EFFECTS OF PIMOZIDE DERIVATIVES ON pSTAT5 IN K562 CELLS

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Abstract: *STAT5 is a transcription factor, component of the STAT family of signalling proteins. STAT-5 is involved in many types of cancer, including chronic myelogenous leukemia (CML), where this protein is found constitutively activated as a consequence of the BCR-ABL expression. Recently, literature has reported that neuroleptic drug pimozide can act as inhibitor of STAT5 phosphorylation and is capable to induce apoptosis in CML cells in vitro. Our group has synthesized simple derivatives of pimozide, with cytotoxic activity and able to reduce the levels of phosphorylated STAT5. In this work we continue the search for novel STAT5 inhibitors, synthesizing compounds which maintain or modify the benzoimidazolinone ring of pimozide, in order to obtain further structure-activity relationship of this class of STAT5 inhibitors. Two compounds of the series showed a potent cytotoxic activity against BCR-ABL positive and pSTAT5 overexpressing K562 cells and were able to decrease markedly the levels of phosphorylated STAT5.*

Introduction

Signal transducers and activators of transcription (STATs) are a family of proteins involved in signal transduction which are activated by multiple pathways, including the janus-associated kinases (JAKs), FLT3, and inflammatory cytokines.[1,2] There is evidence of an important and complex role of JAK2/STAT5 signalling pathway in some prostate and breast cancers evolution.[3] Moreover, STAT5 and many intracellular signalling cascades are activated by the constitutively active cytoplasmic tyrosine kinase BCR-ABL, characteristic for chronic myeloid leukemia (CML) and BCR-ABL-positive acute lymphoblastic leukemia (ALL).[4-6] For this reason, the system JAK2/STAT5 has been identified as a plausible target to inhibit BCR-ABL positive CML,[7,8] especially in case of resistance to treatment with tyrosine kinase inhibitors. More recently, it has been reported that BCR-ABL is able to differently affect the two isoforms $STAT5_A$ and $STAT5_B$.[9] Src-homology 2 domain (SH2) is a crucial part of the STAT5 factor, docked and phosphorylated by cytokine activated receptors or non receptors kinases. When phosphorylated, STAT5 is able to homo- or heterodimerize before nucleus translocation and induction of genes transcription.[10]

As a matter of fact, in recent years, literature has reported the development of some interesting classes of different compounds that interact with the STAT5 function, with a mechanism related to inhibition of the SH2 portion, and sometimes discriminating between A and B isoforms.[11-13] There is however an ongoing interest in finding new structures able to interact with STAT5 signal.

Using a chemical library of compounds known to be safe in humans, with a cell-based screening, Nelson and coworkers identified pimozide (**1**), a neuroleptic drug used to treat Tourette syndrome, as a STAT5 inhibitor. They reported that pimozide decreases levels of STAT5 activated by BCR/ABL, being effective against both imatinib-sensitive and -resistant cells. [14-16] Then, in a recent work,[17] we demostrated that simple modifications of pimozide structure allowed to obtain derivatives (as compound **3c**) more potent in decreasing activated STAT5 levels in BCR-ABL expressing cells. Modifications were in part suggested by our previous work on compound TR120 that strongly decreased STAT5 expression in K562 cells.[18] Moreover, considering that 4,4-di(*p*-fluorophenyl)butyl chain of pimozide plays a role in promoting the CNS effects of the drug, we changed the piperidine nitrogen substitution from the original 4,4-di(*p*-fluorophenyl)butyl chain into analogue 4,4-diarylbutanoyl groups and obtained derivatives with a comparable ability to inhibit activated STAT5. In the present study, a new series of pimozide analogues lacking the original 4,4-di(*p*-fluorophenyl)butyl chain have been synthesized in order to find more effective compounds on BCR-ABL expressing leukemia cell lines. Simple modifications were introduced in previously reported active compounds **3a,c**, in haloacyl

appendage or in the benzimidazolone ring. Moreover, we tested the role of piperidine portion in a molecule simplification strategy. In fact, in the piperidine-containing derivatives, we choose to maintain the amide function instead of amine for the piperidine nitrogen and acylating it with different halogenated substituents. This led to the discovery of two derivatives endowed with a more potent cytotoxic and STAT5 inhibitory activity where compared to pimozide and compound **3c**.

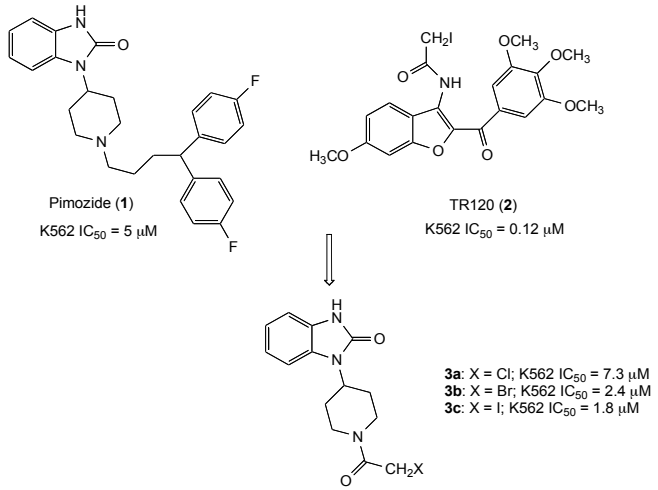
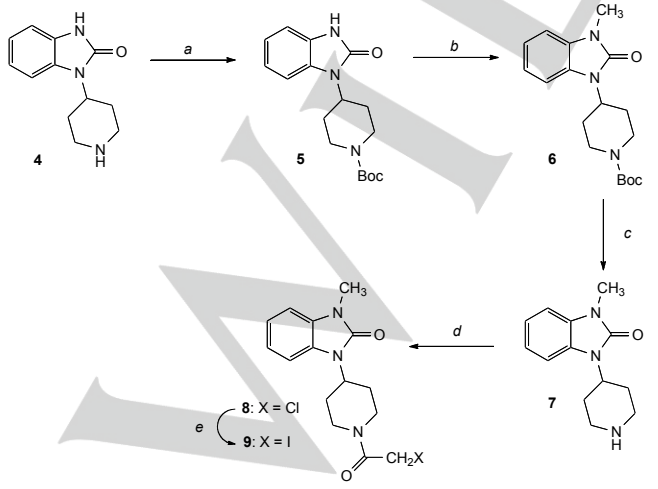


Figure 1. Chemical structure of pimozide, TR120 and first derivatives acting as p-STAT5 inhibitors.

Results

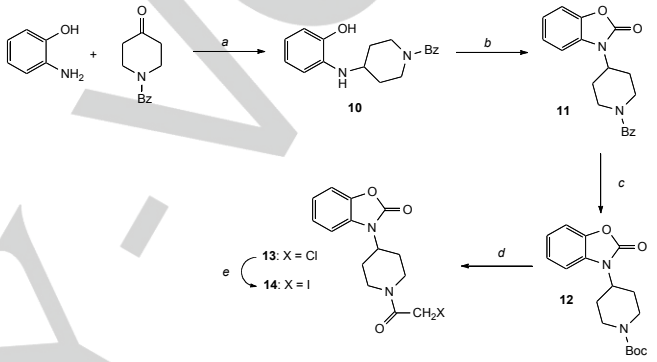
Chemistry.

The synthesis of compounds modified at benzimidazolinone ring is displayed in Schemes 1 and 2. Starting from commercially available compound **4**, the *N*-3 methylation was conducted with methyl iodide after Boc protection at piperidine ring and subsequent deprotonation with sodium hydride. After Boc deprotection of intermediate **6** with trifluoroacetic acid, a simple acylation of piperidine nitrogen with chloroacetyl chloride led to compound **8**, that was in part transformed to compound **9** by halogen exchange reaction adding sodium iodide in acetone solution.



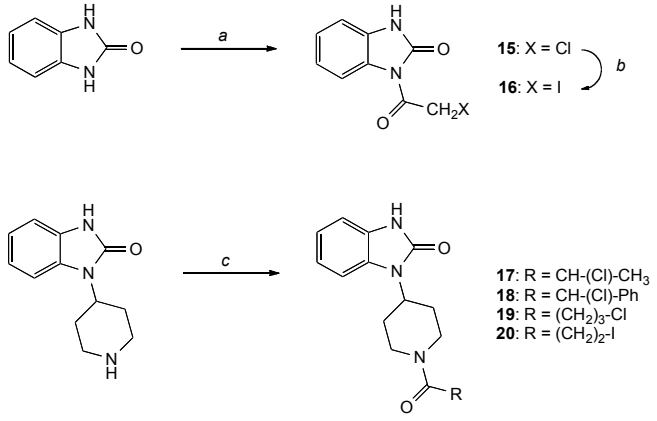
Scheme 1. Synthesis of compounds **8** and **9**. Reagents and conditions: a) $(Boc)_2O$, MeOH; b) DMF, NaH then CH_3I; c) TFA, CH_2Cl_2; d) chloroacetyl chloride, TEA, $CHCl_3$; e) NaI, acetone.

Benzoxazolinone compounds **13** and **14** were instead obtained starting from 2-aminophenol, which was reacted with N-benzyl-4-piperidone in the presence of sodium triacetoxyborohydride to yield intermediate **10**, then transformed into **11** with carbonyl diimidazole. Piperidine nitrogen of intermediate **11** was debenzylated and Boc protected with hydrogen atmosphere in the presence of catalytic amounts of 10% Palladium on carbon and Boc_2O. Boc protection allowed a much easier recovery of debenzylated intermediate. Then transformation of **12** into **13** and **14** was performed following the same procedures as described for the preparation of **8** and **9** from **6**.



Scheme 2. Synthesis of compounds **13** and **14**. Reagents and conditions: a) $NaH(AcO)_3$, CH_2Cl_2; b) carbonyl diimidazole, CH_2Cl_2; c) H_2, 10% Pd/C, Boc_2O, MeOH; d) TFA, CH_2Cl_2, then chloroacetyl chloride, TEA, $CHCl_3$; e) NaI, acetone.

In Scheme 3 are reported the syntheses of the derivatives without the piperidine ring (**15** and **16**), obtained by direct acylation with chloroacetyl chloride of commercial benzimidazolinone after sodium hydride deprotonation and the possible ion exchange as described before for compound **9**. Moreover, in Scheme 3 are also reported the compounds **17**-**20**, obtained by simple acylation of piperidine nitrogen of starting **4**, with acyl halides or carboxylic acid with condensing carbodiimide reagent.



Scheme 3. Synthesis of compounds **15** - **20**. Reagents and conditions: a) DMF, NaH then chloroacetyl chloride; b) NaI, acetone; c) opportune acyl chloride, TEA, $CHCl_3$ or, in case of **20**, 3-Iodopropanoic acid, EDC hydrochloride, TEA, $CHCl_3$.

Biological results.

Table 1 and Figure 2a show the cytotoxic effects of new pimozide derivatives in K562 cells after 48 h of treatment.

Compounds **15**, **17**, **19** and **20** did not show important cytotoxic effects on K562 cells (IC_{50} higher than 30 μM). Compounds **8** and **14** displayed a cytotoxic activity similar to pimozide. Compounds **9**, **16** and **13** were more active than pimozide, with the benzoxazolinone derivative **13** that showed a cytotoxic activity higher than our lead pimozide analogue **3c**.[18] The most active compounds (**8**, **9**, **13**, **14**, **16** and **18**) were tested in K562R, a cell line selected by exposure of K562 cells to increasing concentrations of imatinib and 10 fold more resistant to imatinib mesylate than parental cells.[18] As shown in Figure 2b, the cytotoxic effects of our most active compounds on K562R cells were similar to those observed in parental K562 cells.

Table 1. IC_{50}[a] (μM ± SE) and AC_{50}[b] (μM ± SE) of pimozide and analogues evaluated in K562 cells after 48 h of treatment.

Compound	*IC_{50} (μM)*	*AC_{50} (μM)*
Pimozide	5±0.8	10±2.3
3c[c]	1.8±0.22	5±0.7
8	4±0.7	7.5±1.1
9	1.6±0.2	3.6±0.4
13	0.75±0.12	1.8±0.2
14	5±0.6	11±3
15	>30	>30
16	2.5±0.4	5.8±0.7
17	>30	>20
18	10±2.5	22±5
19	>30	>30
20	>30	>30

[a] Concentration able to inhibit 50% cell growth. [b] Concentration able to induce apoptosis in 50% of cells. [c] Ref. 18

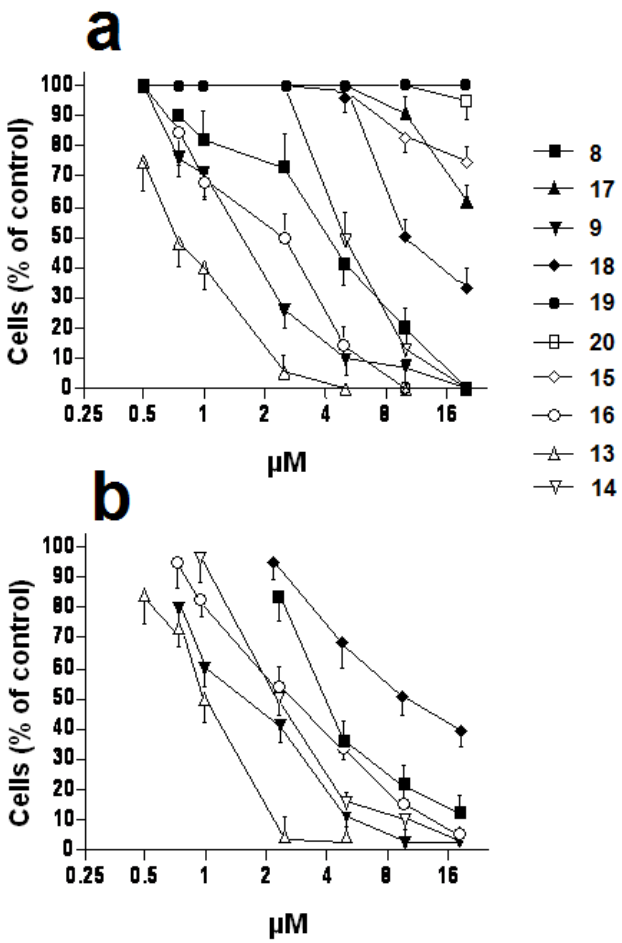


Figure 2. Cytotoxic activity of new pimozide derivatives used at different concentrations in K562 cells (a) and K562R cells (b) after 48 h of treatment.

Then, we examined the influence of the most active compounds (showing an $IC_{50} \leq 5$ μM) on pSTAT5 expression (Figure 3, Table 2) and cell cycle distribution in K562 cells (Fig. 4, Table 3). pSTAT5 expression was determined by flow cytometry after staining cells with a fluorochrome-conjugated anti-pSTAT5 monoclonal antibody (MoAb); analysis of cell cycle effects was carried out by flow cytometry after staining cells with propidium iodide. In Figure 3 the curves expressing the fluorescence of cells stained with a fluoresceinated anti-pSTAT5 after 24 h exposure to each compound (thick lines) were compared to those expressing the fluorescence of untreated cells stained with an anti-pSTAT5 (dotted lines) and to those stained with an isotype monoclonal antibody (thin line).

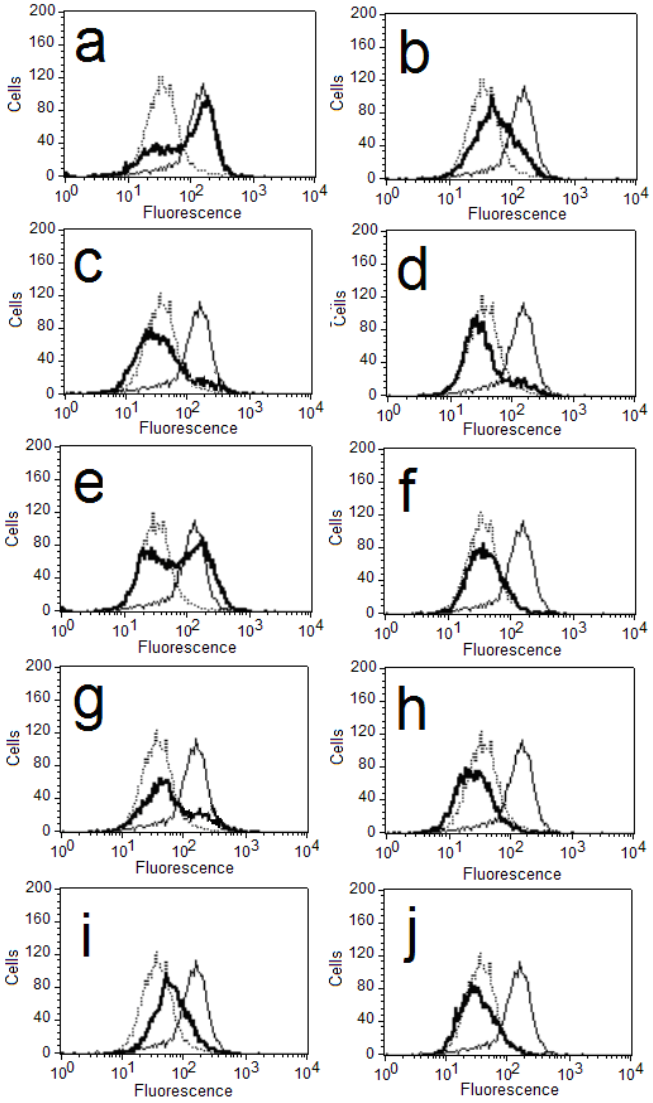


Figure 3. Intracellular levels of phosphorylated STAT5 in K562 cells evaluated by flow cytometry after 24 h exposure to each compound. Dotted line: cells stained with an isotype monoclonal antibody; thin line: cells stained with an anti-STAT5 monoclonal antibody; thick line: cells stained with an anti-STAT5 monoclonal antibody after 24 h exposure to each compound. (a) **8** (10 μM); (b) **8** (15 μM); (c) **9** (10 μM); (d) **9** (15 μM); (e) **16** (10 μM); (f) **16** (15 μM); (g) **13** (10 μM); (h) **13** (15 μM); (i) **14** (10 μM); (j) **14** (15 μM).

All compounds tested decreased the expression of pSTAT5 in a dose dependent manner (Figure 3, Table 2). Compound **9** was the most potent pSTAT5 inhibitor of the series. In fact, as shown in Table 2, the median fluorescence value of K562 cells stained with anti-pSTAT5 MoAb after 24h exposure to compound **9** at 10 μM was similar to that observed in the control stained with an isotypic MoAb, indicating the ability of this compound to inhibit completely the phosphorylation of STAT5. Previously, we observed that compound **3c** was markedly more potent than pimozide in reducing the levels of pSTA5 in K562 cells. However, compound **9** revealed an inhibitory activity on pSTAT5 higher than that observed with our lead compound **3c**. Also compounds **13** and **14** used at the concentration of 10μM showed a potent inhibitory effect on phosphorylation of STAT5. Compounds **16** and **8** were slightly less active on pSTAT5 than **9**, **13** and **14**.

Table 2. Median fluorescence values of K562 cells stained with an anti-pSTAT5 after 24 h exposure to pimozide analogues (10μM)

Compound	Median fluorescence
Control (Isotypic MoAb)[a]	45.7
Control (pSTAT5 MoAb)[b]	172.29
Pimozide	117.2
3c	71.3
8	133.29
9	49.87
13	67.51
14	62.18
16	123.19

[a] Control (Isotypic MoAb) = K562 cells stained with an isotypic MoAb. [b] Control (STAT5 MoAb) = K562 cells stained with a STAT5 MoAb.

Cell cycle analysis (Figure 4 and Table 3) revealed that all compounds that decrease the levels of p-STAT5 caused a prevalent block in G_1. These data are consistent with our previous observations and with the function of STAT5 to promote cell cycle progression[18].

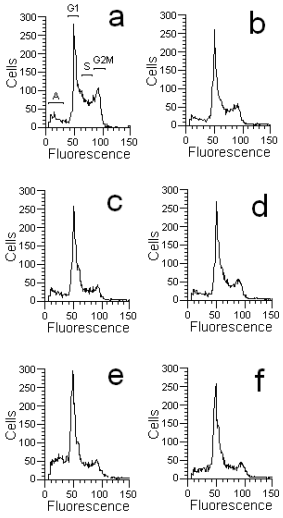


Figure 4. Effects of pimozide derivatives on DNA content per cell following exposure of K562 cells for 24 h. (a) control; (b) **8** (6 μM); (c) **9** (2 μM); (d) **16** (3 μM); (e) **13** (8 μM); (f) **14** (2 μM).

Table 3. Cell cycle distribution (%) of K562 cells after 24 h exposure to pimozide derivatives.

Compound	G_1 (%)	S (%)	G_2/M (%)
Control	34.24	48.04	17.72
8	44.37	50.52	5.11
9	50.67	44.92	4.42
13	48.04	47.84	4.12
14	49.01	46.03	4.96
16	45.42	50.57	4.01

Discussion

On the basis of previous promising results, we explored further modifications of compound **3c,** by maintaining the amide group on the piperidine nitrogen and acylating it with different halogenated substituents in order to study the structure-activity relationship of the STAT5 inhibitors. Compounds **17**-**20** demonstrated in our tests that the substitution of haloacetyl portion with other haloacyl groups is generally detrimental for the activity. Only phenylchloroacetic derivative **18** retained an appreciable IC_{50} (10 μM) and AC_{50} toward K562 cells. More interesting results were obtained investigating the role of the nitrogen N3 of the benzoimidazolinone core in the activity of the compounds.

Two derivatives **8** and **9** have been synthesized with methylated N3 and can be directly compared with **3a** and **3c**. In this case, the growth inhibition and apoptotic potencies are in the same range of concentrations. In the immunofluorescence test, both compounds **8** and **9** are able to inhibit pSTAT5 signal when added at 15 μM but only **9** is effective also at 10 μM concentration, showing a median fluorescence value close to the one obtained with Isotypic MoAb control.

Replacement of the nitrogen at 3 position of the benzoimidazolinone core with an oxygen atom (compounds **13** and **14**) allow to maintain a high growth inhibition and pro-apoptotic activity. Indeed, the cytotoxic activity of compound **14** was similar to pimozide while, of note, compound **13** showed a marked increase of cytotoxic activity (IC_{50} 0.75 μM). Interestingly, on one hand, chloro-substituted compound **13** resulted more potent than iodo-substitued analog **14,** and on the other hand compounds **3c** and **9** (iodo-substituted) were found more potent than respective chloro substituted analogs **3a** and **8**. Therefore, the peculiar relative potency of compounds demonstrated in the **13** *vs* **14** system is in contrast with a trend that could be envisaged in the other cases, for example, considering a role of different alkylating power of iodoacetyl *vs* chloroacetyl groups. At least in this system, a more complex and specific mechanism seems to be important for the biological potency. In the anti-STAT5 monoclonal antibody test, both **13** and **14** showed the ability to act at 10 μM concentration, resulting almost equal or slightly more active than **3c**.

Finally, two compounds, **15** and **16**, were synthetized without the piperidine ring but directly functionalized with halogenated acetyl substituents at N1 of benzimidazolone ring in order to obtain information about the relative importance of the piperidine portion. Despite we had poor expectations for this strategy, results were somehow surprising. As a matter of fact, compound **15** was found not active, while **16** demonstrated a better activity than pimozide. The anti-STAT5 monoclonal antibody test of **16**, moreover, displayed a moderate activity at 10 μM that rose to very good at higher concentration (15 μM). This is not a trivial finding since the piperidine ring has been linked to the determinism of QT prolongation by antipsychotic agents making a piperidine-less structure a desirable feature for a STAT5 inhibitor compound.[19]

Conclusions

In the search of new molecules able to inhibit the uncontrolled proliferation of BCR-ABL expressing cell lines, pimozide was identified as a promising, although unexpected, structure able to interfere with the pSTAT-5 constitutive overexpression. In our previous and present studies, we discovered that important modifications of pimozide structure lead to compounds still or even more active than pimozide itself. Apart from di(p-fluorophenyl)-butyl side chain, in the present study we found that also the benzoimidazolinone ring or the piperidine portion can be chemically modified resulting in compounds highly active as growth inhibitors and apoptosis inducers and able to lower the phosphorylated STAT-5 content.

The presence of a haloacetyl group gives the idea of an important possible contribute of covalent bonds formations in the mechanism of these derivatives, but in the case of the most active compound **13**, a chloroacetyl bearing molecule that overcomes the corresponding iodoacetyl **14** activity, there is the idea that other specific mechanisms can be preferably involved.

We have no specific evidence that our compounds alkylate intracellular proteins, thereby interfering with STAT activation, and we feel this possibility is too speculative to discuss at this time. As reported in the previous literature of Nelson and coworkers, pimozide action toward pSTAT5 levels is not yet supported by a known mechanism, and direct interaction with the STAT5 protein or its SH2 domain is still not demonstrated. At the same time, also the mechanism by which the present new molecules modulates STAT5 expression remains to be elucidated and it is an intriguing problem currently under investigation.

Experimental Section

Chemistry

General procedures

Reagents were purchased from commercial suppliers and used without further purification. Nuclear magnetic resonance spectra (^{1}H-NMR e ^{13}C-NMR) were determined in d_6-DMSO or $CDCl_3$ solutions and recorded with a Bruker AC-200 or a Varian Mercury Plus 400 spectrometers. Peak positions are given in parts per million downfield from tetramethylsilane as the internal standard; J values are expressed in hertz. Mass analyses were performed on Finnigan MAT 95 instrument with BE geometry using double focusing electrospray (ESI) technique. Thin layer chromatographies (TLCs) were performed on silica gel F-254 to evaluate reaction courses and mixtures. Flash chromatography was performed on

Merck 60 silica gel (0.063-0.200mm). The anhydrification of solvents was performed following standard techniques. All non-aqueous reactions were run in argon atmosphere. Solutions containing the final products were dried over Na_2SO_4, then filtered and concentrated *in vacuo* using rotatory evaporator.

Tertbutyl-4(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-carboxylate (5)

To a solution of **4** (436 mg, 2 mmol) in methanol (10 mL) was added di tertbutyl dicarbonate (480 mg, 2.2 mmol) and the solution was kept overnight under stirring. The solvent was then evaporated, the residue solubilized in ethyl acetate, and washed with aqueous citric acid 0.5 M, water, saturated solution of $NaHCO_3$ and brine. After drying and solvent evaporation the product **5** was obtained in 85% yield.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.45 (s, 9H), 1.75-1.90 (m, 2H), 2.20-2.40 (m, 2H), 2.75-2.95 (m, 2H), 4.25-4.4 (m, 2H), 4.35-4.6 (m, 1H), 6.9-7.15 (m, 4H), 9.25 (s, 1H).

Tertbutyl-4(3-methyl-2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidin-1-carboxylate (6)

To a dispersion of NaH 60% (94 mg, 2.2 mmol) in dry DMF (3 mL) was added dropwise a solution of **5** (637 mg, 2 mmol) in dry DMF, and stirring was kept for 30 min. Then, CH_3I (101 µL, 2 mmol) was added and the solution was stirred for 6h. The reaction, was quenched with water and the solvent evaporated. H_2O and AcOEt were then added and the organic solution was separeted. Three extractions of the aqueous phase were performed with AcOEt, while the combined organic phases were washed with, brine, then dried over Na_2SO_4 and evaporated. Chromatographic purification gave the product **6**, 577 mg. Yield 87%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.45 (s, 9H), 1.75-1.90 (m, 3H), 2.20-2.45 (m, 2H), 2.75-2.95 (m, 2H), 3.41 (s, 3H), 4.27-4.34 (m, 2H), 4.35-4.57 (m, 1H), 6.90-7.17 (m, 4H).

1-[1-(2-Chloroacetyl)piperidin-4-yl]3-metil-1H-benzo[d]imidazol-2(3H)-one (8)

To a solution of compound **6** (331 mg, 1 mmol) dissolved 3 mL of CH_2Cl_2, trifluoroacetic acid (3 mL) was added. The solution was kept under stirring at room temperature for 30 minutes. After removal of the solvent under reduced pressure, 5 mL of $CHCl_3$ were added and then triethylamine (0.35 µL, 2.5 mmol). A solution of chloroacetyl chloride (135 mg, 1.2 mmol) in $CHCl_3$ (3 mL) was finally added dropwise under stirring at 0° C. The solution was kept under stirring at room temperature overnight. The mixture was washed with HCl 5%, H_2O, saturated solution of $NaHCO_3$ and brine. After removal of the solvent under reduced pressure, the solution was concentrated using AcOEt. The chromatography purification furnished the desired product **8**, 121 mg. Yield 40%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.945 (m, 2H), 2.23-2.60 (m, 2H), 2.70-2.90 (m, 1H), 3.20-3.40 (m, 1H), 3.413 (s, 3H), 3.98-4.10 (m, 1H), 4.12-4.15 (m, 2H), 4.30-4.50 (m, 1H), 4.75-4.90 (m, 1H), 6.95-7.10 (m, 4H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 27.22, 28.92, 29.68, 41.14, 42.19, 46.14, 50.58, 107.84, 180.97, 121.26, 121.26, 127.85, 130.22, 153.77, 165.15;

MS[ESI]: 308.32 [M+1].

1-[1-(2-Iodoacetyl)piperidin-4-yl]3-methyl-1H-benzo[d]imidazol-2(3H)-one (9)

To a solution of **8** (100 mg, 0.324 mmol) in acetone, NaI was added (97 mg, 2 eq.). The solution was kept under stirring for 24 h. After a filtration the solution was concentrated *in vacuo*. Residue was dissolved in ethyl acetate and washed with H_2O and brine. After the removal of the solvent under reduced pressure and chromatographic purification, the desired product **9** is obtained (90 mg). Yield 70%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.80-1.95 (m, 2H), 2.22-2.60 (m, 2H), 2.70-2.90 (m, 1H), 3.20-3.40 (m, 1H), 3.40 (s, 3H), 3.75-3.88 (m, 2H), 3.90-4.10 (m, 1H), 4.50-4.70 (m, 1H), 4.70-4.90 (m,1H), 6.97-7.13 (m, 4H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 3.98, 27.15, 28.74, 28.92, 42.07, 46.96, 50.48, 107.77, 108.95, 121.17, 127.77, 130.17, 153.78, 166.39;

MS(ESI): 399.71 [M+1].

N-(1-Benzylpiperidin-4-yl)-2-aminophenol (10)

To a solution of 2-aminophenol (436 mg, 4 mmol) in dry CH_2Cl_2, N-benzyl-4-piperidone (757 mg, 4 mmol) and sodium triacetoxyborohydride (1.27 gr, 6 mmol) were added, obtaining a yellow solution. The solution was kept under stirring at room temperature for 14 h. The mixture is washed with saturated $NaHCO_3$ and brine. After removal of the organic solvent *in vacuo* and purification on column chromatography, the desired product **10** was obtained as beige crystalline solid, 850 mg. Yield 75 %.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.40-1.65 (m, 2H), 2.03-2.20 (m, 4H), 2.93 (d, J = 18, 2H), 3.20-3.38 (m, 1H), 3.63 (s, 2H), 6.56-6.80 (m, 4H), 7.25-7.40 (m, 5H).

3-(1-Benzylpiperidin-4-yl)benzo[d]oxazole-2(3H)-one (11)

To a solution of **10** (841 mg, 2.97 mmol) dissolved in dry CH_2Cl_2, (15 mL), carbonyldiimidazole (528 mg, 1.1 eq) was added. The obtained solution was yellow-orange and it was kept under stirring for 16 h. After dilution with more CH_2Cl_2 and washing with H_2O and brine, the organic phase was concentrated *in vacuo*. The chromatography column furnished product **11** as white crystal solid (810 mg). Yield 88%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.82-1.87 (m, 2H), 2.10-2.25 (m, 2H), 2.25-2.57 (m, 2H), 2.98-3.17 (m, 2H), 3.57 (s, 2H), 4.10-2.35 (m, 1H), 7.05-7.35 (m, 9H).

Tertbutyl-4-(2-oxobenzo[d]oxazole-3-(2H)-yl)piperidin-1-carboxylate (12)

To a solution of **11** (440 mg, 1.42 mmol) in EtOH, di-*tert*-butyl dicarbonate (370 mg, 1.2 eq) and catalytic amount of 10% Palladium on carbon were added. The mixture was kept under stirring in a hydrogen atmosphere for 2 h. Catalyzer was then filtered out and the solution was concentrated *in vacuo* obtaining a yellow oil. The chromatography column furnished white crystals of **12**, 380 mg. Yield 84%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.49 (s, 9H), 1.81-1.96 (m, 2H), 2.17-2.40 (m, 2H), 2.75-2.95 (9mm, 2H), 4.20-4.40 (m, 3H), 7.07-7.23 (m, 4H).

3-(1-(2-Chloroacetyl-piperidin-4-yl)benzo[d]oxazol-2-(3H)-one (13)

The procedure was similar as described for **8**, starting from **12** (380 mg, 1.18 mmol) Chromatography column furnished product **13** as white solid, 225 mg. Yield 65%.

^{1}H NMR ($CDCl_3$, 200MHz): δ 1.90-2.12 (m, 2H), 2.17-2.52 (m, 2H), 2.66-2.85 (m, 1H), 3.20-3.60 (m, 1H), 4.06-4.24 (m, 3H), 4.35-4.50 (m, 1H), 4.78-4.85 (m, 1H), 7.04-7.24 (m, 4H).

^{13}C NMR ($CDCl_3$, 400 MHz): δ 28.53, 29.26, 41.05, 41.85, 45.81, 51.99, 109.47, 110.52, 122.60, 123.92, 129.60, 142.72, 153.87, 165.19;

MS(ESI): 295.44 [M+1].

3-(1-(2-Iodoacetyl-piperidin-4-yl)benzo[d]oxazole-2-(3H)-one (14)

The procedure was similar as described for **9**, starting from compound **13** (50 mg, 0.17 mmol). Chromatographic purification gave the desired product **14** as white solid, 45 mg. Yield 68%.

^{1}H NMR ($CDCl_3$, 200MHz): δ 1.85- 2.05 (m, 2H), 2.21-2.38 (m, 1H), 2.38-2.63 (m, 1H), 2.67-2.85 (m, 1H), 3.15-3.37 (m, 1H), 3.75-3.85 (m, 2H), 3.86- 4.10 (m, 1H), 4.37-4.57 (m, 1H), 4.75-2.95 (m, 1H), 7.08-7.22 (m, 4H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 28.42, 28.56, 41.79, 46.69, 51.96, 109.53, 110.53, 122.59, 123.92, 129.56, 142.72, 153.90, 166.51;

MS(ESI): 387.305 [M+1]

1-(2-Chloroacetyl)-1H-benzo[d]-imidazol-2(3H)one (15)

To a mixture of NaH (60%, 80 mg, 2 mmol) in dry DMF (4 mL), 2(1*H*)-Benzimidazolone (268 mg, 2 mmol) was added, giving a milky white solution. The solution was kept under stirring for 30 min. After cooling with ice bath, chloroacetyl chloride (0.18 mL, 2.2 mmol) was then cautiously added. The solution was kept under stirring for 24h. The mixture was then poured into crushed ice, acidified with 5% HCl and extracted three times with small amounts of ethyl acetate. The combined organic phases were washed with water and brine, dried and concentrated *in vacuo*. The solid was then purified in chromatography column to obtain product **15** as white solid (135 mg). Yield 32%.

^{1}H NMR (DMSO, 200MHz): δ 5.05 (s, 2H), 7.03-7.22 (m, 3H), 7.97-8.01 (m, 1H), 11.51 (s, 1H);

^{13}C NMR (DMSO, 400 MHz): δ 45.45, 109.29, 114.61, 121.79, 124.73, 126.64, 129.29, 151.98, 166.24.

MS (ESI): 177.15 [M -Cl +1].

1-(2-Iodo acetyl)-1H-benzo[d]-imidazol-2(3H)one (16)

The procedure was similar as described for **9**, starting from compound **15** (125 mg, 0.6 mmol). After chromatographic purification, **16** was obtained as a white solid (70 mg). Yield 40%.

^{1}H NMR (DMSO, 200 MHz): δ 4.57 (s, 2H), 7.035-7.22 (m, 3H), 7.94-7.98 (m, 1H), 11.54 (s, 1H);

^{13}C NMR (DMSO, 400 MHz): δ 109.27, 114.86, 121.81, 124.83, 126.89, 128.89, 151.60, 168.28.

MS (ESI): 177.15 [M -I +1].

(*R*, *S*) 1-[1-(2-Chloropropanoyl)-piperidin-4-yl]-1H-benzo[d]imidazol-2(3H)-one (17)

To a solution of compound **4** (217 mg, 1 mmol) in CH_2Cl_2 (4 mL) was added triethylamine (0.140 µL, 1 mmol) and, after cooling in ice bath, 2-chloropropanoyl chloride (145 µL, 1.5 mmol) was added dropwise. The solution was kept under stirring at room temperature for 24 h. Afterwards, the mixture was diluted with CH_2Cl_2 and washed with 5% HCl, saturated $NaHCO_3$ solution and brine. After removal of the solvent under reduced pressure, the chromatography purification furnished **17** as a white solid, 205 mg. Yield 66%.

^{1}H NMR ($CDCl_3$, 200MHz): δ 1.723 (d, J = 6.2, 3H), 1.959 (s, 2H), 2.26-2.60 (m, 1H), 2.65-2.90 (m, 2H), 3.19-3.40 (m, 2H), 4.134 (m, 1H), 4.07-4.25 (m, 1H), 4.67 (d, J = 6.4, 1H), 4.79-4.94 (m, 1H), 7.073 (s, 1H), 8.843 (s, 1H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 21.05, 28.38, 28.79, 28.97, 42.42, 49.63, 50.18, 109.64, 109.99, 121.46, 121.61, 128.03, 128.67, 154.98, 167.33;

MS (ESI): 308.544 [M+1].

(*R*, *S*) 1-[1-(2-Chloro-2-phenylacetyl)-piperidin-4-yl]-1H-benzo[d]imidazol-2(3H)-one (18)

(R, S)-Mandelic acid (304 mg, 2 mmol) was dissolved in thionyl chloride (6 mL). The solution was kept under stirring at room temperature for 24 h and then it was concentrated *in vacuo* to obtain the phenylchloroacetic acid chloride, as a colorless oil, used with no further purification. To a solution of compound **4** (414 mg, 2 mmol) dissolved in chloroform (7 mL) and cooled in ice bath, triethylamine (280 µL, 2 mmol) was added. Phenylchloroacetic acid chloride dissolved in chloroform was then added dropwise and the solution was kept under stirring at room temperature for 24 h. The mixture was washed with HCl 5%, H_2O, saturated solution of $NaHCO_3$ and brine. After removal of the solvent under reduced pressure, the chromatography purification gave the desired product **18** as a white solid, 104 mg. Yield 14%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.7-2.98 (m, 2H), 2.05-2.40 (m-2H), 2.63-2.87 (m, 1H), 3.02-3.23 (m, 1H), 2.9-4.10 (m, 1H), 2.40-2.55 (m, 1H), 5.80-5.95 (m, 1H), 5.78-5.81 (d, J=7.4, 1H), 6.90-7.15 (m, 4H), 7.30-7.60 (m, 5H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 28.62, 28.69, 29.03, 42.76, 45.63, 49.76, 109.36, 109.87, 121.04, 121.49, 121.68, 121.76, 127.90, 128.40, 128.61, 128.85, 129.02, 129.25, 154.76, 165.52;

MS (ESI): 370.349 [M+1].

1-[1-(4-Chlorobutyryl)piperidin-4-yl]-1H-benzo[d]imidazol-2(3H)-one (19)

The procedure was similar as described for **17**, from compound **4** (217 mg, 1 mmol) and 4-chlorobutyryl chloride (170 µL, 1.5 mmol). After chromatography purification **19** was obtained as a white solid, 118 mg. Yield 36%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.80-2.00 (m, 2H), 2.09-2.24 (m, 2H), 2.28-2.37 (m, 2H), 2.50-2.65 (m, 2H), 2.65-2.80 (m, 1H), 3.10-3.30 (m, 1H), 3.60-3.75 (m, 2H), 4.05-4.18 (m, 1H), 4.41-4.61 (m, 1H), 4.80-4.97 (m, 1H) 7.03-7.22 (m, 4H), 9.43 (s, 1H);

^{13}C NMR ($CDCl_3$, 400 MHz): δ 27.84, 28.98, 29.77, 31.09, 41.49, 44.94, 45.05, 50.71, 109.24, 109.94, 121.32, 121.58, 127.88, 128.78, 154.94, 170.17;

MS (ESI): 322.420 [M+1].

1-[1-(3-Iodopropanoyl)piperidin-4-yl]-1H-benzo[d]imidazol-2(3H)-one (20)

To a solution of compound **4** (217 mg, 1 mmol) dissolved in $CHCl_3$ (7 mL), 3-iodopropionic acid (200 mg, 1 mmol), EDC·HCl and HOBT (17 mg, 1.1 mmol) were added. The solution was kept under stirring at room temperature for 24 h. The mixture was then washed with HCl 1M, H_2O,

saturated solution of $NaHCO_3$ and brine. After removal of the solvent under reduced pressure, the chromatography purification (AcOEt) furnished 55.5 mg of the desired product **20**. Yield 15%.

^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.9 (m, 2H), 2.20-2.35 (m, 2H), 2.60-2.80 (m, 2H), 3.0-3.13 (m, 2H), 3.16-3.37 (m, 2H), 3.97-4.10 (m, 1H), 4.30-4.63 (m, 1H), 7.05-7.13 (m, 4H), 9.54 (s, 1H);

MS (ESI): 400.556 [M+1].

Cell lines and culture

K562 human myeloid cell line was used in this study. K562 express the anti-apoptotic oncogene BCR-ABL and high levels of phosphorylated STAT5. Cell lines were grown in RPMI 1640 (Gibco Grand Island, NY, USA) containing 10% FCS (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), and 2 mM l-glutamine (Sigma Chemical Co., St. Louis, MO) in a 5% CO2 atmosphere at 37 °C.

Cytotoxicity assays and determination of drug interactions

Cytotoxicity was evaluated by the Trypan blue dye exclusion test. To determine the growth inhibitory activity of the drugs tested, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 mL of complete medium and treated with different concentrations of each drug. After 48 h of incubation, the number of viable cells was determined and expressed as percent of control proliferation.

Apoptosis evaluation

Drug induced apoptosis and necrosis was determined morphologically after labeling the cells with acridine orange and ethidium bromide and by the Annessine V detection test. In the morphological test cells (2×10^5) were centrifuged (300g) and the pellet was resuspended in 25 µL of the dye mixture. Ten microliters of the mixture was examined in oil immersion with a 100× objective using a fluorescence microscope. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange (100 µg/mL) or ethidium bromide (100 µg/mL), respectively, and by the formation of apoptotic bodies. The percentage of apoptotic cells was determined after counting at least 300 cells. For the Annessine V test, cells (1 × 106) were washed with PBS and centrifuged at 200g for 5 min. Cell pellets were suspended in 100 µL of staining solution containing FITC-conjugated Annexine-V and propidium iodide (Annexine-V-Fluos Staining Kit, Roche Molecular Biochemicals, Mannheim, Germany) and incubated for 15 min at 20 °C: Annexine V positive cells were evaluated by flow cytometry (Becton–Dickinson).

Flow cytometry analysis of cell cycle and apoptosis.

Cells were washed once in ice-cold PBS and resuspended at 1×10^6 ml in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 µg/mL in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After 30 min of incubation the fluorescence of each sample was analyzed as single-parameter frequency histograms using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). The distribution of cells in the cell cycle was determined using the ModFit LT program (Verity Software House, Inc.). Apoptosis was determined by evaluating the percentage of hypoploid nuclei accumulated in the sub-G0–G1 peak after labeling with propidium iodide.

Flow cytometric evaluation of intracellular proteins.

About $1x10^6$ cells were washed twice with PBS (Sigma) and resuspended in 100 µL cytofix/cytoperm solution (Becton-Dickinson) at 4˚C. After 20 min the cells were washed twice with BD Perm/Wash™ buffer solution (Becton-Dickinson) and incubated with 20 µL of the specific fluorochrome(PE)-conjugated monoclonal antibody anti-p-STAT5 (Becton-Dickinson) at 4˚C. After 30 minutes the cells were washed twice and analysed by flow cytometry. Alternatively, the cells were incubated with 2 µL PE-conjugated rat anti-mouse IgG1 monoclonal antibody (Becton-Dickinson) at 4˚C. After 30 minutes the cells were washed twice and analysed by a FACScan flow cytometer (Becton–Dickinson).

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Keywords: STAT5 inhibitors • Pimozide • BCR/ABL expressing leukemia • Apoptosis • Antiproliferation

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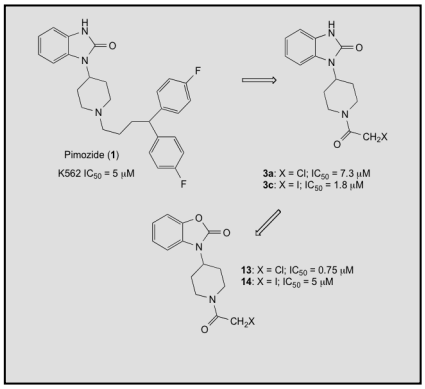

New derivatives of pimozide were obtained with potent growth inhibitor and apoptotic activity in K562 BCR-ABL expressing leukemia cell lines. Moreover, detection of overexpressed phosphorylated STAT5, with fluorochrome-conjugated monoclonal antibody anti-p-STAT5 resulted in strong inhibition when derivatives are used in 10-15 μM concentration, instead of pimozide, active at 30 μM.