Brief Articles

Pyrrolo[1,2-*b*][1,2,5]benzothiadiazepines (PBTDs): A New Class of Agents with High Apoptotic Activity in Chronic Myelogenous Leukemia K562 Cells and in Cells from Patients at Onset and Who Were Imatinib-Resistant

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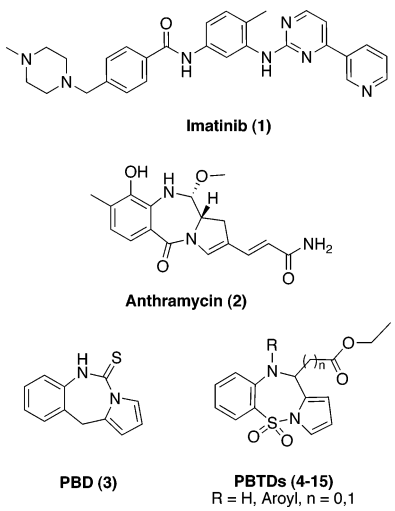
Pyrrolo[1,2-*b*][1,2,5]benzothiadiazepine 5,5-dioxides (PBTDs) induced apoptosis in human BCR-ABL-expressing leukemia cells. The apoptotic activity was also observed in primary leukemic blasts, obtained from chronic myelogenous leukemia (CML) patients at onset or from patients in blast crisis and who were imatinib-resistant. Compounds **5** and **14** induced apoptosis before BCR-ABL protein expression and tyrosin phosphorylation were affected and activated different caspases in the apoptotic pathway. PBTDs are a new class of valid candidates for the treatment of CML.

Chronic myelogenous leukemia (CML) is a clonal disease of hemopoietic progenitor cells.[1] CML is characterized by the expression of the BCR-ABL fusion gene, which is derived from the fusion of the cellular breakpoint cluster region (BCR) gene and the Abelson murine leukemia oncogene (ABL).[2] BCR-ABL-transformed cells activate multiple signal transduction pathways responsible for increasing proliferation. The BCR-ABL protein also activates downstream survival pathways[3] that collectively provide BCR-ABL cells with a survival advantage over the normal cells, thereby contributing to the leukemic phenotype[4] and conferring higher resistance against conventional cytotoxic drugs.[5]

Imatinib (IM, imatinib mesilate, STI-571, Gleevec) (**1**) is now the first-choice drug for all newly diagnosed CML patients[6] (Chart 1). IM is a potent inhibitor in all of the ABL tyrosine kinases, including BCR-ABL. There is a lot of evidence that the oncogene BCR-ABL would protect growth-factor-dependent hemopoietic cells from apoptosis. According to this role, inhibition of BCR-ABL expression by IM showed restoration of susceptibility to apoptosis and enhancement of cell death.[7] Exposure to IM alone induced apoptosis in HL-60/BCR-ABL and K562 cells.[8] Despite its initial efficacy, IM selects drug resistance due to the emergence of mutation at the kinase domain and overexpression of BCR-ABL.[9] To overcome resistance, some strategies have been adopted, such as dose escalation and synergistic combination with conventional drugs (cytarabine, homoharringtonine, IFN), alternative ABL inhibitors, or BCR-ABL protein down-regulating agents.[7]

Chart 1



The antitumor activity of pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) related to anthramycin (**2**) was extensively studied by our research group, as it is well documented in Thurston's excellent review.[10] A correlation between antitumor activity and apoptosis was well demonstrated. It was hypothesized that anthramycin, like other DNA-alkylating agents, would induce apoptosis through a mythocondrial pathway.[11] In addition, in

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Table 1. Apoptotic Activity of Compounds **4**−**18** and Reference Compound **1** in K562 Cells at 10 μM[a]

	% of apoptosis (±SD)[b]			
compd	8 h	16 h	24 h	48 h
4	45.0 ± 2.0	66.6 ± 1.5	80.0 ± 1.0	89.6 ± 0.6
5	45.6 ± 1.5	67.6 ± 1.5	79.6 ± 1.5	91.7 ± 0.6
6	44.3 ± 1.5	67.0 ± 2.0	79.7 ± 1.5	91.7 ± 0.6
7	45.3 ± 2.1	67.6 ± 1.5	81.0 ± 1.0	92.0 ± 1.0
8	43.7 ± 1.7	66.0 ± 1.0	77.0 ± 1.0	87.6 ± 1.1
9	45.3 ± 1.1	68.0 ± 1.0	79.5 ± 2.5	92.3 ± 2.1
10	45.0 ± 2.6	67.6 ± 2.1	74.3 ± 2.5	87.6 ± 1.5
11	43.0 ± 2.6	65.5 ± 2.1	78.0 ± 1.0	90.0 ± 3.6
12	44.6 ± 1.5	66.3 ± 1.5	79.0 ± 2.0	90.7 ± 1.7
13	47.0 ± 2.0	69.0 ± 1.0	80.0 ± 0.0	92.6 ± 3.0
14	34.0 ± 1.7	61.3 ± 0.6	76.3 ± 1.1	89.7 ± 2.1
15	29.3 ± 0.6	53.0 ± 1.0	72.0 ± 2.6	88.3 ± 0.6
16	4.8 ± 0.6	5.7 ± 1.5	6.8 ± 1.5	6.2 ± 2.1
17	4.5 ± 0.6	5.5 ± 1.5	6.4 ± 1.5	6.1 ± 2.1
18	5.3 ± 1.5	19.3 ± 1.5	19.6 ± 0.6	23.0 ± 1.7
1[c]	66.7 ± 1.5	70.0 ± 1.0	71.0 ± 1.5	71.2 ± 2.1
control[d]	4.4 ± 0.6	5.3 ± 1.5	6.4 ± 1.5	6.1 ± 1.2

[a] Compounds **4**−**18** were tested in parallel with the reference compound **1** as described in Supporting Information. [b] Arithmetic mean ± standard deviation (SD) for triplicate experiments. [c] Imatinib, Gleevec. [d] Untreated K562 cells.

Table 2. Apoptotic Activity of Compounds **5** and **14** in Cells from CML Patients at Onset at 10 μM

				% of apoptosis			
				5		**14**	
patient	sex	age (years)	source[a]	24 h	48 h	24 h	48 h
1	M	45	PB	77	85	64	70
2	M	60	BM	70	85	50	70
3	F	73	PB	66	82	65	79
4	M	83	BM	50	75	50	70
5	F	46	PB	60	80	50	70
6	F	27	PB	60	80	50	70
7	F	45	PB	64	80	60	80
8	M	35	PB	65	85	60	80
9	M	66	PB	60	80	60	80
10	F	38	PB	70	80	50	70
11	F	65	PB	55	78	52	71
12	F	27	PB	55	78	52	73

[a] PB: peripheral blood cells. BM: bone marrow cells.

the past few years the activity of some PBDs (i.e., **3**)[12] in leukemia cell lines, such as K562 and Jurkat cells, was reported.

Our extensive studies on anti-AIDS agents led to the discovery of pyrrolo[1,2-*b*][1,2,5]benzothiadiazepine 5,5-dioxides (PBTDs) as potent non-nucleoside inhibitors of the HIV-1 reverse transcriptase.[13] Given the high structural similarity between PBTDs and PBDs, we selected two PBTDs (**5** and **14**) for a preliminary screening aimed to discover new proapoptotic and antileukemia agents. In fact, to our knowledge the anti-CML properties of PBTDs have not been investigated until now.

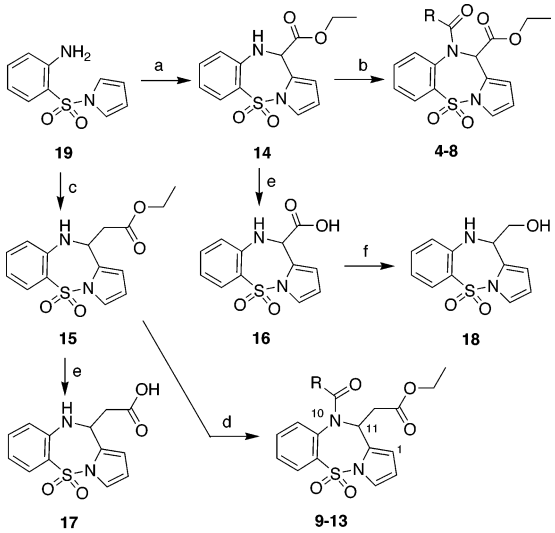
In our first experiments, PBTDs **5** and **14** induced apoptosis in K562 cells (Table 1). In addition, they efficiently induced cell death in BCR-ABL-positive leukemia cells taken from patients who were at onset or were IM-resistant (Tables 2 and 3). These results prompted us to conduct apoptotic mechanism studies on PBTDs **5** and **14**. At the same time we planned the synthesis of new PBTD derivatives to investigate the effect of (i) substituents at the 10-aroyl group, (ii) a 1-naphthoyl group at position 10, and (iii) the introduction of a methylene spacer group at position 11. Our findings indicate that the PBTDs are a new class of potential agents for the treatment of CML.

Table 3. Apoptotic Activity of Compounds **5** and **14** at 10 μM in Cells from CML Patients in Blast Crisis and Who Were Imatinib-Resistant

				% of apoptosis			
				5		**14**	
patient	sex	age	source[a]	24 h	48 h	24 h	48 h
13	M	38	PB	60	80	40	60
14	F	70	PB	55	78	50	70

[a] PB: peripheral blood cells.

Scheme 1[a]



[a] Reagents and reaction conditions: (a) ethyl glyoxylate dimethoxyacetal, PTSA, absolute EtOH, reflux, overnight; (b) aroyl chloride, $NaHCO_3$, 1-bromo-3-chloropropane, reflux, overnight; (c) ethyl 3,3-diethoxy-propionate, $CH_3COOH \cdot H_2O$, overnight; (d) aroyl chloride, $(i\text{-}Bu)_3N$, dry dioxane, reflux, 48 h; (e) LiOH, $THF \cdot H_2O$, room temp, 24 h; (f) $LiAlH_4$, $AlCl_3$, anhydrous THF, room temp, 3 h.

Chemistry

Aroylation of the ester **14** with aroyl chlorides in boiling 1-bromo-3-chloropropane in the presence of sodium hydrogen carbonate afforded the corresponding amides **4**−**8** (Scheme 1). The starting ester **14** was prepared by treatment of **19**[14] with ethyl 2,2-diethoxyacetate in the presence of 4-toluenesulfonic acid (PTSA) as a catalyst in boiling absolute ethanol via a Pictet−Spengler type reaction.[15] Derivatives **9**−**13** were prepared by aroylation of **15** in boiling dioxane in the presence of triisobutylamine. The starting acetate **15** was obtained by reaction of **19** with ethyl 3,3-diethoxypropionate in aqueous glacial acetic acid at 100 °C. Lithium hydroxide hydrolysis of the esters **14** and **15** afforded the corresponding acids **16** and **17**. Lithium aluminum hydride−aluminum chloride reduction of acid **14** gave alcohol **18**. The racemic mixtures **5** and **14** were separated using analytical and semipreparative enantioselective HPLC to give enantiomers **20** and **21**, and **22** and **23**, respectively, with >98.0% ee and in 80−90% yield.

Apoptotic Activity in K562 Cells

The characteristic morphological features of apoptosis, such as cytoplasmic vacuolization, patterns of chromatin condensation and micronuclei, and negligible necrosis, were observed upon treatment of the cells with tested compounds. The chromatin appeared clumped and was observed at the nuclear periphery. The samples showed micronuclei or the nuclear body surrounded by a double membrane and cytoplasmic vacuolization. Large plasmalemmal blebbing was visible in the cells incubated with

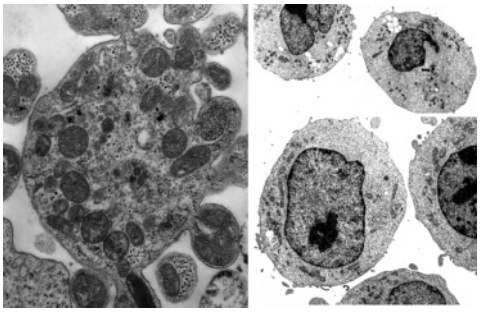


Figure 1.

5 (Figure 1, left (×18 000)), whereas such morphological changes did not appear in the control cells (Figure 1, right).

The apoptotic activity of **5** and **14** in K562 cells was time-dependent at 5, 10, and 15 μM (Figure 1S in Supporting Information). At 10 μM, **4**−**15** showed high apoptotic activity at every time point. A preliminary structure−activity relationship (SAR) inspection indicated that the apoptotic activity of **4**−**13** was weakly affected by the aroyl substitutents at position 10 of the PBTD nucleus or by the methylene linker at position 11 (compare carboxylates **4**−**8** and acetates **9**−**13**). Compounds **14** and **15** deprived of the 10-aroyl portion also showed potent apoptotic activity with greater difference after 8 h (compare **4**−**13** with **14** and **15**). Replacement of the ester **14** with the alcohol **18** caused a neat decrease of apoptotic activity. Free acids **16** and **17** did not show the morphological features of apoptosis, showing an apoptotic activity that was comparable to that of control cells. We hypothesized that these compounds would not be able to penetrate the K562 cell membrane and to activate the cellular mechanisms. To evaluate the influence of the chiral center at position 11 of the PBTD ring, the racemates **5** and **14** were separated by chiral HPLC. Both (+)-(*S*)-enantiomers **21** and **23** and the corresponding (−)-(*R*)-counterparts **22** and **24** showed slight differences of activity in the range 3−5% (Table 1S in Supporting Information).

Apoptotic Activity in Bone Marrow and Peripheral Blood Cells from CML Patients

We harvested bone marrow and peripheral blood cells from 12 consecutive CML patients at onset. We also harvested peripheral blood cells from 2 patients in blast crisis evolved under IM treatment. Cell samples were cultured in vitro, treated with **5** or **14** for 24 and 48 h, and then subjected to DNA fragmentation assay. Tested PBTDs induced apoptosis in all of the samples, including those from the two patients in blast crisis and who were IM-resistant (Tables 2 and 3). The typical appareance of endonucleosome DNA after treatment with **5**, **6**, or **14** in patients cases 1, 11, and 14 is shown in Figure 2S in Supporting Information.

Caspase Activation Activity in K562 Cells

We analyzed the activated apoptotic pathways by performing Western blot analyses of caspase-8 and -9 proforms. Upon drug treatment caspase-8 and -9 became activated in most apoptotic models in receptor-activated caspase-8 and -3 pathways or in cytochrome c release-dependent caspase-9 and -3 pathways.

In both pathways, caspase-3 activation is the final event of the caspase cascade. After 24 h, **14** significantly reduced the procaspase-8 immunoreactive band without changing the procaspase-9 intensity. On the other hand, lysates from **5**-treated K562 cells showed no evidence of alteration in procaspase-8 concentration and paralleled a dramatic decrease of procaspase-9 expression levels. Compound **14** seemed to activate the direct extrinsic caspase-8 mediated pathway, resulting in faster proteolysis induction.

In contrast, **5** seemed to induce apoptosis through the mitochondrial intrinsic pathway with a relatively delayed proteolytic effect (Figure 3S in Supporting Information).

Compounds 5 and 14 Induced Apoptosis by Bypassing the Apoptotic Suppressor BCR-ABL

Western blot analyses of BCR-ABL and tyrosine phosphorylated proteins at different intervals showed that some down-regulation of the BCR-ABL immunoreactive band was detected only after 16 h upon treatment with **14** and after 24 h upon treatment with **5**. One point worth noting was that at regular intervals we noticed a slight diminution of ABL and actin proteins. At the same time, no significant early change in the pattern of tyrosine phosphorylated proteins was detected (at least not until the same BCR-ABL protein level started to fade). This confirmed that no inhibition of a putative BCR-ABL-mediated major phosphorylative process was induced within the cell. These observations suggested that down-regulation of BCR-ABL is not involved in the early events associated with PTDB-induced apoptosis. Accordingly, both **5** and **14** induced apoptosis before the levels of BCR-ABL protein expression and its tyrosin phosphorylation were affected. Both actin protein degradation and whole-cell phosphorylation pattern changes (caused by widespread proteolysis) were dependent on the apoptotic process rather than the inactivation of BCR-ABL kinase (Figure 4S in Supporting Information).

In conclusion, our preliminary results demonstrated that PBTDs **4**−**15** induced apoptosis in human BCR-ABL-expressing leukemia cells. The apoptotic activity was also observed in primary leukemia blasts, obtained from CML patients at onset or from patients in blast crisis and who were IM-resistant. Apoptotic mechanism studies conducted on PBTDs **5** and **14** showed that they activated the caspase activity through two different pathways. These compounds also induced apoptosis before the BCR-ABL protein expression and tyrosine phosphorylation levels were affected. These findings suggest that PBTDs are endowed with powerful growth inhibition on leukemia cells.

Besides the high activity displayed by some PBTDs discovered in the present preliminary work, we are aware that further results are obtainable to evaluate the chemotherapeutic potential of this novel class of anti-CML agents. For this reason, the synthesis of new PBTD derivatives is now ongoing in our laboratories as part of more in-depth structure−activity relationship (SAR) studies guided by molecular modeling.

Experimental Section

Chemistry. Melting points (mp) were determined on a Büchi 510 apparatus and are uncorrected. Infrared spectra (IR) were run on a SpectrumOne FT spectrophotometer. Band position and absorption ranges are given in cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on Bruker 200 and 400 MHz FT spectrometers in the indicated solvent. Chemical shifts are expressed in δ units (ppm) from tetramethylsilane. Column chromatography was performed on columns packed with alumina from Merck (70−230 mesh) or with silica gel from Merck (70−230 mesh). Aluminum oxide TLC cards from Fluka (aluminum oxide precoated aluminum cards with fluorescent indicator at 254 nm) and silica gel TLC cards from Fluka (silica gel precoated aluminum cards with fluorescent indicator at 254 nm) were used for thin-layer chromatography (TLC). Elemental analysis results were found within ±0.4% of the theoretical values.

General Procedure for the Prepapartion of Derivatives 4−8. Example. Ethyl (±)-10,11-Dihydro-10-benzoylpyrrolo[1,2-*b*]-[1,2,5]benzothiadiazepine-11-carboxylate 5,5-Dioxide (4). A mixture of **14** (1.00 g, 0.0033 mol), benzoyl chloride (0.48 g, 0.0034 mol), $NaHCO_3$ (0.31 g, 0.0037 mol), and 1-bromo-3-chloropropane (50 mL) was refluxed overnight. After cooling, the mixture was filtered and the solvent evaporated. The residue was purified by column chromatography (silica gel, chloroform). Yield 40%, mp 172−180 °C (ethanol). ^{1}H NMR (DMSO-d_6): δ 1.30 (t, J = 7.0 Hz, 3H), 4.28 (m, 1H), 4.44 (m, 1H), 6.16 (m, 1H), 6.28 (br, 1H), 6.34 (m, 1H), 7.06 (d, J = 6.9 Hz, 1H), 7.25−7.51 (m, 8H), 7.91 ppm (dd, J = 1.5 and 7.4 Hz, 1H). IR (neat): ν 1185, 1650, 1745 cm^{-1}. Anal. ($C_{21}H_{18}N_2O_5S$ (410.44)), C, H, N, S.

General Procedure for the Prepapartion of Derivatives 9−13. Ethyl (±)-10,11-Dihydro-10-benzoylpyrrolo[1,2-*b*][1,2,5]benzothiadiazepine-11-acetate 5,5-Dioxide (9). A mixture of **15** (1.00 g, 0.0031 mol), benzoyl chloride (0.56 g, 0.0040 mol), triisobutylamine (0.74, 0.0040 mol), and dry dioxane (50 mL) was refluxed for 48 h. After concentration the mixture was extracted with ethyl acetate, washed with 1 N HCl and then with brine, and dried. Removal of the solvent furnished a residue that was purified by column choromatography (silica gel, dichloromethane). Yield 35%, mp 178−181 °C (ethanol). ^{1}H NMR (DMSO-d_6): δ 1.19 (t, J = 7.1 Hz, 3H), 2.81 (m, 1H), 2.91 (m, 1H), 4.12 (m, 2H), 6.35−6,40 (m, 2H), 6.56 (br, 1H), 7.22−7.58 (m, 9H), 8.00 ppm (m, 1H). IR (neat): ν 1186, 1315, 1655, 1725 cm^{-1}. Anal. ($C_{22}H_{20}N_2O_5S$ (424.47)), C, H, N, S.

Ethyl (±)-10,11-Dihydropyrrolo[1,2-*b*][1,2,5]benzothiadiazepine -11-carboxylate 5,5-Dioxide (14). A mixture of **19** (9.4 g, 0.042 mol), dimethoxyacetal of ethyl glyoxylate (11.10 g, 0.063 mol), and 4-toluenesulfonic acid monohydrate (PTSA) (8.0 g, 0.042 mol) was refluxed in absolute ethanol (20 mL) overnight. PTSA (3.7 g, 0.021 mol) was added while refluxing for an additional 4 h. After cooling, the mixture was poured onto crushed ice and extracted with ethyl acetate, washed with brine, and dried. Evaporation of the solvent furnished the crude product, which was purified by column chromatography (silica gel, chloroform). Yield 51%, mp 131−134 °C (toluene/ligroin) (lit.[16a] mp 121−122 °C).

Ethyl (±)-10,11-Dihydropyrrolo[1,2-*b*][1,2,5]benzothiadiazepine-11-acetate 5,5-Dioxide (15). 15 was prepared as **14** using ethyl 3,3-diethoxypropionate by heating in aqueous acetic acid. Yield 45%, mp 121 °C (from benzene/petroleum ether) (lit.[16b] mp 118−119 °C).

(±)-10,11-Dihydropyrrolo[1,2-*b*][1,2,5]benzothiadiazepine-11-carboxylic Acid 5,5-Dioxide (16). Lithium hydroxide monohydrate (0.25 g, 0.006 mol) was added to a solution of **14** (0.61 g, 0.002 mol) in THF (20 mL) and water (20 mL). Then the reaction mixture was stirred at room temperature for 24 h. After dilution with water, the mixture was acidified with 1 N HCl until pH 2 was reached. The acid was extracted with ethyl acetate, washed with brine, and dried. Removal of the solvent gave pure **16**. Yield 98%, mp 161−163 °C (aqueous ethanol) (lit.[16c] mp 159−162 °C).

(±)-10,11-Dihydropyrrolo[1,2-*b*][1,2,5]benzothiadiazepine-11-acetic Acid 5,5-Dioxide (17). 17 was prepared as **16** starting from **15.** Yield 90%, mp 208−210 °C (ethanol) (lit.[16b] mp 209 °C).

(±)-10,11-Dihydro-11-hydroxymethylpyrrolo[1,2-*b*][1,2,5]-benzothiadiazepine 5,5-Dioxide (18). To anhydrous aluminum chloride (0.71 g, 0.005 mol) in THF (30 mL) at 0 °C was added lithium aluminum hydride (8.0 mL of 1 M in THF) while stirring for 15 min. Then a solution of **16** (0.25 g, 0.000 92 mol) in THF (10 mL) was added and the mixture was stirred at room temperature for 3 h. After cooling at 0 °C, the reaction mixture was quenched with 4 N sodium hydroxide and extracted with dichloromethane. The organic layer was separated, washed with brine, and dried. Removal of the solvent gave crude **19**, which was passed through a chromatographic column (silica gel, chloroform). Yield 87%, mp 136−138 °C (ethanol) (lit.[13] mp 135−136 °C).

Supporting Information Available: Additional chemical and biological information. This material is available free of charge via the Internet at http://pubs.acs.org.

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