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## DRUGS THAT INTERACT IN THE DNA MINOR GROOVE

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### Abstract

Recognition of DNA by ligands and the interactions with the *minor groove* of DNA is a topic of growing interest in the fields of Chemistry, Biology and Medicine. Some of these compounds are *sequence-selective DNA-interactive agents* that bind covalently to guanine bases within the minor groove of DNA, modulating the activity of transcription factors or genes. In this regard, pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are of particular interest, as they recognize specific sequences of DNA and they have both antibacterial properties and selective cytotoxicity towards tumour cells. Naturally occurring PBDs (e.g. anthramycin) were originally isolated from a fermentation broth of various species of *Streptomyces*, although recently discovered limazepines A-F come from *Micrococcus* species. The second generation of PBDs, which is formed by dimers, has enhanced sequence selectivity and form both interstrand and intrastrand cross-links that are more difficult for tumour cells to repair, resulting in an increased *cytotoxicity*. As PBDs have attracted many chemists over the last few years, a wide range of analogues have been synthesized. It becomes necessary to outline the PBD dimer named SJG-136, which is the lead candidate currently undergoing pharmacology and toxicology studies due to its *in vitro* and *in vivo* activity against cancer cells.

**Keywords:** minor groove, PBDs (pyrrolobenzodiazepines), cytotoxicity, sequence-selective, synthesis.

### Resumen

La determinación de las interacciones entre fármacos y el DNA, particularmente en el surco menor, constituye un área de creciente interés en química, biología y medicina. Algunas moléculas presentan selectividad de secuencia, uniéndose a secuencias específicas del DNA covalentemente a través de la base guanina y modulando la actividad de genes y factores de transcripción. En este contexto, las pirrolo[2,1-c][1,4]benzodiazepinas (PBDs) reconocen secuencias específicas del DNA localizadas en el surco menor y presentan propiedades antibióticas y citotoxicidad. Las primeras pirrolobenzodiazepinas investigadas fueron monómeros aislados en cultivos de bacterias del género *Streptomyces* (por ejemplo, antramicina), aunque recientemente se han encontrado algunas cepas del género *Micrococcus* que también son capaces de sintetizarlas, por ejemplo las denominadas limazepinas A-F. La segunda generación de PBDs está constituida por dímeros, los cuales presentan una mayor selectividad de secuencia respecto al DNA, ya que forman enlaces intracatenarios e intercatenarios de difícil reparación. Recientemente se han sintetizado análogos, entre los que destaca el dímero SJG-136, en la actualidad en fase clínica por su actividad como anticanceroso. En la última década, el interés biológico de estas moléculas ha atraído a numerosos químicos, gracias a lo cual se han sintetizado análogos de los que se ha determinado la actividad. Resulta interesante el dímero SJG-136, que se encuentra en fase clínica como posible fármaco útil en la terapia del cáncer.

**Palabras clave:** Surco menor, PBDs (pirrolobenzodiazepinas), citotoxicidad, selectividad de secuencia, síntesis.

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## Resum

La determinació de les interaccions entre fàrmacs i l'ADN, particularment en el solc menor, constitueix una àrea de creixent interès en química, biologia i medicina. Algunes molècules presenten selectivitat de seqüència, unint-se a seqüències específiques del DNA covalentment a través de la base guanina i modulant l'activitat de gens i factors de transcripció. En aquest context, les pirrolo [2,1-c] [1,4] benzodiazepines (PBD) reconeixen seqüències específiques del DNA localitzades en el solc menor i presenten propietats antibiòtiques i citotoxicitat. Les primeres pirrolobenzodiazepines investigades van ser monòmers aïllats de cultius de bacteris del gènere *Streptomyces* (per exemple, antramicina), encara que recentment s'han trobat algunes soques del gènere *Micrococcus* que també són capaces de sintetitzar, per exemple les anomenades limazepines AF. La segona generació de PBD està constituïda per dímers, els quals presenten una major selectivitat de seqüència respecte al DNA, ja que formen enllaços intracatenaris i intercatenaris de difícil reparació. Recentment s'han sintetitzat anàlegs entre els quals destaca el dímer SJG-136 en fase clínica per la seva activitat com a anticancerós. En l'última dècada, l'interès biològic d'aquestes molècules ha atret nombrosos químics, de manera que s'han sintetitzat anàlegs dels quals s'ha determinat l'activitat. Resulta interessant el dímer SJG-136, que es troba en fase clínica, com un possible fàrmac útil en la teràpia del càncer.

**Paraules clau:** solc menor, PBD (pirrolobenzodiazepines), citotoxicitat, selectivitat de seqüència, síntesi.

## 1. Introduction

DNA has long been a molecule of high complexity and growing biological interest, and in terms of human disease it has become an extremely specific drug target.

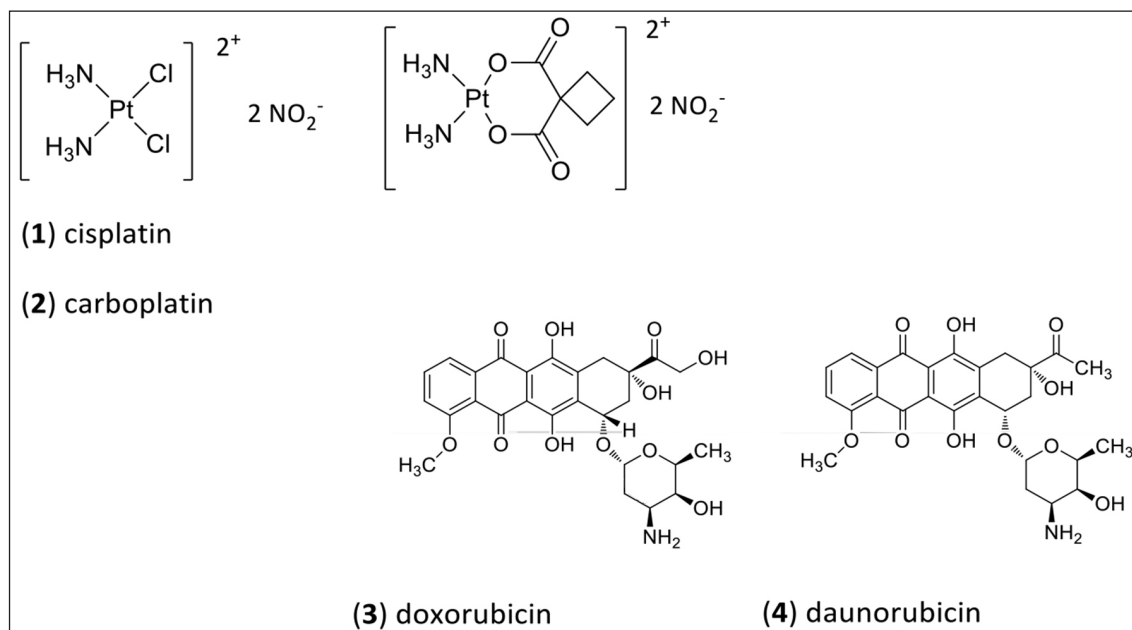
There are two major limitations in cancer chemotherapy: a lack of tumour specificity and the design of molecules that achieve DNA by crossing both cell and nuclear membranes. In fact, both of them are currently a challenge for drug design. These drawbacks can be successfully overcome by using DNA-interactive agents that selectively interfere with DNA by preventing transcription and subsequent translation of oncogenic genes, thereby inhibiting tumour progression. In this context, minor groove binders constitute an important class of derivatives in anticancer therapy. DNA-targeting strategies require the development of molecules that recognize unique sequences in the minor groove of DNA with a reasonable affinity.

As far as drug-DNA interactions are concerned, drugs can be classified into three major categories: alkylators, intercalators and groove binders. All of them can interact with DNA, either covalently or non-covalently. In this respect, several drugs have found use in therapeutics and are currently the basis of a wide range of cancer treatments, either in monotherapy or in combination regimens. In order to better understand interactions between drugs and DNA, some of the most representative compounds in each of the three groups are described below.

Alkylators are electrophilic compounds that react with nucleophilic groups on DNA to form covalent bonds through nucleophilic substitution. The resulting adducts are irreversible inhibitors of transcription and translation. For instance, platinum complexes [cisplatin (**1**) or carboplatin (**2**)] (**Figure 1**) are alkylating drugs that form primarily intra-strand cross-link adducts which lead to cell death, in which the most commonly involved bases are guanines and adenines.

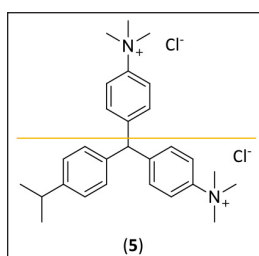
Intercalators act by inserting their coplanar aromatic ring within the DNA backbone. (Braña *et al.*, 2001) They stabilize the DNA without disrupting base pairing. Their effect consists of lengthening the duplex by 3 Å per bound drug molecule, thus causing DNA unwinding and preventing transcription and translation. Among intercalators there are remarkable anthracyclines such as doxorubicin (**3**) (also known as adriamycin) and

daunorubicin (**4**) (also known as daumomycin) (**Figure 1**). They bind to DNA in a non-covalent manner through the protonation of the amino group, which is capable of interacting with the negatively charged DNA backbone.



**Figure 1.** Structures of alkylators and intercalators.

Groove binders include both major and minor groove binders. Among them, this work is mainly focused on the latter. An example of a major groove binding agent is methyl green (**5**) (**Figure 2**). Minor groove binders include a highly representative family of molecules called pyrrolo[2,1-*c*][1,4]benzodiazepines, which constitute the core of this work. In terms of pharmacology, they have shown remarkable biological activities, particularly in anticancer and anti-infective therapies.



**Figure 2.** Major groove binder methyl green.

This work is focused primarily on the detailed description of the structure of the DNA and the atoms involved in the interactions with drugs and proteins. Secondly, minor groove binders are considered explaining the mechanism of their action at a molecular level. Furthermore, the substitution pattern of these drugs concerning structure-activity studies is analyzed. Finally, several methods of synthesis of PBDs are described based on different chemists' proposals. All of these synthetic methods are based on the use of racemic structures as starting materials. Interestingly, an experimental work and a discussion in order to access the enantiomerically pure pyrrolidine scaffold is described.

## 2. Materials and methods

This work is based initially on the bibliographic research in *Sci-Finder* (<https://scifinder.cas.org>) and the National Cancer Institute (NCI) (<http://www.cancer.gov>). Organic Chemistry and Biochemistry text books have been useful as a point of support for the acknowledgement of basic concepts and notions of each subject that are taken for granted (Berg, Tymoczko and Stryer, 2006),(Boyle, 2008).

This work is focused on gathering and studying the bibliographic information on drugs that interact with the DNA minor groove. This implies two chapters: first, the description of the structure of DNA, and, second, the search for the published bibliography on the PBDs by several authors. Therefore, it becomes easier to understand the mechanism of interaction between both of them and provides a reasonable explanation of the biological activity of these compounds. Finally, there is a description of the patterns concerning the structure of naturally occurring and synthetic PBDs.

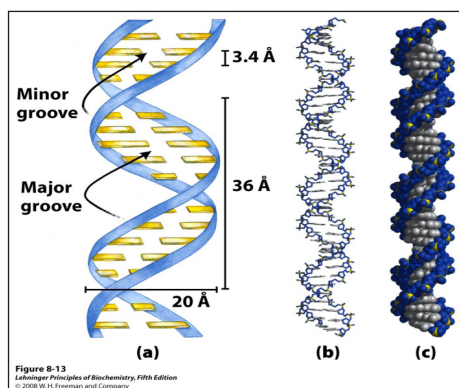
## 3. Results and discussion

### 3.1. General structure of DNA

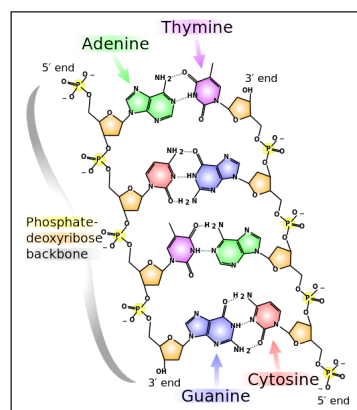
The actual structure of DNA was postulated in 1953 by James Watson and Francis Crick. They reported that DNA consists of two helical DNA chains around the same axis that form a double helical and right-handed structure. Nucleotides are its building blocks, which are formed of three components: a nitrogenous base (purine, such as adenine and thymine, or pyrimidine, such as cytosine and guanine), a pentose (in DNA there is always a deoxyribose) and a phosphate that forms the DNA backbone (Watson and Crick, 1993).

Three forms of duplex nucleic acid have been described: B-DNA, A-DNA and Z-DNA. B-DNA is the most common at neutral pH and physiological salt concentration and it is the form that is going to be described in this section. In B-DNA, the hydrophilic groups deoxyribose and phosphate are placed on the outside of the double helix, while the nitrogenous bases are placed on the inside with the hydrophobic and nearly planar ring structures very close and perpendicular to the long axis. Therefore, the model proposed by Watson and Crick considers 10.5 base pairs or 36 Å per helical turn (**Figure 3**), giving place to a major and a minor groove in which nitrogenous base from one strand interacts through hydrogen bonds with the other. From Chargaff's rules, depicted in **Figure 4**, the base pairs that are formed are A · T and C · G, taking into consideration that two and three hydrogen bonds are formed, respectively. The third hydrogen bond is between the additional exocyclic amino group on G and the *keto* group on C. The base pairings mentioned above are known as complementary base pairs (Kresge, Simoni and Hill, 2005).

The orientation of the polynucleotide chains is an additional feature to consider, as the chains of DNA are antiparallel (the 3', 5'-phosphodiester bonds are orientated in an opposite direction), a hypothesis that was later confirmed by X-ray diffraction analysis. Hydrogen bonds and base-stacking interactions are the main interactions involved in the double helix. Hydrogen bonds, as commented above, are responsible for the complementarity between base pairs, while base-stacking interactions account for the high stability of the double helix.



**Figure 3.** Minor and major grooves in DNA.

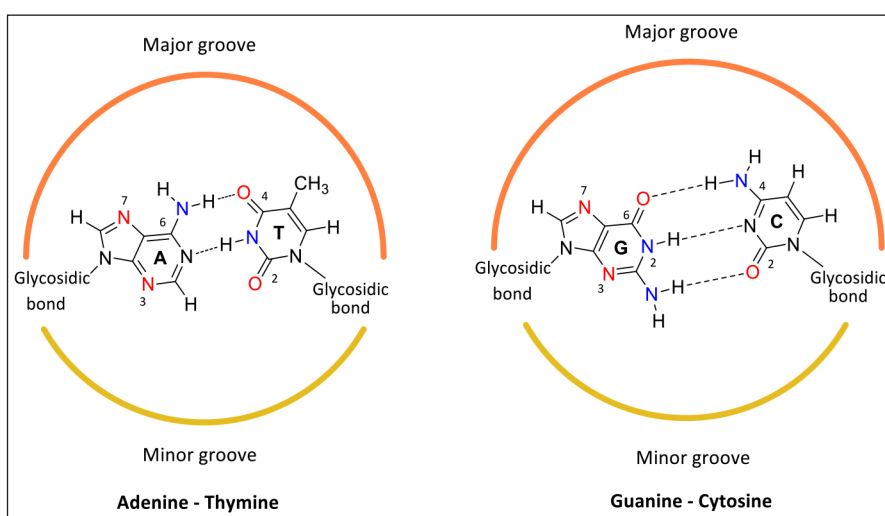


**Figure 4.** Hydrogen bonds between complementary bases.

### 3.2. DNA-interactive agents

Complexes between drugs and DNA play a major role in anticancer and anti-infective therapies, either as single agents, in combination drug regimens or as components of targeted therapies (Barrett, Gemmell and Suckling, 2013). DNA has been regarded as a first outstanding target due to the close relation between genes expression and the progression of a disease. Furthermore, when thinking of proteins as possible drug targets, they are also encoded by the information contained in DNA (Spitzer et al., 2007).

When considering DNA interaction, the structure of the alpha helix is particularly important in all of the previously mentioned groups (alkylators, intercalators and groove binders). Double helical DNA is not uniform, as the two *N*-glycosidic bonds are not diametrically opposite each other and base pairs have a larger and a smaller side that define the major and minor groove, respectively (**Figure 3**). In general terms, the major groove is deeper (8.5 versus 7.5 Å) and wider (12 versus 6 Å). Therefore, the minor groove is normally inaccessible by molecules of more than 1000 Da.



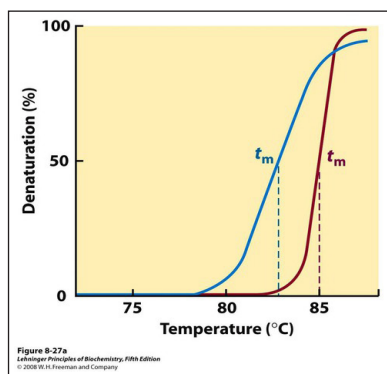
**Figure 5.** Hydrogen bonds between nitrogenous bases in DNA (hydrogen bond acceptors are depicted in red and hydrogen bond donors are depicted in blue).

When focusing specifically on hydrogen bonds that form nitrogenous bases of DNA in the major and in the minor groove, in the major groove hydrogen bond donating groups are C6 amino group of adenine and the C4 amino group of cytosine, while hydrogen bond accepting groups are positioned at adenine N7, thymine O4, guanine N7 and O6. Similarly, in the minor groove, the adenine N3, thymine O2, guanine N3 and cytosine O2 can act as hydrogen bond acceptors while the C2 amino group of guanine can act as a donating group (**Figure 5**).

### 3.3. Methods for the characterization of the interactions

Drugs can interact with different minor groove atoms giving place to a broad range of binding motifs. The types of interactions can be divided into two main groups, covalent and non-covalent. Simultaneously, non-covalent interactions involve hydrogen bonding to base pair edges, van der Waals interactions and electrostatic interactions.

This work primarily focuses on covalent interactions between DNA and PBDs, which are strong and alter DNA to a greater extent than non-covalent binding. In this context, the objective is to compare interactions of several drugs with DNA using the following methods (Palchaudhuri and Hergenrother, 2007). It is important to outline that the biochemical experiments described below are not specific for PBDs as DNA minor groove-interactive agents, as they have been extrapolated from general procedures.



**Figure 6.**  $T_m$  of different DNA specimens in which denaturation occurs at different temperature.

- DNA binding is evaluated *in vitro* in thermal denaturation studies by observing the midpoint denaturation temperature ( $T_m$ ) with *calf thymus* (CT) duplex DNA at pH 7.0 employing protocols reported in previous studies (Kamal et al., 2010).
- Interactions between drugs and DNA stabilize the double helix and reasonable increase in the  $T_m$  (**Figure 6**), which is labelled as  $\Delta T_m$ . Therefore, the higher the  $T_m$  is, the greater biological activity is attributed to the compound.
- Sequence-selective binding of small molecules to DNA is determined by DNA footprinting assay. It consists of 3'-<sup>32</sup>P labelled DNA fragment of known sequence composition, which is incubated with DNase I in the presence of increasing concentration of compound. The differential cleavage pattern indicates that bases are prevented from enzymatic digestion, and thus the precise sequence of bases of DNA that interact with the compound (Bailly et al., 2005) the method is described and technical details are given for performing deoxyribonuclease (DNase).
- Cytotoxicity is evaluated *in vitro* and *in vivo* by using different human tumour cell lines. The cell growth is detected by calculating the 50% growth inhibitory

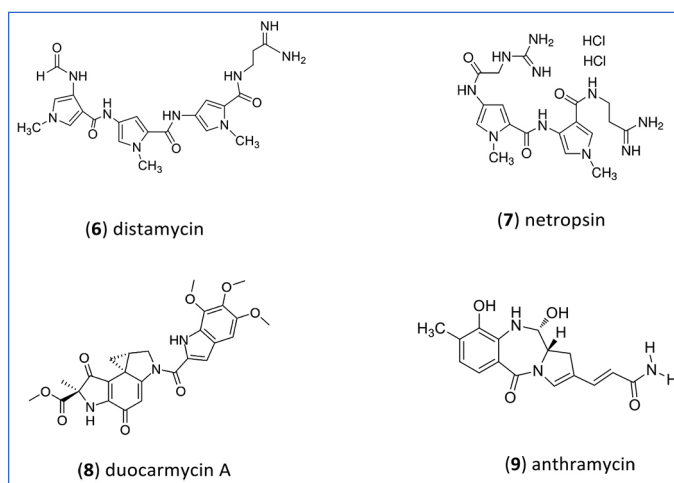


concentration ( $GI_{50}$ ) and the 50% lethal concentration ( $LC_{50}$ ). The protocols followed in the assays have previously been reported in other studies (Monks *et al.*, 1997).

### 3.4. Minor groove-interactive drugs

Several natural and synthetic compounds have been recognized as minor groove binders (Denny, 2001) where attachment of the mustard unit to carrier molecules can change the normal patterns of both regio- and sequence-selectivity, from reaction primarily at most guanine N7 sites in the major groove to a few adenine N3 sites at the 3'-end of poly(A/T). The type of binding between these drugs and DNA can be either covalent or non-covalent. Two minor groove binders that interact in a non-covalent manner are distamycin (**6**) and netropsin (**7**) (Figure 7). (Finlay *et al.*, 1951) The main difference between them lies in the way they establish interaction with the DNA: on the one hand, netropsin binds to DNA as a single molecule, while on the other hand distamycin does it in a combination of two identical molecules.

Other compounds typically form covalent adducts with their pharmacological target, which is the case of duocarmycin A (**8**) and anthramycin (**9**) (Figure 7). The latter will be extensively explained and analyzed, as it is a naturally occurring pyrrolo[2,1-c][1,4]benzodiazepine that links to guanine.



**Figure 7.** Structures of naturally occurring minor groove binders.

As far as the mechanism of action is concerned, drugs can interact with different DNA atoms placed in the minor groove, giving place to binding motifs, either covalently or non-covalently. Moreover, some of the evaluated drugs have proven to be sequence-selective DNA binding agents, with a different range of affinities for unique sequences of DNA and recognition fidelities.

Minor groove binders have a preference to interact with the electronegative pockets of A · T sequences, probably due to better van der Waals interactions between the drug and the groove walls in this region, since A · T sites are narrower and steric hindrance is lower because of the lack of C2 amino group compared to guanine base. It has been found that the atoms which play an important role in the interaction are mainly purine

N3, pyrimidine O2 (in which polar contacts are dominant), guanine N2 and deoxyribose O4' (mainly hydrophobic) (Morávek, Neidle and Schneider, 2002). These findings are in agreement with the more polar character of the base pairs G · C compared to A · T, which results from a larger electronegative potential in the minor groove of B-DNA compared to the major groove (Rohs *et al.*, 2010).

Specifically in anticancer therapy, the development of covalently binding minor groove binders is strongly progressing. The most effective anticancer therapy involves binding covalently and irreversibly to DNA with a long permanence of lesion that finally leads to cell death. For instance, the PDB dimer named SJG-136 (**10**) (Figure 8) has found use in the treatment of solid tumours and it has already undergone Phase I clinical trials, showing therapeutic benefit to warrant access to Phase II studies. (Janjigian *et al.*, 2010).

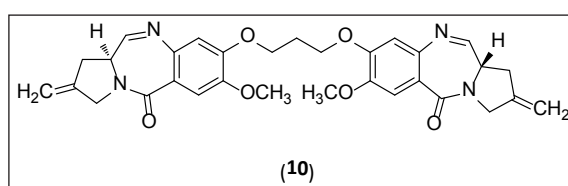


Figure 8. Structure of SJG-136.

Sequence selectivity is undoubtedly a point to take into account when dealing with drugs of application in cancer chemotherapy, as it becomes highly important to design molecules that can deliver critical DNA damage with minimal disturbance of the whole DNA architecture.

### 3.5. PBDs monomers (naturally occurring PBDs)

Discovered in the 1960s by Leimgruber and co-workers, the pyrrolo[2,1-c][1,4]benzodiazepines (PBDs) are important sequence-selective DNA-interactive agents that bind covalently to guanine bases within the minor groove of duplex DNA but not to RNA. They are classified into two different groups, PBDs monomers and dimers. Each of them differ in the substitution patterns in both their aromatic A-ring and pyrrolidine C-ring and in the saturation degree of the latter, which can be fully saturated or unsaturated at either the C2-C3 (endocyclic) or C2 (exocyclic) positions, as it is depicted in Figure 9.

The first representative is anthramycin (**9**) (Figure 9), which was isolated, characterized and eventually synthesized by Leimgruber (Leimgruber, Batcho and Schenker, 1965) along with a wealth of 13 different types of monomers. This finding suggests that PBDs structure is more widely distributed than firstly thought. As for PBDs structure, they differ in the substitution patterns in the A-ring and the saturation of the C-ring, which can be either fully saturated [neothramycin A (**20**) and neothramycin B (**21**)], C2/C3-*endo*-unsaturated [anthramycin (**9**), mazathramycin (**11**), porothramycin (**12**), sibiromycin (**13**)] or C2-*exo*-unsaturated [tomaymycin (**22**), prothracarcin (**23**)] (Figure 9). Experimentally, it has been found that the most biologically active are the ones in which C-ring is C2/C3-*endo*-unsaturated or C2-*exo*-unsaturated. As a drawback, anthramycin (**9**) and sibiromycin (**13**) have proven to be cardiotoxic and produce tissue necrosis at the site of injection, while neothramycin A (**20**) and neothramycin B (**21**) have demonstrated little efficacy in cancer therapy (Hurley *et al.*, 1988).



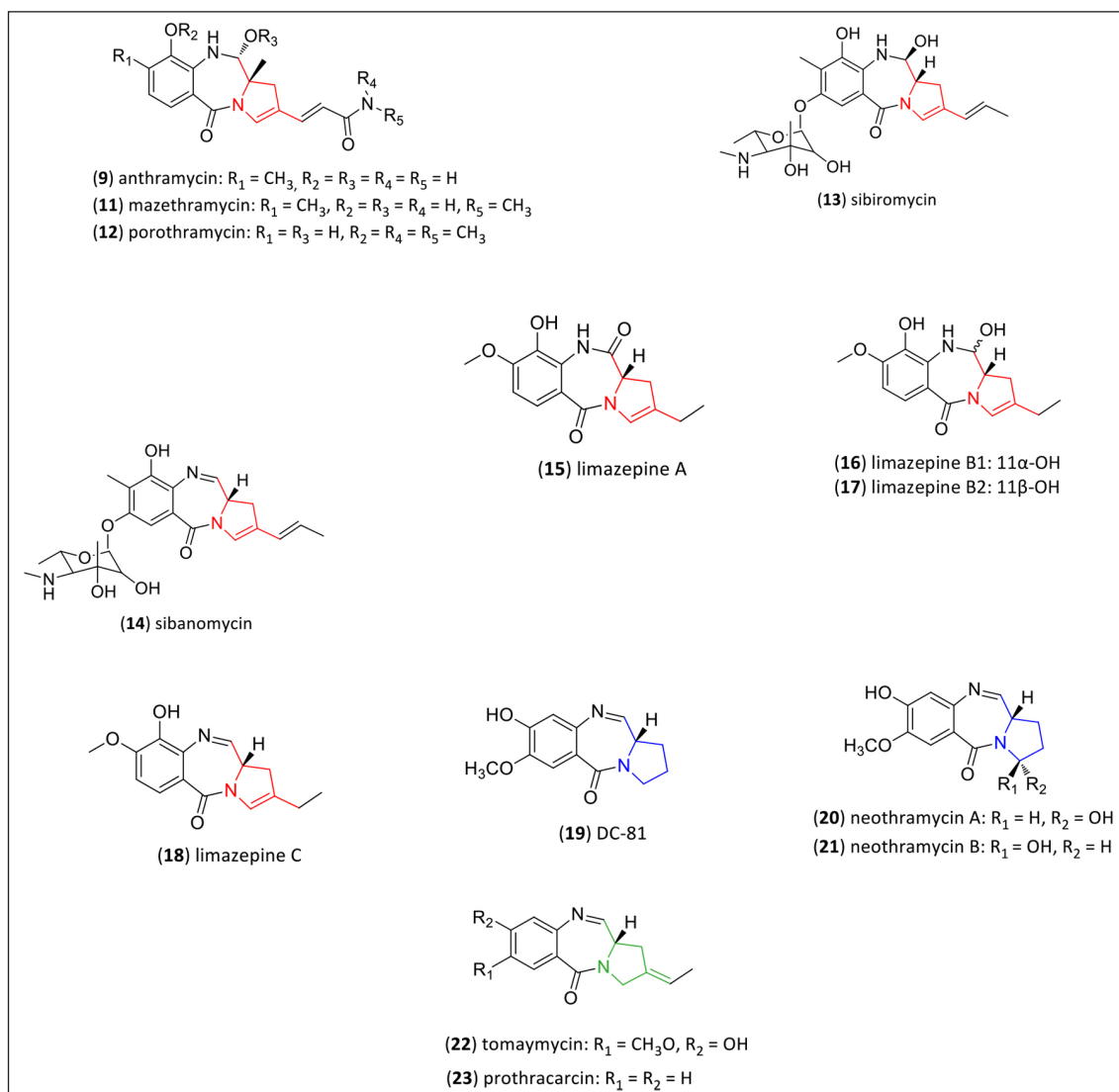


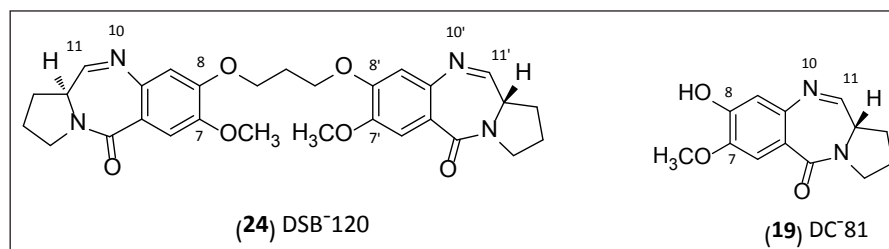
Figure 9. Structures of naturally occurring PBDs.

### 3.6. PBDs dimers

Following the molecular duplication strategy, many chemists envisaged the possibility to design new compounds that result from a conjugation of two identical monomers through a covalent binding. Thereby, molecules have two pharmacophores and thus an increased ability to bind simultaneously to two guanine bases from both DNA strands and establish interstrand and intrastrand cross-links. As far as the metabolic course of dimers is concerned, they are not regenerated metabolically. This means the pharmacological target necessarily has a symmetric region (in this case, both strands of DNA) that increases binding affinity of the drugs, and thereby they exert a greater pharmacological effect.

The dimers reported as the most biologically active are those with C8/C8'-linkage. Among the first generation of PBDs dimers it is remarkable the synthesis of DSB-120 (**24**) in the early 1990s, which is formed by linking two naturally occurring PBDs (DC-81) through C8 position (**Figure 10**).

It is worthy of attention that the type of adducts appear to depend on (a) the length of the C8/C8'-linker connecting the two PBD units, (b) the positioning of the two reactive guanine bases on the same or opposite strands, and (c) their separation (i.e. the number of base pairs, usually A · T, between them) (Rahman, James and Thurston, 2011).



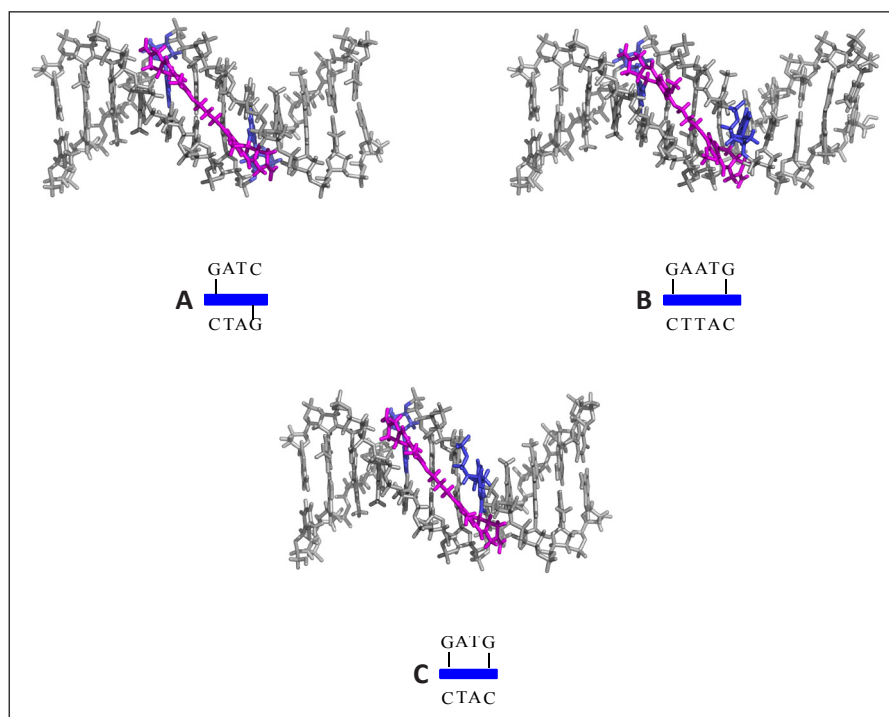
**Figure 10.** Dimer DSB-120, which is formed by linking two monomers of DC-81.

As expected, its cytotoxicity profile *in vitro* is enhanced and its sequence selectivity (Jenkins *et al.*, 1994) is greater than PBDs monomers, as it can span six base pairs within the minor groove of the DNA, with a preference for 5'-GATC-3' as a central sequence (Jenkins *et al.*, 1994),(Smellie *et al.*, 2003)1-c][1,4]benzodiazepine (PBD). As regards its mechanism of action, this molecule binds covalently to DNA by a nucleophile attack via NH<sub>2</sub> groups, giving place to interstrand cross-links.

It is remarkable that interstrand cross-links cause little distortion of DNA, which provide a greater persistence on cells and thus a greater biological activity. Furthermore, DSB-120 (**24**) interstrand cross-links have an influence on DNA repair pathways, thereby contributing to its cytotoxicity. After successful *in vitro* assays in human cell lines, it was tested *in vivo*, its therapeutic index being poor due to high affinity to proteins and extensive metabolism (S J Gregson *et al.*, 2001).

A second generation of PBD dimers includes the dimer SJG-136 (**10**) (**Figure 8**), predicted from molecular models and synthesized based on the monomer tomaymycin (**22**) depicted in **Figure 9**. The dimer SJG-136 differs from DSB-120 (**24**) (**Figure 10**) because of its unsaturation at C2 and C2' positions. This feature significantly reduces electrophilicity at N10-C11 moiety, so the resulting molecule, named as SJG-136 (**10**, C2-*exo*-methylene PBD dimer), was less reactive to nucleophilic molecules (Morris, Thurston and Nevell, 1990).

As for *in vitro* cytotoxicity, it has proven to be more biologically active than DSB-120 (**24**) against human tumour cell lines due to its increased ability to form interstrand cross-links of exocyclic N2 groups of guanine-guanine between opposite DNA strands (a unique mechanism of action which has never been described before). Therefore, its mechanism of action consists of the formation of different types of adducts depicted in **Figure 11**: interstrand (A) and intrastrand (B) cross-linked adducts, and mono-alkylated adducts (C) following the mechanism further described in section 4.7. Mono-alkylated adducts are formed in binding sites where neither intra on interstrand cross-links are possible because of the inappropriate distribution of guanines, where the dimer is inaccessible. Binding affinity to double stranded DNA and cytotoxicity data for C8/C8' dimers in which DSB-120 (**24**) and SJG-136 (**10**) are included are depicted in **Table 1**. Furthermore, it is compared to the monomer DC-81 in order to see the higher cytotoxicity of dimers against neoplasms (Rahman, James and Thurston, 2011).



**Figure 11.** Three different kinds of adducts formed by SJG-136 and DNA.  
A, interstrand cross-linked adduct; B, intrastrand cross-link adduct;  
C, mono-alkylated adduct.

**Table 1.** DNA binding affinity and cytotoxicity data of DC-81, DSB-120 and SJG-136

Compound	Cytotoxicity		$\Delta T_m$ ( $^{\circ}\text{C}$ ) <sup>a</sup>			
	Cell type	$GI_{50}$ ( $\mu\text{M}$ )	0 h	4 h	18 h	36 h
DC-81 (19)	ADJ/PD6	0.33	0.3	0.5	0.7	0.7
	ADJ/PD6	0.0005				
DSB-120 (24)	K562	0.2	10.2	13.1	15.1	15.7
	K562	0.043				
SJG-136 (10)	K562	0.043	25.7	31.9	33.6	-

<sup>a</sup> DNA binding affinity is evaluated in *calf thymus* DNA duplex.

When dealing with *in vivo* assays against different tumours, it exhibits a broad-spectrum of antitumour activity with multiple dosing regimens and relatively wide dose ranges. It has been tested in mice ovarian cancer xenograft models based on cisplatin-resistant tumours, indicating its potential use in drug-resistant disease (Alley *et al.*, 2004).

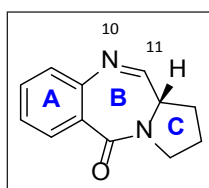
After observing favourable results both *in vitro* and *in vivo*, SJG-136 (10) is reported as a broad-spectrum anticancer agent that requires further clinical pharmacology and toxicology studies. As depicted in **Figure 12**, in 2004 it was proposed as a lead candidate suitable to undergo Phase I clinical trials in patients with advanced carcinomas showing satisfactory results that warrant future pharmacological development. At present it is in Phase II clinical trials, where it is demonstrating anticancer activity against platinum resistant or refractory ovarian carcinoma (Hochhauser *et al.*, 2009) (Janjigian *et al.*, 2010), (Puzanov *et al.*, 2011).



**Figure 12.** SJG-136 in clinical trials as a drug in anticancer therapy.

### 3.7. Mechanism of action

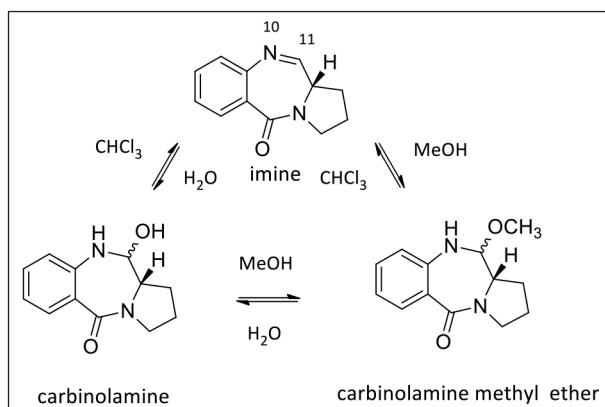
PBDs monomers bind to double-stranded DNA and experimental models have successfully given enough evidence of their sequence selectivity, which gives these molecules the property to downregulate genes' expression. Their placement within the minor groove and their interaction with nitrogenous base guanine give a reasonable explanation for their biological activity. Therefore, it is important to highlight the role of rings A, B and C that constitute the tricyclic core depicted in **Figure 13**, which is described below.



**Figure 13.** Structure of a PBD with A, B and C rings.

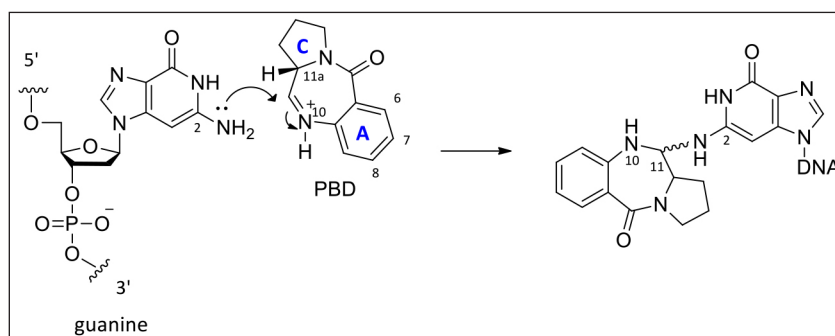
As regards the **A-ring**, it is necessary to remark that PBDs place themselves pointing the A-ring towards the 3' end of the strand and that the substitution patterns of C6, C7 and C8 do not take part in the interaction between the DNA double strand and the binding agent, as they point to the outside of the minor groove (**Figure 15**). As far as PBDs monomers are concerned, the substituent at C7 in sibiromycin (**13**) stabilizes the adduct through hydrogen bonding and Van der Waals forces, as well as the hydroxyl group in anthramycin (**9**), as it is a polar functional group. This finding gives a successful explanation why anthramycin (**9**) and sibiromycin (**13**) stabilize DNA to a greater extent rather than tomaymycin (**22**), neothramycin A (**20**) and neothramycin B (**21**), all of them lacking a hydroxyl group in position C9 (**Figure 9**). In contrast, analogues of anthramycin (**9**) and sibiromycin (**13**) containing a 9-OCH<sub>3</sub> group generally decrease the degree of binding to DNA because of steric hindrance, and they have demonstrated to be biologically inactive.

**B-ring** contains the N10-C11 moiety, which is responsible for PBDs biological activity due to their ability to bind covalently to the C2-NH<sub>2</sub> group of the guanine nitrogenous base. This group can exist in three different and interchangeable forms, all of them able to give alkylation to DNA: imine, carbinolamine and carbinolamine methyl ether (**Figure 14**). The mechanism of action implies the previous protonation of the N10-C11 iminium species as shown in **Figure 15** (Hurley et al., 1988).



**Figure 14.** Possible forms of PBDs are capable of alkylating DNA (imine, carbinolamine and carbinolamine methyl ether).

Another architectural feature to consider in PBDs scaffold is the chirality of C11a position (*S* configuration), which allows the molecule to have a longitudinal right-handed twist that locates the N10 position pointing to the floor of the minor groove, a prerequisite for the formation of a covalent bond between the C11 position and the amine group of the DNA through an equatorial attachment (**Figure 15**). As far as stereochemistry is concerned, a C11aR drug would only fit in a left-handed DNA double helix by an axial attachment, thus obstructing the fitting of the drug into the minor groove. Therefore, C11aS stereochemistry in guanine adducts is strongly favoured over C11aR stereoisomers as suggested by several theoretical and experimental studies. Moreover, it is important to outline that the racemization of C11a significantly reduces the biological activity (Rao, Singh and Kollman, 1986).



**Figure 15.** Diagram showing the mechanism of covalent binding of an electrophilic PBD unit with the nucleophilic C2-NH<sub>2</sub> group of a guanine base resulting in formation of an aminal bond.

**C-ring** enables us to distinguish PBDs monomers in the saturation degree as mentioned above. Experimental data has proven that both the stereochemistry and the size of the substituents in positions C2 (which places following the wall of the minor groove and establishes both hydrogen bonds and van der Waals interactions) determine the degree of binding to DNA. Therefore, large substituents prevent PBDs from binding to DNA as the accommodation inside the minor groove is impaired because of steric hindrance. This finding provides the explanation why anthramycin (**9**) is capable of stabilizing to a greater extent the DNA double strand rather than tomaymycin (**22**). Anthramycin (**9**) has a polar functional group (-OH) in the C9 position and a C2-acrilamide tail, which suggests a

hydrogen-bonding ability to DNA. Thermal denaturation experiments of *calf thymus* (CT) DNA after 18 h have proven that anthramycin (**9**) experimented  $\Delta T_m = 13.0^\circ\text{C}$ , whereas in the case of tomaymycin (**22**) it was only  $2.6^\circ\text{C}$ . Therefore, anthramycin (**9**) is more biologically active than tomaymycin (**22**).

Apart from substitution patterns in C2, substituents in the C3 position also play a remarkable role in adduct formation. Focusing on C3 substituents, it is important to outline the differences in neothramycin A (**20**) and neothramycin B (**21**). While neothramycin A (**20**) binds strongly to DNA, in the case of neothramycin B (**21**) the bond strength is weaker due to the change of the stereochemistry of 3-OH group. Regarding *O*-methoxy and *O*-butoxy derivatives from neothramycin A (**20**) and neothramycin B (**21**), they do not bind to DNA. Overall, it is important to consider the remarkable role of the carbinolamine group, as *N*-acetyl derivatives have shown negligible binding affinity to DNA, and variation in the structure of this functional group gives unreactive species towards DNA. Based on these results, the implication of carbinolamine to the alkylation of DNA is reaffirmed.

One specific feature to be considered about PBDs is their preference of binding to specific sequences of DNA, being in the majority of cases the consensus motif 5'-Pu-G-Pu-3' binding sites (Hurley et al., 1988). DNA-footprint experiments have proven that other binding to sequences is also likely to occur, although less frequently. The preference of sequences is, in decreasing order, 5'-Pu-G-Py > Py-G-Pu > Py-G-Py 3', inherently due to the helical twist characteristics (Kopka et al., 1994). Apart from experimental evidence providing an explanation for the adduct formation, *in vitro* transcription stop assays have evaluated the ability of these small molecules to attach DNA and block transcription in the coding region of genes. Moreover, other biochemical experiments have demonstrated that they can also inhibit the transcription binding factor. Both hypotheses have a point in common: regardless of their mechanism of impairing transcription of genes, both of them exert an effect against cancer cells, particularly towards those genes that are overexpressed. Another reasonable explanation is that the PBD-DNA adduct may be challenging because cancer cells are usually deficient in various DNA repair pathways compared to healthy cells. Therefore, this has also been thought of as a likely target.

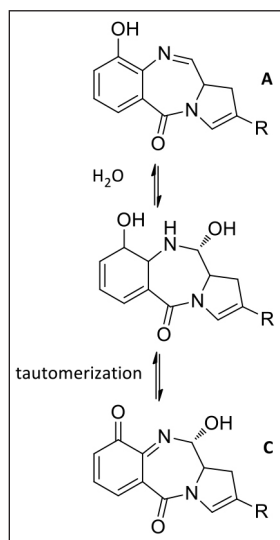
### 3.8. Toxicity

PBDs have been reported as strong anticancer agents. As a drawback, their toxicity cannot be ignored. Both anthramycin (**9**) and sibiromycin (**13**) have been tested clinically showing a wide range of activity against neoplasms, but also affecting healthy cells from the organism.

In particular, all these compounds are significantly cardiotoxic (Cargill, Bachmann and Zbinden, 1974). Cardiotoxicity has been closely associated with a quinone structure, which is not found in natural PBDs in the form they are isolated depicted in **Scheme 1, A**. As explained before, the imine moiety can be in equilibrium with the carbinolamine structure, which can tautomerize with the ortho quinone imine (**C**, **Scheme 1**). Thus, the ortho quinone imine scaffold is responsible for cardiotoxicity (Cipolla et al., 2009) agents which target and can recognize discrete sequences of DNA have the potential to offer selective therapies by modulating the activity of specific transcription factors or genes. For this reason, a number of sequence-selective DNA binding agents have been evaluated with a range of affinities and recognition fidelities. In this respect, the pyrrolo[2,1-c][1,4]benzodiazepines (PBDs. In the case of tomaymycin (**22**), which lacks of the C9-OH



group, it is considered to be negligibly cardiotoxic. According to these findings, the presence of the 9-OH group is therapeutically undesirable, as it leads to an increase in cardiotoxicity.



**Scheme 1.** Mechanism of formation of the ortho quinone imine structure.

### 3.9. Structure-activity relationship (SAR) studies

After the precise and unique mode of action of PBDs was extensively studied, it becomes presumably easier to design and synthesize a large number of novel and often much more potent PBDs analogues that offer additional biological and chemical information. Such analogues include chemical modifications in substitution patterns of the three rings.

Structure-activity relationships are divided into two major groups depending on the ring considered: A-ring modifications if the scaffold of the PBDs is changed in carbons 6, 7, 8 or 9, which are placed in the aromatic ring and C-ring modifications if the changes are made over the carbons 1, 2 or 3. As explained below, both of them influence in overall binding affinity, although C-ring exerts a broader impact on the PBDs biological activity (Thurston et al., 1999).

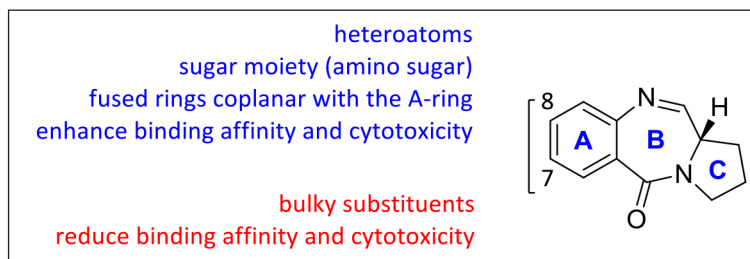
#### 3.9.1. Modifications on A-ring

Concerning the A-ring, it is important to take into account several features regarding the PBD skeleton. To begin with, electron-donating substituents at positions 7, 8 or 9 are a prerequisite in the A-ring in order to exert biological activity. The presence of electron-withdrawing groups in the A-ring results in an increase in the carbinolamine reactivity, with a consequent loss of DNA specificity. (Hurley and Boyd, 1988).

The observations that have been carried out are the following (**Figure 16**):

- Substituents with heteroatoms in positions C7 and C8 provide greater *T<sub>m</sub>* and increase reactivity, suggesting that polar contacts contribute to DNA binding.
- Moreover, bulky alkyl substituents significantly reduce the binding affinity, probably due to steric hindrance, as the penetration into the minor groove is a prerequisite in PBDs for later alkylation of guanine.

- As for the C7 position in particular, the presence of a sugar moiety (precisely, an amino sugar) in C7 position of the tricyclic core enhances both DNA affinity and cytotoxicity.



**Figure 16.** Structure-activity relationship of the A-ring system.

After analyzing the three forms in which N10-C11 moiety can exist, it is remarkable the influence of A-ring substituents upon it. Electron-donating substituents ( $\text{R-OCH}_3$  and  $\text{R-OH}$ ) on C7 promote the stabilization of the imine form (which is the vast majority of the cases). In contrast, electron-deficient substituents usually give place to the formation of the carbinolamine form. The explanation is simple: the withdrawal effect significantly reduces the availability of the lone pair of electrons on N10, which is directly involved in the further alkylation of guanine. Moreover, electron-withdrawing substituents also prevent the imine group from protonation, a prerequisite for nucleophilic attack.

### 3.9.2. Modifications on C-ring

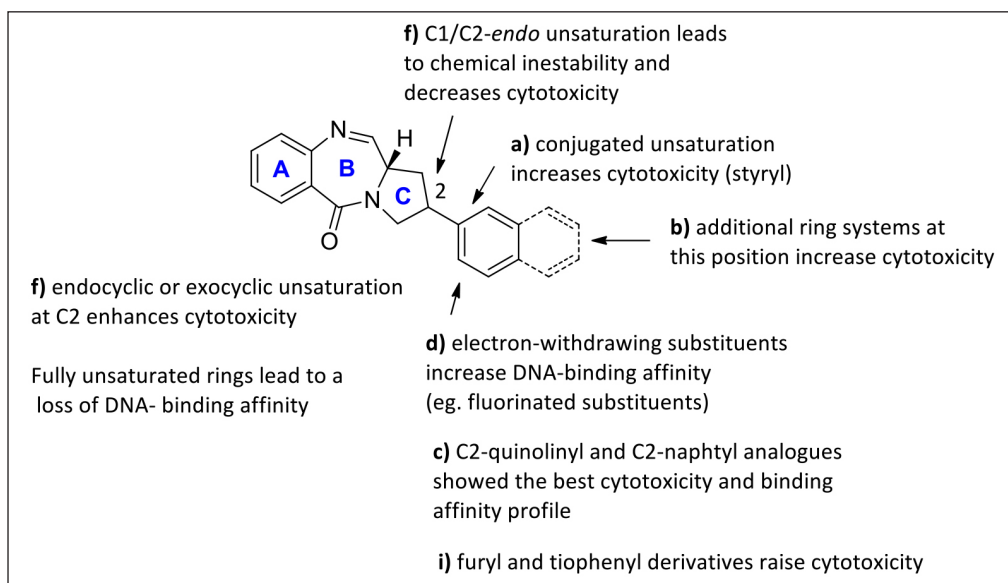
Different type of substitutions in the C-ring can provide a greater stabilization of the DNA duplex and, at the same time, enhance cytotoxicity (**Figure 18**).

#### 3.9.2.1. C2 substitution patterns

Regarding C2 substituted PBDs, binding affinity and cytotoxicity were studied by Thurston and co-workers in several *in vitro* experiments. These molecules exhibit more cytotoxicity than the analogues in which the substituent has been removed.

- The incorporation of an aryl or styryl substituent in the C2 position results in a significant increase in cytotoxicity.
- Molecules that showed good profiles of binding affinity and cytotoxicity were C2-quinolinyl ( $\text{GI}_{50} = 0.0004 \mu\text{M}$ ) and C2-naphtyl ( $\text{GI}_{50} = 0.0006 \mu\text{M}$ ) analogues (Antonow et al., 2010) 1-c[1,4]benzodiazepine (PBD).
- Regarding fluorine as a substituent in C2, it provides a greater stabilization of the DNA duplex and thus an increased binding affinity ( $\Delta T_m = 2.10^\circ\text{C}$ , after 18h of incubation) compared to its analogues in which fluorine has been removed ( $\Delta T_m = 0.74^\circ\text{C}$ , after 18 h of incubation).
- Furthermore,  $\Delta T_m$  shifts are slightly higher than the analogues lacking of a C2-substituent, which means it binds with higher affinity to double stranded DNA.

As a conclusion, all compounds that show a potent biological activity have a planar conformation of the C2-substituent ( $Csp^2$ ) (it is coplanar with C-ring) instead of having a tetrahedral conformation ( $Csp^3$ ), which is in most of them with less cytotoxicity. This architectural feature undoubtedly the best fit into the minor groove of DNA through hydrophobic interactions. In this way, it is demonstrated that the clue point that determines the potency of molecules remains on the planar configuration of substituents in C2, which allow substituents to fit perfectly within the minor groove of DNA (Cipolla et al., 2009).



**Figure 18.** Structure-activity relationships of the C-ring system.

### 3.9.2.2. Effect of C2/C3-*endo* unsaturation

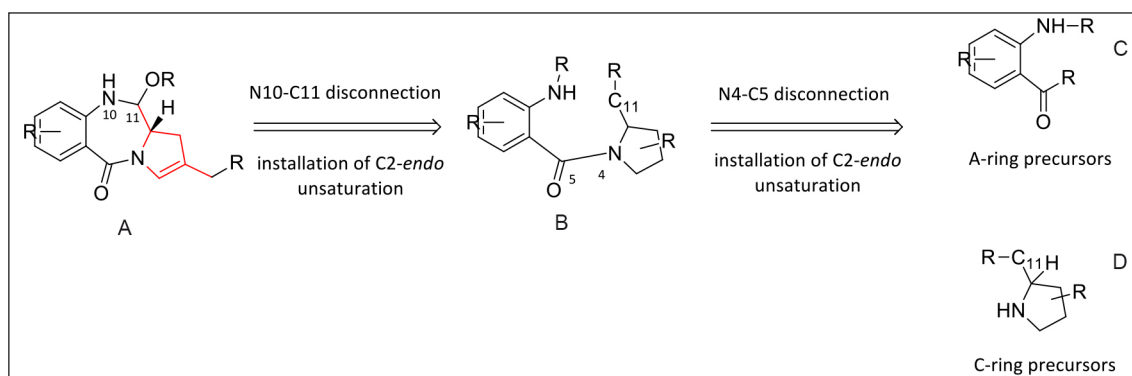
The introduction of C2/C3-*endo* unsaturation enhances the DNA binding affinity and cytotoxicity rather than its respectively unsaturated analogues. An attempt to synthesize unsaturated C2/C3 novel PBD analogues has been carried out by Thurston and co-workers. They compared the cytotoxicity of these analogues with anthramycin (**9**), and came up with the following conclusions (Cipolla et al., 2009):

- The introduction of C2-*exo* or C2/C3-*endo* unsaturation results in a modest cytotoxicity and in an increased binding affinity to DNA ( $GI_{50} = 1.28 \mu M$ ), with respect to the reference compound which is fully saturated ( $GI_{50} = 2.39 \mu M$ ).
- The best potency profile is shown by compounds with C2/C3-*endo* unsaturation with additional substitution in position C2.
- The introduction of C1/C2-*endo* unsaturation decreases cytotoxicity.
- The presence of C2/C3-*endo*-unsaturation with additional C2 substitution with heteroaryl groups such as thiophenyl ( $GI_{50} = 0.02 \mu M$ ) or furyl ( $GI_{50} < 0.01 \mu M$ ) raises cytotoxicity by 10-fold, compared to anthramycin ( $GI_{50} = 0.029 \mu M$ ) (Thurston et al., 1990).

### 3.10. Synthesis of PBDs

Since the isolation of anthramycin (**9**) and as the first PBD with potential use as a lead molecule for anticancer and antibacterial drug therapy, a great effort to synthesize PBDs has been made.

Retrosynthetic analysis of the PBDs skeleton reveals that the tricyclic core containing the imine moiety (**I**, **Scheme 2**) is formed by reaction A- and C-ring precursors (**III** and **IV**). A-ring starting materials are usually anthranilic acids or isatoic anhydrides. In other cases, the presence of a nitro group as the key functionality is also frequent, which leads through reduction to a secondary amine that reacts with the electrophilic C11-carbon from C-ring. C-ring precursors are pyrrolidine-derived building blocks.



**Scheme 2.** Retrosynthetic analysis of the tricyclic core of PBD from A- and C-ring precursors.

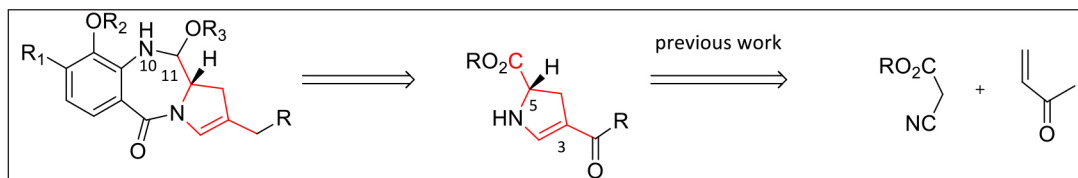
In the PBDs scaffold there are two remarkable points to consider:

1. The *S* configuration of the stereocenter at C11a position is a prerequisite for PBDs to be biologically active. Regarding C-ring precursors, the ones derived from L-proline fragments obtained from natural sources are of particular interest. They can provide directly the C11a*S*-stereochemistry required in the tricyclic core of the PBDs.
2. As for the saturation degree of the C-ring, it has previously been outlined that the most biologically active PBDs are unsaturated in positions C2-*exo* or C2/C3-*endo* (**Figure 9**). The installation of the double bond has been proposed in different synthetic routes, as it is explained below.

Many chemists have reasonably proposed the generation of the C2/C3-*endo* and C2-*exo* unsaturation in C-ring in several steps. In all synthetic procedures envisaged up to date, the introduction of the double bond arises after the formation of the tricyclic core, from a highly developed compound. In this field, synthetic pathways carried out by Gregson and co-workers, and Chen and co-workers, among others, require careful strategic planning around the final synthetic steps due to the chemical sensitivity of the N10-C11 moiety (Gregson *et al.*, 2000), (Stephen J Gregson *et al.*, 2001), (Thurston and Howard, 2000).

In order to avoid the cumbersome installation of the double bond after the formation of the tricyclic core, our research group in the Laboratory of Organic Chemistry in the Faculty of Pharmacy is investigating a new synthetic strategy that relies on the unprecedented use of enantiopure 3-acyl-5-alcoxycarbonyl-2-pyrrolines as starting materi-

als. Our group has recently synthesized them in enantiomerically pure form (Arróniz et al., 2011). Interestingly, the methylketone appendage in position 3 and the ester substituent in position 5 are the most suitable to give access to the PBDs compounds with the general structure depicted in **Scheme 3** through known organic transformations.



**Scheme 3.** Retrosynthetic analysis of the installation of the double bond proposed by our research group.

#### 4. Conclusions

After performing a bibliographic research into the drugs that are able to interact with DNA to exert their activity, this work allows us to draw the following conclusions:

- DNA is a target in cancer chemotherapy and, in fact, plenty of compounds exert their anticancer activity by interacting with DNA. They can be divided into three groups: alkylators, intercalators and groove binders, depending on their mechanism of action.
- There are several atoms in DNA minor and major grooves that are likely to interact with drugs through hydrogen bonding, Van Der Waals interactions and electrostatic interactions, leading to binding motifs.
- PBDs are sequence-selective DNA-interactive agents whose antitumour activity is due to their covalent interaction with specific A · T regions within the DNA minor groove. They have a tricyclic core formed by rings A, B and C and are divided into two main groups: PBDs monomers and dimers. It is necessary to outline the role in therapeutics of the PBD dimer named SJG-136 (**10**), which is currently undergoing Phase II clinical trials.
- The mechanism of action consists of a nucleophile attack of guanine PBD prior to the protonation of the imine moiety, with the consequent formation of the DNA adduct.
- Synthetic strategies have been described, and our research group has recently developed an enantiomeric synthetic approximation for the preparation of the C2/C3-unsaturated C-ring framework included in PBDs structures.

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