not able to form such stabilizing interaction with the corresponding G3 residue. According to this observation, it seems that the linking chain chosen for this dimer was still not appropriate enough in order to increase the drug affinity. Probably a different distribution of the protonated nitrogen atoms would be more suitable.

Mechanism of Action Studies. The DNA binding properties of compound **4** as a model of a monointercalator and **8** as a model of a bisintercalator have been studied by viscosimetric titration with calf thymus DNA as well as by UV−vis spectrometry. It is known that DNA length increases when a drug behaves as an intercalator.[19] When the relative increase in contour length, L/L_0, versus r is plotted, the slope m of this plot has different values depending on the functionality of the intercalator. Monofunctional intercalators such as ethidium bromide, proflavine, and aminoacridines usually have values of m between 0.8 and 1.5.[20] In our case, when L/L_0 is plotted versus r for compound **4**, the least-squares fitting gives a slope of 1.3, indicating that this compound provokes DNA helix extension as a monofunctional intercalator. In the case of compound **8**, a slope of 2.2 has been obtained, showing that **8** provokes DNA extension as a bifunctional intercalator. For bisintercalators, the slope is expected to be twice the value observed for monointercalators, as verified for a variety of intercalating compounds.[21] Finally, a similar study with bisnaphthalimide **10** led to a slope of 0.8, a value that is in accordance with a monointercalative mode of interaction with DNA for this compound.

The data obtained from UV−vis spectrometry confirm that compounds **4** and **8** behave as intercalators. Addition of these compounds to a sample of sonicated calf thymus DNA in Tris-HCl (pH = 7.5) induces hypochromic and bathochromic shifts, the shift being higher as the temperature decreases. This is the expected behavior for an intercalator.[22]

To further evaluate the mechanism of action, compound **5** was selected for the assay of single-cell gel electrophoresis (comet assay). The comet assay detects DNA damage in individual cells embedded in agarose. It is based on the property of negatively charged DNA fragments to migrate when an electric field is applied to the gel after cell lysis.[23] Doxorubicin was chosen as

a positive reference, and PBS (phosphate-buffered saline, pH = 7.4) was used as a negative control. One hour after the treatment, the samples were observed using a fluorescence microscope and the DNA damage of compound **5** was similar to the damage of the positive reference. This result suggests that the anitumor activity of these mononaphthalimides may be related to their ability to induce DNA damage.

Conclusions

In conclusion, according to the results presented in Table 1, the presence of an imidazole ring fused to the naphthalimide system increases the cytotoxic activity of mononaphthalimides with respect to the unfused compounds against HT-29 cell lines. Dimerization of the chromophore, using either the linker present in elinafide (compound **8**) or a methylated linker (compound **9**), has not resulted in an increase of activity. A molecular modeling study of these compounds has shown that the linker is too short to form stable complexes with the DNA molecule. This study has also shown that the addition of a methylene unit, as in compound **12**, increases the stability of the complex. Consequently, bisnaphthalimide **12** was synthesized and tested for activity. Disappointingly, we did not find any improvement in the antitumor activity. This result prompted us to propose that this type of chromophore is not suitable for dimerization, and further modifications in the chromophores will be undertaken in future studies.

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Supporting Information Available: General experimental procedure for preparation and physical and spectral characterizations (^{1}H and ^{13}C NMR, mass spectrometry, and IR data) of all the synthesized compounds; Scheme 2 showing synthesis of **18**; experimental procedure for viscosimetric titrations (Figure 4) and UV−vis spectrometry for compounds **4** and **8** (Figure 5); alkaline single-cell gel electrophoresis assay for compound **5** (Figure 6); model building, energy minimizations, and molecular dynamics simulations details for compounds **4**, **8**, **9**, and **12** (Figures 3 and 7−9); and AMBER parameters and partial atomic charges for compounds **4**, **8**, **9**, and **12** (Tables 2 and 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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