Published in final edited form as:

Med Res Rev. 2012 March ; 32(2): 254–293. doi:10.1002/med.20212.

Biosynthesis, Synthesis and Biological Activities of Pyrrolobenzodiazepines

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Abstract

Pyrrolobenzodiazepines (PBDs) are sequence selective DNA alkylating agents with remarkable antineoplastic activity. They are either naturally produced by actinomycetes or synthetically produced. The remarkable broad spectrum of activities of the naturally produced PBDs encouraged the synthesis of several PBDs, including dimeric and hybrid PBDs yielding to an improvement in the DNA binding sequence specificity and in the potency of this class of compounds. However, limitation in the chemical synthesis prevented the testing of one of the most potent PBDs, sibiromycin, a naturally produced glycosylated PBDs. Only recently the biosynthetic gene clusters for PBDs have been identified opening the doors to the production of glycosylated PBDs by mutasynthesis and biosynthetic engineering. The present review describes the recent studies on the biosynthesis of naturally produced pyrrolobenzodiazepines. In addition, it provides an overview on the isolation and characterization of naturally produced PBDs, on the chemical synthesis of PBDs, on the mechanism of DNA alkylation, and on the DNA binding affinity and cytotoxic properties of both naturally produced and synthetic pyrrolobenzodiazepines.

Keywords

pyrrolobenzodiazepines; DNA alkylation; anticancer; biosynthesis; anthramycin; sibiromycin; tomaymycin; SJG-136; lincomycin; DSB-120

1. INTRODUCTION

The 1,4-benzodiazepine ring system characterizes a class of compounds with diverse chemical structures and biological activities (Fig. 1). Four pharmacophore groups compose this class, the 1,4-benzodiazepine-2-ones, 1,4-benzodiazepine-2,5-diones, the dibenzodiazepinones and the pyrrolobenzodiazepines (PBDs). The most widely used members of the 1,4- benzodiazepines are the 1,4-benzodiazepine-2-ones, whose most prominent member is diazepam (valium®, Hoffmann-La Roche). At least 50 members of this group are clinically approved psychoactive drugs used for the treatment of insomnia, alcohol withdrawal, epileptic seizures, muscles spasms, and anxiety as anxiolytic¹. Some members of the second group, the 1,4- benzodiazepine-2,5-ones, have been tested as panxiolytic² and anticonvulsant³ as well as peptidomimetic inhibitors^{4,5}. In addition to their medicinal properties they also possess herbicidal activity⁶. Cyclopenin, the only naturally produced 1,4-benzodiazepine-2,5-one isolated, is particularly interesting as the biosynthetic

intermediate of viridicatin⁷, an inhibitor of replication of HIV⁸. The naturally produced TLN-4601 (formerly ECO-4601) is the sole member of the dibenzodiazepinones' group⁹ with a broad spectrum of antitumor activity in cancer cells and human xenografts¹⁰. Phase I/II trials (Thallion Pharma company web site: <http://www.thallion.com/en/drug-development/tln-4601.php>) are underway. The last group contains several naturally and synthetically produced PBDs. These compounds are minor groove sequence specific DNA alkylating agents with remarkable potency against cancer cells^{11–13} and are the subject of this review. Excellent reviews have recently covered advances in the synthesis of PBDs^{12,14–16}. Hence, we will report only on significantly bioactive synthetic PBDs as well as recently synthesized PBDs not covered by the above-mentioned review. The main focus of this review will be on recent discoveries in the biosynthesis of naturally produced PBDs and on the biological activities of these compounds.

2. ISOLATION AND CHARACTERIZATION OF NATURALLY PRODUCED PYRROLOBENZODIAZEPINES

Since the discovery of anthramycin in 1963¹⁷ 18 others PBDs produced by micrococci and streptomyces have been isolated (Table I, Fig. 2). Sixteen of these possess moderate antimicrobial activity (Fig. 2.A). The three C11-oxo-PBDs (Fig. 2.B) do not display any biological activity and, as discussed in section 3, are believed to be the product of a resistance mechanism present in the RK1441A, tomaymycin and limazepines producers. More recently 10 new pentacyclic pyrrolobenzodiazepines, the circumdatins, have been isolated from terrestrial and marine *Aspergillus* fungi^{18–22} (Fig. 3). The chemical structure of the circumdatins have been revisited based on the crystal structures of circumdatins A and B²¹. Only the isolation, spectroscopic and crystallographic characterization, and chemical synthesis^{23–25} but not the biosynthetic gene clusters have been reported on the circumdatins. Therefore, this review will not cover the pentacyclic pyrrolobenzodiazepines but it will focus on the bacterial PBDs, hereafter referred to as PBDs.

All PBDs contain the tricyclic ring system formed by an anthranilate (A), a diazepine (B) and a hydropyrrole (C) moieties shown in figure 4. Different degrees and types of substituents at the A- and C-rings provide chemical diversity among PBDs. For example, PBDs such as sibiromycin and sibanomicin are glycosylated at C7 of the A-ring (Fig. 2). In addition, the ring C can be fully saturated, unsaturated at the C2-C3 bond or exocyclically unsaturated at C2 as in neothramycin, anthramycin and tomaymycin, respectively. The PBDs can be in three different forms depending on the storage conditions (Fig. 4). In aqueous solution the imine form is in equilibrium with the carbinolamine form and it is stabilized by the presence of electron-withdrawing substituents at C8^{26–28}. The carbinolamine ether form is formed in protic solvent such as alcohols and it is in equilibrium with the carbinolamine form^{29,30}. The PBDs are most stable in the carbinolamine ether forms and as such they are usually stored in methanol at –20 °C²⁸. The crystalline imine and the carbinolamine methyl ether forms can be respectively obtained from boiling acetone and methanol-water²⁸. The literature, especially on the naturally produced PBDs as on tomaymycin³¹ and abbemycin³², has led to significant confusion by naming the methoxy form and referring to the carbinolamine and imine forms, respectively, as the 11-hydroxy

and anhydro derivatives. Particularly indicative are the cases of chicamycin and porothramycin. The methoxy derivative of chicamycin was named chicamycin A, while the imine form of chicamycin was named chicamycin B³³. The methoxy derivative of porothramycin was named porothramycin B, while the carbinolamine form was named porothramycin A³⁴. In most cases the carbinolamine form is considered the parent compound such as in mazethramycin³⁵, sibiromycin³⁶ and anthramycin²⁸. However, adding to this confusion, in other cases the imine form is considered such as in sibanomicin³⁷ and neothramycin³⁸. Equilibrium between the three forms, imine, carbinolamine, and methoxy of PBDs not only depends on the solvent used but also on the conditions of the analysis. For example, LC-ESI spectra of PBDs shows three peaks corresponding to the three forms and the ratio of the peaks can be changed by changing the acquisition parameters and the solvent composition^{39,40}. I am proposing to revisit the nomenclature by naming the imine form of PBDs as the parent form considering, as we will see later, that it is the biologically active form of this class of compounds. Figure 2 was drawn to follow this convention.

The literature on many naturally produced PBDs is limited to the initial isolation and characterization referenced in table I. Sibiromycin, tomaymycin, anthramycin and, to some extent, neothramycin are the only exceptions with several studies devoted to these compounds. Isolation of naturally produced PBDs entails a general protocol that includes an initial extraction using chloroform or ethyl acetate, followed by silica and gel filtration chromatography. The absorbance spectrum of PBDs has a characteristic peak at ~320 nm. 1D ¹H- and ¹³C-NMR have mainly been used for structural determination of PBDs. I refer to the references of table I for detailed conditions and structural characterization of the naturally produced PBDs. The crystal structure of anthramycin^{41,42} and tomaymycin⁴³ were pivotal not only to confirm the structure of these compounds but, more importantly, to assign the S-stereochemistry of C11a, which gives the characteristic right-handed twist of the PBD scaffold. The assignment of the structure of sibiromycin was not as straightforward. Spectroscopic characterization of the degradation products of sibiromycin obtained by harsh acidic hydrolysis and, subsequently, by catalytic hydrogenation and base hydrolysis lead to the wrong assignment of the aglycone of sibiromycin with an aromatic pyrrole ring³⁶ further confirmed by synthesis of the degradation products⁴⁴. Facile oxidation of the dihydropyrrole under the harsh acidic degradation conditions⁴⁵ used resulted in the incorrect assignment. 1D and 2D NMR studies of sibiromycin lead to the final assignment of the aglycone of sibiromycin⁴⁶ with a hydropyrrole moiety (Fig. 2). Similarly, the original structure³⁶ of sibirosamine moiety was revisited by detailed spectroscopic⁴⁷ analysis and further confirmed by synthesis of L-sibirosamine⁴⁸.

3. BIOSYNTHESIS OF THE PYRROLOBENZODIAZEPINES

Excellent reviews on precursors feeding experiments of the biosynthesis of anthramycin, sibiromycin and tomaymycin are available^{49,50}. In summary, the following conclusions were drawn:

1. L-Methionine, L-tyrosine and L-tryptophan are the immediate biosynthetic precursors of anthramycin, sibiromycin and tomaymycin^{51,52} (Fig. 5).

2. The indole nitrogen of tryptophan and the α -amino nitrogen of tyrosine are conserved in PBDs (Fig. 5)⁵³.
3. The biosynthesis of the hydropyrrole moiety of anthramycin, sibiromycin and tomaymycin proceed through a common pathway shared also by the biosynthesis of lincomycin, a natural product produced by *Streptomyces lincolnensis*^{54,55} (Fig. 6.A and Fig. 7). The pathway includes DOPA formation from L-tyrosine followed by extradiol cleavage to a 4-alanyl-2-hydroxy-muconate-6-semialdehyde, which undergoes cyclization to yield the cyclic α -ketoacid shown in figure 6.A. Removal of two carbon atoms from the side chain results in 4-vinyl-2,3-dihydropyrrole-2-carboxylic acid, considered the branch point of all these biosynthetic pathways⁵³.
4. Transformation of L-tryptophan to kynurenine by a common biosynthetic pathway for the anthranilate moiety of anthramycin, sibiromycin, and tomaymycin is proposed. For sibiromycin, kynurenine undergoes hydroxylation at C3, methylation at C4 and then transformation to the 3-hydroxy-4-methylantranilic acid (Fig. 6.B)⁵². A similar pathway was proposed for anthramycin due to the similarity of the anthranilate moiety between these compounds but it could not be verified based on the impermeability of *S. refuineus* to anthranilate analogs⁵². A very speculative pathway in which kynurenine is first hydroxylated at C4, then C5 and eventually converted to the 4,5-dihydroxyanthranilic acid was proposed for tomaymycin (Fig. 6.B)⁵⁶.
5. The tomaymycin to oxotomaymycin transformation is attributed to a constitutively enzyme. It is likely not co-regulated with the biosynthetic enzymes because this reaction occurs independently from the time of growth and in a strain incapable of producing tomaymycin⁵⁷. This can be a resistance mechanism of some PBDs producers.
6. Resistance to PBDs in producing strain is mainly achieved by changes in cell permeability to the antibiotic and it is regulated with the biosynthesis⁵⁸.

A quarter of a century has passed before another study on the biosynthesis of PBDs was published^{39,40,59}. In the mean time progress on the lincomycin biosynthesis was laying the foundation for future assignments of the PBDs biosynthetic pathways. Characterization of a nonproducing *S. lincolnensis*, yielded to the isolation from the growth media of 4-propylidene-3,4-dihydropyrrole-2-carboxylic acid, proposed as an intermediate in the lincomycin pathway (Fig. 7)⁶⁰. Accumulation of this intermediate is due to the inability in this strain to synthesize the F420 cofactor. Thus, this compound is the substrate of an F420-reductase whose gene was identified when the biosynthetic gene cluster was sequenced⁶¹. Limited functional assignment of the enzymes coded by the other genes of the cluster was possible due to the low or no similarity to characterize proteins in Protein Data Bank. Currently, the activities of only two enzymes, LmbB1 and LmbB2, involved in the tyrosine to hydropyrrole transformation in lincomycin biosynthesis have been assigned (Fig. 7). LmbB1 contains the catechol-2,3-dioxygenase signature sequence Hx₇FYx₂DPxGx₃E⁶² and shares very low sequence similarity to hypothetical glyoxylases. The reaction of purified LmbB1 with DOPA yields a yellow product with absorbance at 414 nm⁶³, which was later assigned to 4-(3-carboxy-3-oxopropenyl)-2,3-dihydropyrrole-2-carboxylic acid by capillary

electrophoresis coupled to ESI-MS⁶⁴. The formation of a transient product with absorbance at 378 nm followed by the 414 nm product has been observed spectrophotometrically⁶⁵. ¹H-NMR study of the reaction and spectral comparison with chemical standards support the enzymatic formation of the semialdehyde followed by non-enzymatic cyclization to the cyclic α -hydroxyacid⁶⁵ (Fig. 7). The activity of LmbB2 was indirectly assigned *in vivo* by observing conversion of tyrosine to the yellow colored compound only in cells transformed with a plasmid containing *lmbB1* and *lmbB2*⁶³.

The gene clusters for anthramycin⁵⁹, sibiromycin³⁹ and tomaymycin⁴⁰ have been recently identified and contain 25, 26 and 17 open reading frames, respectively (Tables II, III and IV). Functional assignments of the enzymes coded by the gene clusters were made possible by homology analysis, gene inactivation, chemical complementation, and comparison of the sibiromycin, tomaymycin, anthramycin and lincomycin gene clusters. The presence of genes in the sibiromycin and anthramycin gene clusters coding for proteins homologous to characterized enzymes involved in degradation of L-tryptophan to kynurenine confirms the tryptophan origin of the anthranilate moiety in these PBDs proposed by Hurley^{51,52,66}. Chemical complementation experiments support the intermediacy of L-3-hydroxykynurenine, 3-hydroxyanthranilic and 3-hydroxy-4-methylanthranilic acid (Table V). The failure of 4-methyl-3-hydroxyanthranilic acid to rescue production of anthramycin is simply due to the known impermeability of *S. refuineus* towards this compound⁵² and, therefore, it is not inconsistent with the intermediacy of this compound in the biosynthetic pathway. Adenylation by the ORF21 A-T didomain of 4-methyl-3-hydroxyanthranilic acid confirms the intermediacy of this compound⁶⁷. ORF24 based on inactivation and feeding experiment was proposed to be involved in the biosynthesis of the anthranilate moiety of anthramycin⁵⁹. Hu *et al.* speculated that ORF24 could be involved in the formation of 3-hydroxykynurenine and L-kynurenine⁵⁹. However, the absence of a gene encoding a homologous protein in the sibiromycin gene cluster³⁹ raises question on this assignment, especially considering that all the substituents present on the anthranilate moiety of anthramycin are present on sibiromycin. The precise role of this protein in the anthramycin biosynthesis remains unclear. The anthranilate moieties of sibiromycin is C-methylated at C8 and hydroxylated at C9 as in anthramycin, and is hydroxylated at C7 as in tomaymycin. Hydroxylation at C9 is likely to occur prior to the diazepine ring formation due to the similarity of SibC to kynurenine-3-monooxygenases and based on the chemical complementation experiments, which also support C8 methylation to occur after C9 hydroxylation and before diazepine ring formation³⁹ (Fig. 8.A). Tomaymycin contains the same C7 hydroxyl substitution at the anthranilate moiety found in sibiromycin while anthramycin does not. Gene inactivation and chemical complementation experiments confirm the assignment of SibG and TomO as the C7 hydroxylase⁴⁰ (Table V, Fig. 8.B).

Tryptophan was proposed as the precursor for the anthranilate moiety of tomaymycin based on the feeding experiments⁵², even though higher level of incorporations of radiolabeled were observed in tomaymycin when the cells were fed with labeled anthranilic acid compared to labeled L-tryptophan. In addition, in feeding experiment with tryptophan-[5-³H, 7-¹⁴C]⁵⁶ minimal ³H retention (16%) in tomaymycin was measured compared to anthramycin (100%) and sibiromycin (91%). Notably, only in tomaymycin, and not in

sibiromycin and anthramycin, incorporation of radiolabeled anthranilic acid was detected, which indicates a possible different biosynthetic pathway for the anthranilate moiety. The sequenced tomaymycin gene cluster is consistent with anthranilic acid as the precursor for the anthranilate moiety of tomaymycin. The presence of genes coding for proteins (TomD, TomP and TomC) with high similarity to enzymes involved in the transformation of chorismate to anthranilic acid and to enzymes involved in the shikimate pathway (Fig. 8.B) confirm the chorismate/anthranilic acid origin of the anthranilate moiety in tomaymycin. The biosynthesis of L-tryptophan in plants and microbes proceeds through chorismate, the product of the shikimate pathway, which is then transformed into anthranilic acid by anthranilate synthetase⁶⁸. Gene inactivation of *tomO* and rescue of tomaymycin production with 5-hydroxyanthranilic acid support C7 hydroxylation occurring prior to C8 hydroxylation, assigned by homology search to TomE and TomF. Both reactions occur prior to diazepine ring formation⁴⁰ (Fig. 8.B).

The biosynthetic pathways proposed for the hydropyrrole moiety of tomaymycin, sibiromycin, and anthramycin is mainly based on homology search, gene clusters comparison and the experimental data mentioned above on the lincomycin biosynthesis. We were able to confirm the tyrosine hydroxylase activity of SibU, ORF13 and TomI and of the extradiol dioxygenase activity of SibV, ORF12 and TomH (Fig. 9, unpublished data). 4-Vinyl-2,3- dihydropyrrole-2-carboxylic acid (Fig. 9) represents the branch point of the three biosynthetic pathways. Tomaymycin contains an ethylidene substituent at C2, while anthramycin and sibiromycin have a propylidene substituent at C2 (Fig. 2). As expected, the methyltransferase homologous to SibZ, ORF5 and LmbW necessary to elongate the C2 chain of the 4-vinyl-2,3- dihydropyrrole-2-carboxylic acid intermediate is not found in the tomaymycin gene cluster⁴⁰. The methyltransferase is proposed to catalyze also a methyl transfer coupled to a tautomerization reaction. Accordingly, the tomaymycin gene cluster contains a gene coding for TomN, a putative tautomerase⁴⁰, likely involved in the tautomerization of the 4-vinyl-2,3-dihydropyrrole-2- carboxylic acid intermediate. The presence of homologous proteins SibS, ORF15, TomK and LmbX in all PBDs and lincomycin gene clusters strongly supports a role for these enzymes in the formation of 4-vinyl-2,3-dihydropyrrole-2-carboxylic acid³⁹. Blast search of the Protein Data Bank fails to identify similar characterized proteins. However, a recent gene inactivation experiment showed the requirement of LmbX for production of lincomycin⁶⁹. We have proposed that these enzymes catalyze an unusual C-C hydrolysis of 4-(3-carboxy-3-oxo-propenyl)-2,3-dihydropyrrole-2-carboxylic acid similarly to BphD, a C-C bond hydrolase^{39,70}.

4-propylidene-tetrahydropyrrole-2-carboxylic acid is the last intermediate in common in the anthramycin, sibiromycin and lincomycin A biosyntheses (Fig. 9), which is the product of F420- dependent reduction of 4-propylidene-3,4-dihydropyrrole-2-carboxylic acid and accordingly it accumulates in lincomycin strain unable to produce F420 cofactor⁶⁰. LmbA, ORF6 and SibY contain conserved sequence motifs of F420-dependent reductases. F420 are flavin like cofactors involved in redox reaction substituted with side chain with different number of glutamate residues. Proteins homologous to γ -glutamyltransferases are found in all gene clusters considered consistent with the mechanism proposed (Fig. 9; Tables II, III and IV). The biosynthesis of lincomycin requires an additional reduction of 4-propylidene-

tetrahydropyrrole-2-carboxylic acid to yield the propyl side chain. Instead, the biosyntheses of anthramycin and sibiromycin diverge with the oxidation of 4-propylidene-tetrahydropyrrole-2-carboxylic acid to 4-propenyl-2,3-dihydropyrrole-2-carboxylic acid (Fig. 9). Enzymes involved in this transformation were assigned based on homology search and gene cluster comparison (Tables II and III)^{39,59}. Enzymes involved in the oxidation and amidation of the propenyl side chain should only be present in the anthramycin and, therefore, were assigned by a process of elimination (Table II)⁵⁹. The biosynthetic pathway for the sibirosamine moiety in sibiromycin is mainly based on sequence homology to characterized enzymes involved in sugar biosynthesis (Table III, Fig. 10)³⁹. Sibirosamine loading occurs after the PBD scaffold is formed as shown by the formation of the aglycone in the *sibG* strain (Table V, Fig. 10)³⁹. A clear biosynthetic strategy emerges from these studies in which the anthranilate and dihydropyrrole moieties are fully synthesized before formation of the diazepine ring (Fig. 11). This modular and assembly approach significantly eases future combinatorial biosynthetic studies.

4. SYNTHETICALLY PRODUCED PYRROLOBENZODIAZEPINES

Monomeric PBDs

Chemical syntheses of naturally occurring PBDs such as anthramycin^{71–73}, DC-81^{74–76}, tomaymycin⁷⁷ and chicaymycin⁷⁸ have been reported. Over 60 not naturally occurring monomeric PBDs have been synthesized^{76,78–90} and are shown in figures 12 and 13. Chemical synthesis of naturally occurring PBDs and their analogs is hampered by the stability of the N10-C11 imine bond, the retention of stereochemistry at C11a, the loss of unsaturation in the C-ring under reductive cyclization and dependency on ring A substituents for the diazepine ring closure (reviewed in ^{12,16}). The predominance of DC-81 analogs (Fig. 12) reflects the relatively ease of these synthetic procedures (9 steps and 18% overall yield⁷⁵) compared to those for more chemically diverse PBDs (Fig. 13)^{87,88}, and especially for PBDs with C2-*endo-exo* unsaturation (Fig. 13.B), which are laborious and often plague by modest yields. A common synthetic strategy has been employed for almost all these compounds (Fig. 14) in which the C5-N4 amide bond is formed first then followed by intramolecular cyclization through aza-Wittig reaction of azidocarbonyl compounds⁸⁰, catalytic hydrogenation of nitrocarbonyl compounds^{38,89}, or Swern oxidation of aminohydroxyl compounds^{82–84,91}. The first reaction results in direct formation of the PBD. Reduction of the amide bond with NaBH₄ or of the protected carbinolamine with Pd yields the final PBD in the second and third reactions, respectively. C2-*exo* unsaturation is introduced before the formation of the diazepine ring by Wittig oleifination of the C2 ketone⁸⁴. The C2-*endo* unsaturation is introduced after formation of the diazepine ring by reacting a C2 ketone compound with trifluoromethanesulphonic anhydride forming the C2-C3 enoltriflate⁹¹. A Stille reaction on the enoltriflate PBD allows introduction of the C2-*endo-exo* unsaturation⁸⁸. The C2-C3 enoltriflate was recently used in the synthesis of a 66-member library of C2-aryl PBDs⁸⁷ (Fig. 15). The novelties in this PBD synthesis are the use of solid-supported palladium catalyst and diethanolamine in the Suzuki coupling and in the subsequent work-up, respectively, and the microwave conditions during the Suzuki coupling. Both modifications improved the time of the reaction and the ease of purification. Some members of this family (55 and 56, Fig. 13) display interesting biological properties

(see section 5 and 6)⁹². The general appeal of the synthetic procedures of PBDs is diminished by the need of highly toxic reagents and by the tedious and lengthy syntheses of PBDs with different substituted anthranilate and C2-*endo-exo* pyrrole moieties⁷⁵ such in the synthesis of the sibiromycin aglycone⁴⁴. In addition, synthetic access to sibiromycin, the PBD with highest affinity to DNA among all synthetically or naturally produced monomeric PBDs (see section 5 and Table VI), and to glycosylated PBDs is prevented by difficulties associated with the synthesis of the sibirosamine sugar⁴⁸ and efficient conjugation to the core.

Dimeric and hybrid PBDs

A DNA alkylating compound should be specific for a 10 bp sequence to specifically modify a target double-stranded DNA. Most common DNA alkylating agents, including monomeric PBDs, fell short of this requirement. In order to increase the DNA base specificity of PBDs (see section 5), Thurston's group designed a PBD dimer with two DC-81 unit linked together by 1,3-propanedioldioxy ether linkage named DSB-120⁹³ (Fig. 16). The length of the diether linker was optimized to 3 methylenes by synthesis of DSB-120 analog with 3, 4, 5 and 6 methylenes^{94,95} and by comparison of their biological activities^{96,97} to DSB-120. The synthesized C7-linked dimers have not proven as biologically active as C8-linked^{98,99}. The most noteworthy PBD dimer is SJG-136 (Figure 16). The remarkable biological properties of this compound are discussed in section 6. The increased base sequence specificity and DNA affinity, the interstrand-crosslinking and the good cytotoxicity properties of DSB-120 and SJG-136 encouraged the synthesis of a variety of PBD dimers. Synthesized analogs of DSB-120 and SJG-136 are fluoride substituted dimers (as an example **57**, Fig. 16)^{86,100,101}, mixed imine-amide and imine-amine dimers (as an example **58**, Fig. 16)^{102,103}, and dimers with one of the methylenes of the linker substitutes with a piperazine or anthraquinone moieties (as an example **59** and **60**, Fig. 16)^{104,105}. These compounds and all the other dimeric PBDs synthesized before 2008 are reported in the excellent review by Cipolla *et al.*¹⁴. A new generation of PBD dimers includes C2-fluoro substituted analogs of DSB-120 and SJG-136 with a piperazine linker⁸⁶ (**61–63**, Fig. 16). The biological properties of the C2-aryl monomeric PBDs have recently inspired the synthesis of the dimeric C2/C2'-aryl compound **64** (Fig. 16)¹⁰⁶. Because of the insolubility of **61**, which prevented *in vivo* studies, compound **65** (Fig. 16) was synthesized¹⁰⁶. The slow reactivity of **65** towards DNA suggests that it is acting as a prodrug. Kamal and coworkers have recently synthesized the monomeric and dimeric β -glucuronide prodrugs (Fig. 17) encouraged by the efficient activation by *E. coli* β -galactosidase of a previously produced monomeric PBD β -galactoside prodrug¹⁰⁷ and on the high level of expression of β -glucuronidase in solid tumors compared to healthy tissues¹⁰⁸. Both monomeric and dimeric glucuronide prodrugs are efficiently cleaved *in vitro* by *E. coli* β -glucuronidase (Fig. 17) as determined by HPLC-activity assays¹⁰⁸. Two main strategies have been adopted for the synthesis of dimeric PBDs (Fig. 18). The first synthesis (Fig. 18.A) includes formation of a 2-nitrobenzoic acid dimer core followed by coupling of the pyrrolidine fragment, Swern mediated cyclization and deprotection of the carbinolamine or reduction yielding the imine^{106,109}. The introduction of C2-*exo* and *endo-exo* unsaturation is accomplished through the same synthetic reactions used for the monomers (Fig. 15). The second synthesis (Fig. 18.B) includes coupling of a nitrobenzoic acid compound to a pyrrolidine-2-carboxylate,

esterification with dibromoalkanes yielding the dimeric intermediate, which undergoes cyclization after reduction of the nitro groups^{86,100}. The syntheses of **64** and of the difluoro-DSB-120 (Fig. 18) are examples of the first and second strategies, respectively. The synthesis of the glucuronide prodrug is similar to the one adopted for the difluoro-DSB-120 (Fig. 18.B) with the additional steps required for the addition of the glucuronide promoiety that is added using triphosgene and dibutyltin dilaurate before formation of the diazepine ring and after reduction of the nitro group¹⁰⁸.

The ability to add a second monomeric PBD through a linker to form a dimeric PBD inspired the design of hybrid PBDs in which instead of a second monomeric PBD a different pharmacore is added. The main rationale for making these conjugates is to produce a compound with two pharmacophoric heads with different DNA sequence specificity. Classical example is the distamycin-DC-81 hybrid⁸² designed to exploit the AT and GC rich sequence specificity of distamycin and DC-81, respectively. This review will describe literature on hybrid PBDs published since 2008 (Fig. 19). For coverage of hybrid PBDs reported prior to 2008 I refer to the excellent review of Cipolla *et al.*¹⁴. Hybrid PBDs **66**, **67** and **68** (Fig. 19) with a naphthalimide and phenyl benzimidazole moieties tethered to DC-81 were produced to exploit their DNA intercalating properties and minor groove binding specificity at AT rich sequences, respectively^{110,111}. Additionally, the chromophoric properties of these moieties allow a detailed study of their binding mode to DNA discussed in section 5. Diethylphosphonate (**69**)⁹⁰, triazolebenzothiadiazine (**70**)¹¹², indole (**71**)^{113,114}, and enediyne (**72**, **73**)¹¹⁵ conjugates were synthesized to increase cytotoxicity and in some cases solubility. Two types of hybrid PBDs synthesized by Thurston *et al.* are of particular interest^{116,117}. The first type is a dimeric PBD, **74**, with a tripyrrole linker with a TATAA base pairs specificity¹¹⁶. The length of the linker allows interstrand cross-linking of DNA that covers roughly one turn of the helix. The second type is the fluorescent 7-diethylaminocoumarin PBD conjugates **75**, **76**, and **77**¹¹⁷ (Fig. 19). The fluorescent moiety can be utilized as a fluorescent marker in cellular localization studies allowing for the first time to characterize the cellular distribution of PBDs and to correlate it to the cytotoxicity.

5. DNA ALKYLATING PROPERTIES OF PYRROLOBENZODIAZEPINES

Monomeric PBDs

The inhibition of replication and transcription in cells by anthramycin as well as the high wavelength shift from 333 to 343 nm of the spectrum of anthramycin in the presence of DNA provided the initial clues on the DNA alkylating properties of monomeric PBDs^{118–121}. The reaction of anthramycin with RNA and DNA molecules *in vitro* show a lack of activity with RNA and a preference for double-stranded over single-stranded DNA¹²². The binding of anthramycin with DNA stabilizes the DNA as evident by an increase of the T_m of the complex compared to the unbound DNA^{118,122}. Copurification by gel filtration and coprecipitation in alcohol of PBD and DNA provided the first indication that a covalent bond is formed between PBD and DNA^{118,121}. Although the covalent bond formed is generally stable, the reverse reaction can occur albeit very slowly¹²³ but it can significantly speed up at pH below 3¹²². The reaction of only polydG single stranded DNA molecules, among the four single-stranded DNA molecules polydG, polydC, polydA and

polydT tested, pointed to guanine as the reactive site on DNA¹²⁴. CPK modeling¹²⁵, footprinting^{123,126}, NMR^{127–130}, and crystallographic¹³¹ studies identified as the chemical functionalities involved in the covalent bond the C11 on the PBD scaffold and the exocyclic amino group of guanine base on the DNA (Fig. 20). Alkylation of DNA by anthramycin, sibiromycin and tomaymycin is sequence specific with the following trend for binding preference 5'-Pu-G-Pu>5'-Py-G-Pu>5'-Pu-G-Py>5'-Py-G-Py^{126,132}.

The presence of the imine and carbinolamine forms in aqueous solution prompted the proposal of three different mechanisms^{133,134}. The first mechanism entails a direct S_N2 attack from the guanine nitrogen on the carbinolamine carbon with water elimination. The second mechanism includes an intramolecular cyclization of the Schiff base formed between the guanine nitrogen and the aldehyde resulting from the opening of the diazepine ring. The third mechanism entails a direct reaction between the imine form and the guanine nitrogen (Fig. 20). Lack of adduct formation with aldehyde analogs ruled out the Schiff base mechanism^{133–135}. The third mechanism is strongly supported over the carbinolamine mechanism by the formation of two diastereomeric tomaymycin-DNA adducts (11*S*, 11*aS* and 11*R*, 11*aS*) in amount different to the free diastereomers¹³⁰ (Fig. 21). Chirality is lost in the imine reaction intermediate also in the formation of the anthramycin-DNA adduct¹³¹. Further proof of the imine mechanism is the increased reactivity of PBDs with electron-donating substituents at the aromatic ring, which is consistent with a facile protonation of the imine nitrogen and a favored nucleophilic attack²⁶. Recent quantum mechanics/dynamics simulations of the binding of anthramycin to DNA shows that in the imine bond is polarized facilitating nucleophilic attack from the guanine¹³⁶.

The imine group at N10-C11 and the *S* configuration at C11*a* are the two chemical features always conserved among naturally occurring PBDs and intrinsically linked to the biological properties of these antitumor natural products. Because of the 11*aS* stereochemistry the PBD scaffold adopts a right handed twist from the anthranilate to the hydropyrrole ring that is a perfect fit for the minor groove of B-DNA molecule⁴² (Fig. 22). The right-handed twists of free anthramycin and tomaymycin are 35.4°^{41,137} and 9.1°⁴³, respectively. The right-handed twist increases by few degrees once anthramycin is bound to the DNA¹³¹. Significantly, the 11*aR* PBD with a left-handed twist does not bind to DNA²⁶. Two diastereoisomers can result from the reaction of the imine form of a PBD with DNA, the 11*S*, 11*aS* and the 11*R*, 11*aS*. The substituents on the PBD scaffold and the local sequence of DNA dictate which diastereoisomer is formed and, thus, the mode of binding. There are four possible modes of binding of a monomeric PBD to DNA (Fig. 21). In the first two, the 11*R*, 11*aS* and 11*S*, 11*aS* are bound with the anthranilate moiety pointing towards the 5' end of the modified strand. In the other two, the hydropyrrole moiety is pointing towards the 5' end. Anthramycin mainly binds to DNA with the hydropyrrole moiety ring on the 5' end of the modified strand resulting in the 11*S*, 11*aS* adduct as proposed first by molecular mechanics calculations^{30,138,139} and then shown by 2D-NMR studies^{128,140} and crystallography¹³¹. The formation of one adduct of anthramycin-DNA is likely due to the significant 35° twist of the anthramycin scaffold and to the steric clashes resulting from the 11*R*, whose axial bond would move the molecule away from the minor groove¹³¹. Hydrogen bonding among the anthranilate substituents and the acrylamide tail to the DNA bases seems to contribute to

the stability of the 11*S*, 11*aS* adduct^{131,140}. The C9 hydroxyl group, that in solution has a pK_a of 8.7¹²⁴, is protonated in the DNA adduct¹²⁵.

All four possible orientations have been observed in tomaymycin-DNA complexes as shown by NMR and fluorescence studies using the same DNA oligomer used in the anthramycin studies¹⁴¹ (Fig. 21). Fluorescence studies are possible because of the different fluorescent spectra of the 11*S*, 11*aS* and 11*R*, 11*aS* diastereoisomers¹³⁰. Fluorescence of free tomaymycin is lost at basic pHs when the phenolate ion is formed (pK_a of hydroxyl group at C8 is 8.0)¹⁴². However, at basic pH a quenching of fluorescence was not observed in the tomaymycin-DNA adduct indicating that also in this adduct the pK_a is perturbed and the C8 hydroxyl group is protonated¹⁴². The equilibrium distribution between the different possible adducts seems to be dependent not only on the chemical structure of the PBD such as the modest right-handed twist of tomaymycin but also on the sequence of DNA adjacent to the guanine base, site of the covalent bond^{132,141}. For example, with the d(CICGAATTCICG) duplex tomaymycin mainly forms only the 11*S*, 11*aS* with the hydropyrrole at the 5' end of the modified strand¹²⁹. The 11*R* stereoisomer is partly destabilized by an unfavorable interaction between O4 of tomaymycin and the phosphate oxygen of the DNA backbone¹²⁹ not present in the dATGCAT duplex.

Binding of anthramycin and tomaymycin to DNA causes very little distortion of the double helix^{129,131,136}. Although, formation of the PBD-DNA adduct induces a modest bending of the DNA around the covalent bond^{129,131,132}. The degree of bending is more pronounced in the tomaymycin-DNA (8.2–14.5°) than in the anthramycin-DNA (5–8.9°) adduct as judged by gel mobility assay¹³². Some degree of correlation between the structural flexibility of DNA and the sequence specificity has been observed¹³² with increased flexibility yielding to an increase in sequence specificity and in reactivity. The pseudo-first order rate constant for the formation of PBD-adducts is directly related to the sequence specificity¹³². Pseudo-first order rate constants measured for anthramycin varied from 0.1 to 0.005 min⁻¹ at pH 7.6¹³². The conformational flexibility of the drug and the degree of the right-handed twist, 9.1° for tomaymycin, contribute to explain the lower reactivity of tomaymycin vs. anthramycin and the higher bending of DNA in the tomaymycin covalent adduct^{131,132}. The DNA affinity of anthramycin, sibiromycin, tomaymycin, DC-81 and neothramycin shows the following trend from the highest affinity to the least sibiromycin>anthramycin>tomaymycin>DC-81>neothramycin^{13,143} (Table VI). Table VI lists all naturally and synthetically produced PBD compounds with a T_m above 2.0 °C in complex with calf thymus DNA with the exception of DC-81. Compounds not listed either show a T_m lower than 2.0 °C when bound to DNA or their T_m has not been reported. The high affinity of sibiromycin for DNA is likely due to the additional interactions between the amino sugar and the DNA backbone²⁷ as inferred from the structure of the anthramycin-DNA adduct. Inhibition of transcription *in vitro* correlates well with the DNA binding reactivity of these monomeric PBDs¹. The molecular basis for the 3 bp sequence selectivity of PBDs and the different reactivity of PBDs is still unclear. Among the synthetic monomeric PBDs only **55** (Fig. 13) has a DNA binding affinity comparable to sibiromycin as judged by thermal denaturation experiments with calf thymus DNA⁹² (Table VI). High resolution NMR studies shows that the C11*S*,C11*aS* diastereoisomer of **55** is bound with the

hydropyrrole ring towards 5' end of DNA of the covalently modified strand (Fig. 23, PBD code 2K4L). The C2-hapthyl ring buried in the minor groove of the DNA establishing van der Waals interactions that significantly contributes to the high binding affinity of **55**⁹².

Dimeric and hybrid PBDs

Characterization of a DNA duplex with two molecules of tomaymycin bound to guanine bases separated by six base pairs on opposite strand^{129,144} provided the structural basis for the design of the first PBD dimer, DSB-120⁹³ (Fig. 16). DSB-120 forms interstrand cross-links at 6 base pairs specific sequences 5'-PuGATCPy-3' or 5'-PyGATCPu-3'. When bound to DNA, DSB-120 assumes the 11*S*, 11*aS* stereochemistry^{93,145}. DSB-120, the dimeric equivalent of two DC-81, is much more reactive towards DNA than many monomeric PBDs (Table VI). The linker position at C8 is important for activity as shown by the negligible inhibition of transcription measured for the C7-linked homolog of DSB-120¹⁴⁶. However, as a result of the enhanced biological properties of monomeric PBDs with endocyclic/exocyclic unsaturation and substitution at C2¹⁴⁷ a new PBD dimer, SJG-136, was pursued¹⁴⁸.

Footprinting experiments indicate that SJG-136 forms interstrand cross-links with a similar base pairs selectivity as per DSB-120 by preferentially binding to 5'-GGATCC-3' but with higher affinity (Table VI)¹⁴⁶. Specificity for the AT bp is possibly due to the hydrogen bonding interaction of the N10 hydrogen and the adenine base¹⁴⁸. Recent HPLC-MS studies¹⁴⁹ of the cross-links products of SJG-136 to DNA show that dimeric PBDs such as SJG-136 in addition to the interstrand crosslinks form an intrastrand cross-links (Fig. 24) and monoalkylated adducts in the absence of an appropriately spaced guanine base. These results are apparently confirmed by high-field NMR studies¹⁴⁹ but at the time of writing this report they have yet to be published. Nonetheless, the above-mentioned data signify that the product distribution of these dimeric PBDs is not as simple as previously believed¹⁵⁰ and it can have serious implication for their biological activities.

Among the recently synthesized hybrid PBDs, the binding mode and specificity to DNA of **68** and **74** have been studied. Consistent to the majority of PBDs, the C11*S*,C11*aS* diastereoisomer of **68** is bound to the DNA. However, it assumes an opposite orientation in the minor groove with the anthranilate moiety, instead of the hydropyrrole, towards the 5' end of the covalently modified strand¹⁵¹. Structural distortions are apparent both by CD and NMR studies around the covalent bond in the complex^{111,151}. The binding of the PBD also causes a slight unwinding of the double helix as well as a narrowing of the minor groove. The major contribution to the binding affinity of **68** to DNA comes from van der Waals interactions. The N-methylpiperazine moiety is extremely disordered in the complex and fails to establish interactions with the DNA. As mentioned in section 4, **74** is a very remarkable hybrid dimeric PBD with a high affinity towards 5'**GCTTATAATGG**-3' (in bold are the sites of the covalent bond with the PBD moieties) with a XL_{50} of 0.06 μ M compared to the XL_{50} of 0.23 μ M of SJG-136¹¹⁶. Energy calculations of the binding of **74** to DNA with differently spaced covalently linked guanines indicate that a 7 bp spacer stabilizes interstrand over intrastrand cross-links. Molecular dynamic simulations suggest that very little distortion of the double helix upon binding occurs and that **74** binding site is 11 bp long¹¹⁶ unprecedented among any PBDs.

6. ANTINEOPLASTIC PROPERTIES OF PYRROLOBENZODIAZEPINES

Monomeric PBDs

The antimicrobial properties of monomeric PBDs are not particularly potent to warrant interest in this class of natural products. However, their antitumor properties are remarkable. The first animal¹⁵² and human studies on PBDs have been reported for anthramycin¹⁵³. Among the 219 patients treated some received crystalline pure anthramycin and others a crude extract. All had unresectable or unresponsive tumors such as lymphomas and sarcomas as well as tumors in the gastrointestinal tract and breast. Almost half of the patients were responsive and some showed a significant decrease in tumor size. No side effects were reported besides ulceration at the injection site¹⁵³. Using higher daily doses (0.5–1.0 mg/Kg) than those used in the clinical trials (0.02 mg/kg) intestinal necrosis was observed but it was reversed 36 h after drug administration¹⁵⁴. However, the identification of cardiotoxic side effects in rats at doses between 0.1–0.5 mg/Kg were most discouraging and suggested a disruption of the electron transport chain in mitochondria¹⁵⁵. The diminished lethality of anthramycin *in vivo* in the presence of CoQ points to inhibition of CoQ-dependent enzymes as the cause of the cardiotoxicity¹⁵⁶. The inhibitory form of anthramycin is the quinolinic resonance form of the C9 hydroxyl group. Neothramycin displays broad antitumor activities without bone marrow and neurotoxicity and, more importantly, without any cardiotoxic effects¹⁵⁷. Significantly, neothramycin does not have a C9 hydroxyl substituent (Fig. 2). The lack of side effects of neothramycin resulted in Phase I clinical trials that were not successful due to the lack of potency of this compound¹⁵⁸. However, neothramycin seems particularly effective in the topical treatment of bladder cancer with complete disappearance of the tumor in 36 % of patients and with more than 50% regression of the tumor in 55 % of patients¹⁵⁹. Because the twist of the PBD structure matches very well the twist in the minor groove, it is believed that PBD modification is particularly effective at evading the DNA error correction machinery that would otherwise strip modified nucleobases at damaged positions¹²³. Another attractive property of PBDs is the absence of bone marrow suppression in model organisms, a serious side effect of many chemotherapeutics¹⁶⁰.

Structural-activity relationships studies

The availability of naturally produced PBDs and of synthetic PBDs has allowed the determination of their structure-activity relationships (Tables VI and 7). Change in the stereochemistry of C11a from *S* to *R* results in PBD compounds devoid of the biological properties of the corresponding *S* stereoisomers²⁷. Substituting the anthranilate ring with a heterocycle ring such as pyridine, pyrazine, pyrimidine or pyrazole decreases the binding affinity and potency¹³. The presence of unsubstituted anthranilate moiety in PBDs diminishes their potency¹³. Bulky substituents at C9 of the anthranilate moiety inhibit DNA binding and diminish the potency of PBDs¹³. C9 hydroxyl group has been proposed to be the source of the cardiotoxic properties of anthramycin^{27,155,156}. C9 hydroxyl group is likely not significantly contributing to the potency of anthramycin. Compounds **48–50**, analogs of anthramycin missing the C9-hydroxyl groups, have similar average GI₅₀ in the NCI cancer cells screen (Table VII)⁹¹. Therefore, an unsubstituted C9 is preferred for PBDs with chemotherapeutic properties. The presence of electro-donating groups such as methoxy

substituents at C7 and C8 shifts the equilibrium from the carbinolamine to the imine form, increasing the biological activities of PBDs¹³. As previously mentioned, O-glycosylation at C7 further increases the DNA binding affinity and cytotoxic properties of PBDs as in sibiromycin¹³. All PBDs with high antitumor activities, such as anthramycin, sibiromycin and tomaymycin, share a common endocyclic or exocyclic C2 unsaturation^{83,84,91}. C2-*endo* or *exo* unsaturation is likely to enhance the properties of PBDs by flattening the ring and, thus, improving the fit to the minor groove¹³. Therefore, C2-*endo* or *exo* unsaturation is a prerequisite for optimization of PBDs's properties. Full saturation at the ring C significantly inhibits DNA binding, while complete unsaturation at the ring C prevents any DNA binding¹⁶¹. The presence of an additional unsaturation at the C2 side chain characterizes the most potent PBDs as in the propenyl side chain of sibiromycin and was the basis of the design and synthesis of the C2-aryl PBDs (Fig. 13). A screening of C2-aryl PBDs identified DHR-417 (**52**) with mean IC₅₀ values of 3 nM against melanoma, breast and renal cells carcinoma¹⁶². The maximum tolerated dose by intraperitoneal administration for this compound is 0.5 mg kg⁻¹. DHR-417 shows promising potency only in renal xenografts studies¹⁶².

Dimeric and hybrid PBDs

The modest potency of DSB-120 in *in vivo* studies and its high reactivity with thiols¹⁶³ lead to the development of SJG-136. SJG-136 is a very potent antiproliferating compound *in vitro* with GI₅₀ values between 0.14–320 nM and significant cell selectivity¹⁶⁴. Interestingly, the activity profile of SJG-136 is different from other known alkylating compounds indicative of a novel mode of action¹⁶⁴. Four hours after administration SJG-136 can still be detected in plasma. The half-time for removal of SJG-136 in mice is 0.98 h. Most significantly the remarkable activity of SJG-136 is persistent *in vivo* showing significant tumor regression especially in melanoma, leukemia and lung xenografts tumor¹⁶¹. An LD₅₀ of 9.06 nM for SJG-136 was determined in efficacy studies on tumor cells taken from lymphocytic leukemia (B-CLL) affected patient¹¹. The high potency of SJG-136 is likely due to the failure of recognition of the PBD-DNA complex by repair proteins as for other PBDs¹⁶⁵. The potency, the unique specificity and the low toxicity in animals of SJG-136 prompted the start of phase I clinical trials to evaluate the maximum tolerated dose through intravenous administration and the optimal dosage to be used in phase II clinical trials. The MTD tested for SJG-136 had to be reduced significantly to 45 µg m⁻² due to unforeseen side effects such as edema and ascite in surrounding tissue to the injection site (vascular leak syndrome, VLS) and some degree of hepatotoxicity¹⁶⁶. The VLS symptoms can be decreased by treatment with steroids and diuretics¹⁶⁷. Based on the company web-site (<http://www.spirogen.com/sg2000.php>) at the time of writing this review phase II trials of SJG-136 (SG2000) will begin soon.

Among the most recently synthesized PBD compounds, the coumarin PBD conjugates are of particular interest¹¹⁷. The fluorescence properties of these compounds allowed evaluating their cellular distribution and, specifically, whether they can successfully localized in the nucleus. Compound **75** with the ideal three-carbon linker is the most potent and it is the only one among the three tested that predominantly localizes in the nucleus. The less potency of compound **76** can be attributed to a less efficient localization in the nucleus. Consistent with

this analysis compound **77**, which is only found in the cytoplasm, is the least potent of the three. It is apparent that the cellular distribution of this compounds is a function of the chemical structure of the linker¹¹⁷. Lack of uptake in the nucleus could explain the relatively modest cytotoxic activity of **67** compared to its DNA binding affinity¹¹⁰.

GI₅₀ and TGI₅₀ values support localization of **74** in the nucleus despite its MW of 984¹¹⁶. This compound showed significant cytotoxic activity in the NCI 60 cell lines screen. Other noteworthy compounds recently made are **72** and **73** with GI₅₀ values from 0.28 to 0.92 μ M for leukemia, colon, ovarian, renal and breast cancer cells¹¹⁵. The most noteworthy PBDs recently synthesized is the dimer of DHR-417 (**64**) and its produrg **65**¹⁰⁶ that show cytotoxic activity in the picomolar range (Table VIII). Preliminary data indicates that this compound is similarly active *in vivo*¹⁰⁶. The poor DNA binding affinity of **70** is reflected in modest potency. Weak cytotoxic activity characterizes the difluoro substituted DC-81 dimer with a piperazine linker⁸⁶. The piperazine linker decreases the cytotoxic activity as evident by comparison of **62** to C2- difluoro-DSB-120 (Table VII). However, the C2-difluoro modification has an even more deleterious effect as shown by the very good GI₅₀ average value for **61** compared to those of **62** (Table VII). Among the phosphonate PBD conjugates, only compound **69** with n=3 and m=1 displays significant cytotoxic activity especially for myeloma⁹⁰.

Mode of action of PBDs

It is only recently that researchers have started to address the mechanism of PBD-induced cell death. Inhibition of DNA binding by the Sp-1, NF-Y, NF- κ B and AP-1 transcription factors occurs in the presence of PBD-distamycin¹⁶⁸, of the polyamide GWL-78¹⁶⁹ and of the indole PBD **71**¹⁷⁰, respectively. Recent genetic profiling of sensitive tumors treated with DHR-417 identified many transcriptional factors in the first 150 up-regulated genes but Sp-1 is not included. DHR-417 was proposed to induce cell apoptosis through an insulin-like growth factor signaling pathway¹⁶². Clearly, research aimed at elucidation the mode of action of PBD and, specifically, the identification of the target genes is at its infancy but it is necessary for a successful use of the PBDs as antineoplastic drugs.

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Biography

Barbara Gerratana received her degree in Chemistry from the Università degli Studi di Pavia in Italy. Soon after she pursued a Ph.D. in Biochemistry with Profs. W. W. Cleland and P.A. Frey at the University of Wisconsin-Madison. She worked as a post-doc with Prof. C.A. Townsend in the Chemistry Department at The Johns Hopkins University. She joined the faculty of the Department of Chemistry and Biochemistry at the University of Maryland, College Park, where she is now an Assistant Professor. Her current research focuses on the biosynthesis of pyrrolobenzodiazepines and on the structural and mechanistic characterization of NAD⁺ synthetase, a multifunctional enzyme essential for M. tuberculosis.

1,4-benzodiazepine ring system

1,4-benzodiazepine-2-ones:
Diazepam (Valium)

1,4-benzodiazepine-2,5-ones:
cyclopinin

Dibenzodiazepinones: Diazepinomicin/TLN-4601

Pyrrolobenzodiazepines: Anthramycin

Figure 1.

The 1,4-benzodiazepine ring system and representative members of the 1,4- benzodiazepine class.

Figure 2. Naturally produced pyrrolobenzodiazepines: **(A)** bioactive and **(B)** not bioactive PBDs.

Figure 3.
Revised structures of circumdatins A and B.

Figure 4.
Epimerization of C11 in the presence of protic solvent such as water and methanol.

Figure 5. Labeling patterns in tomaymycin, sibiromycin and anthramycin. Atoms from Ltryptophan, L-tyrosine, and L-methionine are in grey, bold black and italic/shadow black, respectively.

Figure 6. Proposed biosynthetic pathways based on the feeding experiments for the hydropyrrole (**A**) and anthranilate (**B**) moieties.

Figure 7.
The biosynthesis of the tetrahydropyrrole moiety in lincomycin.

Figure 8.
Biosynthetic pathways for the anthranilate moiety of sibiromycin and anthramycin (**A**), and of tomaymycin (**B**).

Figure 9.
Biosynthetic pathways for the hydropyrrole moiety of sibiromycin, anthramycin, lincomycin and tomaymycin.

Figure 10. Biosynthetic pathways for the sibirosamine moiety of sibiromycin and for its transfer to the PBD aglycone.

Figure 11.
Biosynthetic strategy for PBDs.

Figure 12.
Chemically synthesized analogs of DC-81.

Figure 13. Chemically synthesized C2-*exo* (A) and C2-*endo-exo* (B) unsaturated PBDs, and C2-*endo* unsaturated aryl-substituted PBDs (C).

Figure 14.

The common retrosynthetic approach for monomeric PBDs.

Figure 15.

Synthetic procedure for a 66-member C2-endo aryl-PBD library.

Figure 16.
PBD dimers.

Figure 17.
Activation of the dimeric β -glucuronide PBD prodrug.

Figure 18. Syntheses of the compound **64** (A), a C2-aryl dimeric PBD, and difluoro-DSB-120 (B).

Figure 19.
PBD conjugates.

Figure 20.
Mechanism of the formation of the PBD-DNA complex.

Figure 21.

Four possible modes of binding of monomeric PBD to DNA, the example of tomaymycin-DNA.

Figure 22.

Crystal structure of the anthramycin (in black)-DNA (CCACGTTGG decamer, in grey) at 2.3 Å resolution (PDB code 274D). The duplex is represented in CPK in (A) and in sticks in (B).

Figure 23.
NMR structure of **55** (in sticks)-DNA (in CPK) complex (PDB code 2K4L).

Figure 24.
Interstrand (**A**) and intrastrand (**B**) cross-links of SJG-136 to DNA.

Table I

Producers and antimicrobial activity of bacterial PBDs

PBDs	Producer	Antimicrobial activity, two best MICs or inhibition diameter
Anthracycline ^{153,171}	<i>Streptomyces refuineus thermotolerans</i> NRRL 3143 <i>Strept. spadicogriseus</i> ATCC 31179	<i>Staph. aureus</i> : 0.1–5 µg/mL <i>B. subtilis</i> : 0.1–5 µg/mL
Tomaymycin ^{31,172}	<i>Streptomyces achromogenes</i> ATCC 3143	<i>Staph. aureus</i> : 6 µg/mL <i>B. subtilis</i> ATCC-6633: 12.5 µg/mL
Oxotomaymycin ⁵⁷	<i>Streptomyces achromogenes</i> ATCC 3143	No activity
Sibiromycin ¹⁷³	<i>Streptosporangium sibiricum</i>	<i>Staph. aureus</i> : 1 µg/mL <i>B. subtilis</i> : 0.3 µg/mL
Sibanomicin ³⁷	<i>Micromonospora</i> sp. SF2364	<i>Staph. aureus</i> S-424: 12.5 µg/mL <i>B. anthracis</i> 119: 25 µg/mL
Chicamycin A ³³	<i>Streptomyces</i> sp. J576-99	<i>Micrococcus luteus</i> PCI 1001: 50 µg/mL <i>Streptococcus pyogenes</i> A20201: 50 µg/mL
Mazethramycin ³⁵	<i>Streptomyces thioluteus</i> ME561-L4	<i>B. subtilis</i> PCI 219: 1.6 µg/mL <i>Xanthomonas oryzae</i> : 1.6 µg/mL
Prothracarcin ¹⁷⁴	<i>Streptomyces umbrosus</i> subsp. <i>raffinophilus</i> DO-62	<i>B. subtilis</i> 10707: 50 µg/mL <i>E. coli</i> ATCC 26: 50 µg/mL
Abbeymycin ³²	<i>Streptomyces</i> sp. AB-99F-52	<i>B. loeschii</i> ATCC 15930: 16 µg/mL <i>Bacteroides fragilis</i> ATCC 25285: 16 µg/mL
Porothramycin ³⁴	<i>Streptomyces albus</i> K731-113	<i>B. subtilis</i> PCI 219: 1.6 µg/mL <i>Bacteroides fragilis</i> A 22053: 0.8 µg/mL <i>E. coli</i> ATCC 26: 50 µg/mL
DC-81 ^{175,176}	<i>Streptomyces</i> sp.	Not reported
RK-1441A ^{177,178}	<i>Streptomyces</i> sp. RK-1441	<i>E. coli</i> BE 1186: 100 µg/mL
RK-1441B ^{177,178}	<i>Streptomyces</i> sp. RK-1441	No activity
Neothramycin A ^{38,179}	<i>Streptomyces</i> sp. MC916-C4	<i>Aeromonas salmonicida</i> ATCC14174: 25 µg/mL <i>Xanthomonas oryzae</i> : 50 µg/mL
Neothramycin B ^{38,179}	<i>Streptomyces</i> sp. MC916-C4	<i>Aeromonas salmonicida</i> ATCC14174: 50 µg/mL <i>Xanthomonas oryzae</i> : 100 µg/mL
Limazepine A ¹⁸⁰	<i>Micrococcus</i> sp. ICBB8177	No activity
Limazepine C ¹⁸⁰	<i>Micrococcus</i> sp. ICBB8177	<i>E. coli</i> 10 mm (40 µg/disk) <i>Staph. aureus</i> 18 mm (40 µg/disk)
Limazepine E ¹⁸⁰	<i>Micrococcus</i> sp. ICBB8177	<i>E. coli</i> 10 mm (40 µg/disk) <i>Staph. aureus</i> 12 mm (40 µg/disk)
Limazepine F ¹⁸⁰	<i>Micrococcus</i> sp. ICBB8177	No activity

Table IIDeduced functions of ORFs in the anthramycin biosynthetic gene cluster⁵⁹

gene	size ^a	putative function	NCBI accession number of protein homolog	%identity/s imilarity
<i>ORF1</i>	624	amidotransferase	BAB12569	57/68
<i>ORF2</i>	500	aldehyde dehydrogenase	CAD30313	-----
<i>ORF3</i>	354	alcohol dehydrogenase	EAO60654	50/65
<i>ORF4</i>	410	cytochrome P-450 hydroxylase	CAJ23858	49/63
<i>ORF5</i>	352	methyltransferase	ABW71852 (ORF21)	47/58
			TomA	40/53
<i>ORF6</i>	621	γ -glutamyltransferase	LmbBA	79/87
			SibY	56/67
			TomL	50/59
<i>ORF7</i>	487	FAD oxidoreductase	SibW	41/48
<i>ORF8</i>	764	UvrA-drug resistance pump	SibF	61/75
			TomM	66/78
<i>ORF9</i>	256	putative hydroxylase/glyoxylase	CAB55527	27/40
<i>ORF10</i>	377	transporter	EAL16816	41/66
<i>ORF11</i>	89	none	----	----
<i>ORF12</i>	169	extradiol dioxygenase	SibV	45/59
			TomH	44/52
<i>ORF13</i>	302	tyrosine hydroxylase	SibU	36/46
			TomI	44/52
<i>ORF14</i>	297	F420-dependent reductase	LmbY	50/63
			SibT	57/70
			TomJ	47/59
<i>ORF15</i>	276	unknown	LmbX	34/41
			SibS	32/43
			TomK	34/42
<i>ORF16</i>	413	kynureninase	SibQ	45/57
<i>ORF17</i>	261	tryptophan 2,3-dioxygenase	SibP	58/68
<i>ORF18</i>	58	None	----	----
<i>ORF19</i>	348	aromatic C-methyltransferase	SibL	45/54
<i>ORF20</i>	296	aryl formamidase	SibK	45/54
<i>ORF21</i>	600	NRPS	SibE	47/58
			TomA	39/52
<i>ORF22</i>	1146	NRPS	SibD	41/53
			TomB	37/49
<i>ORF23</i>	500	kynurenine 3-monooxygenase	SibC	44/58
<i>ORF24</i>	475	FAD-oxidoreductase	ABF87356	36/50
<i>ORF25</i>	273	repressor response regulator	SibB	33/46

^aNumbers are in amino acids.

Table III

Deduced functions of ORFs in the sibiromycin biosynthetic gene cluster³⁹

gene	size ^a	putative function	NCBI accession number of protein homolog	%identity/s imilarity
<i>sibA</i>	299	putative regulator	BAC79018	31/43
			ORF25	33/46
<i>sibB</i>	94	none	-----	-----
<i>sibC</i>	477	kynurenine 3-monooxygenase	XP_001514157	31/48
			ORF23	44/58
<i>sibD</i>	1504	nonribosomal peptide synthetase	ORF22	41/53
			TomB	42/54
<i>sibE</i>	597	nonribosomal peptide synthetase	ORF21	47/58
			TomA	40/53
<i>sibF</i>	771	UvrA-drug resistance pump	YP_001822519	55/70
			ORF8	61/75
			TomM	61/75
<i>sibG</i>	352	NADH-dependent flavin oxidoreductase	YP_001823080	20/25
			TomO	40/49
<i>sibH</i>	393	glycosyltransferase	ABO28818	48/57
<i>sibI</i>	332	dTDP-glucose synthase	AAS79450	57/69
<i>sibJ</i>	582	dTDP-4-keto-6-deoxyglucose 3,5-epimerase	BAC55217	48/61
<i>sibK</i>	822	esterase/aryl formamidase	YP_001676677	38/53
			ORF20	45/54
<i>sibL</i>	345	C-methyltransferase	AAL33761	38/53
			ORF19	45/54
<i>sibM</i>	417	Sugar C-methyltransferase	AAF01816	54/66
<i>sibN</i>	390	dTDP-4-keto-6-deoxyglucose transaminase	ZP_02842724	51/68
<i>sibO</i>	246	N-methyltransferase	CAA63163	38/49
<i>sibP</i>	262	tryptophan 2,3-dioxygenase	YP_001812683	37/57
			ORF17	58/68
<i>sibQ</i>	394	kynurenine hydrolase	CAJ89348	50/60
			ORF16	45/57
<i>sibR</i>	251	unknown	AAK59995	33/39
<i>sibS</i>	300	unknown	LmbX	41/52
			ORF15	32/43
			TomK	43/52
<i>sibT</i>	293	F420-dependent reductase	LmbY	54/67
			ORF14	57/70
			TomJ	53/64
<i>sibU</i>	318	tyrosine hydroxylase	LmbB2	39/49
			ORF13	36/46
			TomI	39/48
<i>sibV</i>	152	L-DOPA 2,3-dioxygenase	LmbB1	56/67

gene	size ^a	putative function	NCBI accession number of protein homolog	%identity/s imilarity
			ORF12	45/59
			TomH	51/58
<i>sibW</i>	492	FAD-dependent oxidoreductase	ORF7	41/48
<i>sibX</i>	391	Transcriptional regulator	YP_001823517	21/33
<i>sibY</i>	598	γ -glutamyltransferase	LmbA	57/68
			ORF6	56/67
			TomL	52/62
<i>sibZ</i>	337	methyltransferase	LmbW	56/65
			ORF5	60/74

^aNumbers are in amino acids.

Table IV

Deduced functions of ORFs in the tomaymycin biosynthetic gene cluster⁴⁰

gene	size ^a	putative function	NCBI accession number of protein homolog	%identity/similarity
<i>tomA</i>	614	nonribosomal peptide synthetase	ORF21	39/52
			SibE	41/54
<i>tomB</i>	1542	nonribosomal peptide synthetase	ORF22	37/49
			SibD	42/54
<i>tomC</i>	406	putative 3-deoxy-D-arabinose-heptulosonic 7-phosphate (DHAP) synthase	CAL34108	47/58
<i>tomD</i>	677	phenazine biosynthesis protein PhzE	YP_001348741	44/59
<i>tomE</i>	206	Phenol-2-monooxygenase reductase component	YP_707281	36/46
<i>tomF</i>	533	Phenol-2-monooxygenase oxygenase component	YP_702343	63/74
			NP_927609	66/79
<i>tomG</i>	234	O-methyltransferase	YP_001644688	47/60
<i>tomH</i>	145	L-DOPA-2,3-dioxygenase	LmbB1	54/59
			ORF12	44/52
			SibV	51/58
<i>tomI</i>	318	tyrosine hydroxylase	LmbB2	48/61
			ORF13	44/52
			SibU	39/48
<i>tomJ</i>	319	F-420 dependent reductase	LmbY	50/63
			ORF14	47/59
			SibT	53/64
<i>tomK</i>	287	unknown	LmbX	45/56
			ORF15	34/42
			SibS	43/52
<i>tomL</i>	577	γ -glutamyltransferase	LmbA	50/59
			ORF6	49/58
			SibY	52/62
<i>tomM</i>	783	UvrA-drug resistance pump	YP_001822519	60/72
			ORF8	66/78
			SibF	61/75
<i>tomN</i>	66	4-oxalocrotonate tautomerase	ZP_01514863	44/63
<i>tomO</i>	394	salicylylCoA hydroxylase (NADH-dependent flavin oxidoreductase)	YP_117454	25/32
			SibG	40/49
<i>tomP</i>	637	anthranilate synthase	T03800	42/51
			TomD	35/45
<i>tomQ</i>	482	flavin containing amine oxidase	YP_633086	32/47
			ORF24	57/71

^aNumbers are in amino acids.

Table V

Abolishment and rescue of PBD production by gene inactivation and plasmid/chemical complementation^{39,40,59}

Inactivated Gene	Chemical/plasmid complementation	Analog production
<i>ORF1</i>	Not reported	Not reported
<i>ORF12</i>	Not reported	Not reported
<i>ORF19</i>	Rescued with 3-hydrokynurenine and 3-hydroxyanthranilic acid. Not rescued with kynurenine and 3-hydroxy-4-methyl-anthranilic acid.	Not reported
<i>ORF21-22</i>	Not reported	Not reported
<i>ORF23</i>	Rescued with 3-hydrokynurenine and 3-hydroxyanthranilic acid. Not rescued with kynurenine and 3-hydroxy-4-methyl-anthranilic acid.	Not reported
<i>ORF24</i>	Rescued with 3-hydrokynurenine and 3-hydroxyanthranilic acid. Not rescued with kynurenine and 3-hydroxy-4-methyl-anthranilic acid.	Not reported
<i>tomA</i>	Rescued by plasmid complementation	Not reported
<i>tomO</i>	Rescued with 5-hydroxyanthranilic acid	<i>tomO</i> strain produces prothracarcin (Table 1)
<i>sibA</i>	Not reported	Not reported
<i>sibC</i>	Rescued with 3-hydrokynurenine, 3-hydroxyanthranilic acid, and 3-hydroxy-4-methylanthranilic acid. Not rescued with kynurenine.	Not reported
<i>sibE</i>	Not reported	Not reported
<i>sibG</i>	Not reported	<i>sibG</i> strain produces 7-deoxyaglycone of sibiromycin

Table VI

Thermal denaturation (T_m , °C) of calf thymus DNA-PBD complex at 37 °C^{a,b}.

Compound	0 h	4 h	18 h
Sibiromycin ¹³	15.7	15.9	16.3
Anthramycin ¹³	9.4	11.2	13.0
Tomaymycin ¹³	1.0	2.4	2.6
DC-81 ¹³	0.3	0.5	0.7
34 ⁸⁴	0.9	1.6	2.3
35 ⁸³	3.4	4.6	5.9
36 ⁸³	3.1	4.0	4.7
37 ⁸³	1.8	2.3	3.5
38 ⁸³	1.6	2.1	2.5
39 ⁸³	4.0	4.6	4.6
41 ⁸⁴	1.5	2.0	2.4
55 ⁹²	n/a	n/a	15.8
DSB-120 ⁸¹	10.2	13.1	15.1
SJG-136 ¹⁴⁸	25.7	31.9	33.6
61 n=1 ¹¹⁰	37.0	n/a	n/a
61 n=2 ¹¹⁰	38	n/a	n/a
61 n=3 ¹¹⁰	37.0	n/a	n/a
62 n=1 ¹¹⁰	11.0	n/a	n/a
62 n=2 ¹¹⁰	14	n/a	n/a
62 n=3 ¹¹⁰	13	n/a	n/a
63 n=3 ¹¹⁰	28.9	n/a	n/a
67 n=2, m=2 ¹¹⁰	21.9	n/a	22.7
67 n=2, m=3 ¹¹⁰	25.9	n/a	26.5
67 n=2, m=4 ¹¹⁰	23.1	n/a	23.9
67 n=3, m=3 ¹¹⁰	12.0	n/a	12.9
67 n=4, m=4 ¹¹⁰	19.9	n/a	20.8
69 n=1, m=1 ⁹⁰	9.0	n/a	9.0
69 n=1, m=2 ⁹⁰	9.0	n/a	9.0
69 n=2, m=2 ⁹⁰	9.0	n/a	9.9
69 n=3, m=2 ⁹⁰	6.2	n/a	7.1
69 n=3, m=2 ⁹⁰	6.1	n/a	7.2
70 n=5 ¹¹²	2.1	2.4	2.6
75 ¹¹⁷	2.9	3.4	3.6
76 ¹¹⁷	1.1	1.4	2.0

^a A 5:1 = DNA: PBD molar ratio was used at pH 7.00.

^b Only compounds with a T_m higher than 2.0 °C are listed.

Table VII

Average GI₅₀, TGI (dose that inhibits 100% cell growth) and LC₅₀ values of C2-endo-exo PBDs across the 60 cell lines of the NCI screen.

Compound	GI ₅₀ (μM)	TGI (μM)	LC ₅₀ (μM)
Anthramycin ⁹¹	0.029	0.61	12.7
SJG-136 ¹⁶⁴	0.0074	0.00083 for sensitive cells	0.0071 for sensitive cells
42 ⁸⁸	0.023	n/a	n/a
43 ⁸⁸	<0.01	n/a	n/a
44 ⁸⁸	0.123	n/a	n/a
46 ⁸⁸	<0.01	n/a	n/a
48 ⁹¹	0.053	3.72	69.2
49 ⁹¹	0.013	1.32	55.0
50 ⁹¹	0.012	0.096	0.65
52 ¹⁶²	<0.01	0.065	10.5
54 ⁸⁹	<0.01	n/a	n/a
C2-F₂ DSB ⁸⁶	0.42	4.2	39.8
61 ⁸⁶ n=1	0.014	0.76	22.4
62 ⁸⁶ n=1	3.9	16.2	63.1

Table VIII

In vitro cytotoxicity of PBDs with IC₅₀ values lower than 0.1 µM with the exception of DC-81.

Compound		IC ₅₀ µM [cells type]	
Sibromycin ¹³	0.0029 [L1210, leukemia]	0.000017 [ADJ/PC6, plasmacytoma]	0.04 [CH1, ovarian]
Anthracycline ¹³	0.022 [L1210, leukemia]	0.0028 [ADJ/PC6, plasmacytoma]	0.32 [CH1, ovarian]
Tomaymycin ¹³	0.0037 [L1210, leukemia]	0.0018 [ADJ/PC6, plasmacytoma]	0.00013 [CH1, ovarian]
DC-81 ¹³	0.38 [L1210, leukemia]	0.33 [ADJ/PC6, plasmacytoma]	0.10 [CH1, ovarian]
DSB-120 ⁹³	0.01 [L1210, leukemia]	0.0005 [ADJ/PC6, plasmacytoma]	0.003 [CH1, ovarian]
1 ⁸¹	0.045 [A2780, ovarian]	0.047 [CH1 ^{cisR} , ovarian]	0.059 [CH1, ovarian]
32 ⁸⁴	n/a	0.082 [CH1 ^{cisR} , ovarian]	0.017 [CH1, ovarian]
33 ⁸⁴	n/a	0.056 [CH1 ^{cisR} , ovarian]	0.035 [CH1, ovarian]
34 ⁸⁴	n/a	0.031 [CH1 ^{cisR} , ovarian]	0.031 [CH1, ovarian]
36 ⁸³	0.0145 [A2780, ovarian]	0.04 [CH1 ^{cisR} , ovarian]	0.016 [CH1, ovarian]
39 ⁸³	0.07 [A2780, ovarian]	0.037 [CH1 ^{cisR} , ovarian]	0.09 [CH1, ovarian]
41 ⁸⁴	n/a	0.084 [CH1 ^{cisR} , ovarian]	0.066 [CH1, ovarian]
DSB-120 ⁸¹	0.0072 [A2780, ovarian]	0.022 [CH1 ^{cisR} , ovarian]	0.033 [CH1, ovarian]
SJG-136 ¹⁴⁸	0.000022 [A2780, ovarian]	0.00012 [CH1 ^{cisR} , ovarian]	0.0006 [CH1, ovarian]
47 ¹⁸¹	0.005 [KB, epidermal]	0.020 [HCT116, colon]	0.050 [K562, leukemia]
51 ¹⁸¹	0.086 [KB, epidermal]	0.050 [HCT116, colon]	0.085 [K562, leukemia]
74 ¹¹⁶	n/a	n/a	0.037 [K562, leukemia]
67 ¹¹⁰ n=2, m=2	0.5 [A2780, ovarian]	0.8 [PC3, prostate]	1.6 [MCF7, breast]
67 ¹¹⁰ n=2, m=3	0.5 [A2780, ovarian]	0.5 [PC3, prostate]	1.6 [MCF7, breast]
67 ¹¹⁰ n=2, m=4	0.5 [A2780, ovarian]	0.5 [PC3, prostate]	1.6 [MCF7, breast]
69 ⁹⁰ n=3, m=1	0.17 [A2780, ovarian]	0.17 [GURAV, oral]	0.0051 [RPMI8226, myeloma]
64 ¹⁰⁶	0.0024 [A2780, ovarian]	0.0014 [CCRF-CEM, all]	0.028 [RPMI8226, myeloma]
65 ¹⁰⁶	0.0005 [A2780, ovarian]	0.0001 [CCRF-CEM, all]	0.21 [SiHa, cervix]
64 ¹⁰⁶	0.033 [A549, NSCL]	0.015 [DU145, prostate]	0.0027 [LNCaP-FGC, prostate]
65 ¹⁰⁶	0.019 [A549, NSCL]	0.0064 [DU145, prostate]	0.0007 [LNCaP-FGC, prostate]
64 ¹⁰⁶	0.018 [MCF7, breast]	0.022 [LOXIMI, melanoma]	0.0007 [LS174T, colon]
65 ¹⁰⁶	0.031 [MCF7, breast]	0.053 [LOXIMI, melanoma]	0.0043 [LS174T, colon]