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Novel 1,4-benzodiazepine derivatives with antiproliferative properties on tumor cell lines

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Abstract—Novel 1,4-benzodiazepine compounds were synthesized and evaluated for their ability to inhibit the proliferation of tumor cells. Some compounds revealed activities in the micromolar range and were more efficient than reference compound Ro 5-4864. Preliminary SAR helped to identify critical motifs for antiproliferative activity and led to the discovery of a compound selective of a melanoma cell-line, known for its resistance to chemotherapy.

Benzodiazepines are widespread compounds used for the treatment of mental disorders. A few years ago, some of these compounds showed antiproliferative properties against some tumor cell lines. This highlights them as potential anticancer agents.^{1,2}

We are presently resorting to the 1,4-benzodiazepine scaffold, a suitable template for combinatorial chemistry³, to design small inhibitors of STAT3 dimerization. STAT3 (Signal Transducer and Activator of Transcription 3) plays a key role in cancer, by regulating as a dimer the expression of anti-apoptotic or pro-survival genes.⁴ Dimerization occurs upon phosphorylation on its Tyr705, through reciprocal interaction between the SH2 (Src Homology 2) domain of STAT3 and the pTyr residue. On the basis of preliminary molecular modelling based on the X-ray crystal structure of STAT3⁵, the N-1 position on the 1,4-benzodiazepine structure (**Figure 1**) was used to substitute phosphate or phosphonate analogues, mimicking the role of pTyr in its SH2 domain interaction. The C-3 position was used to introduce a basic substituent to probe the effect of a possible ionic interaction with the SH2 domain.

The compounds were evaluated by an ELISA test for their ability to disrupt *in vitro* STAT3 dimerization but showed no significant activity. Nevertheless, they were further evaluated for their possible antiproliferative activities on two STAT3-dependent cell lines, NIH 3T3/v-Src cells⁶ and melanoma cells A2058⁷, as well as on Hela cells, a cervical carcinoma tumor cell line, whose growth is not dependent on STAT3.⁸

Here, we report the synthesis and biological evaluation of our derivatives.

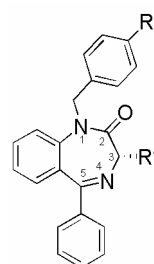
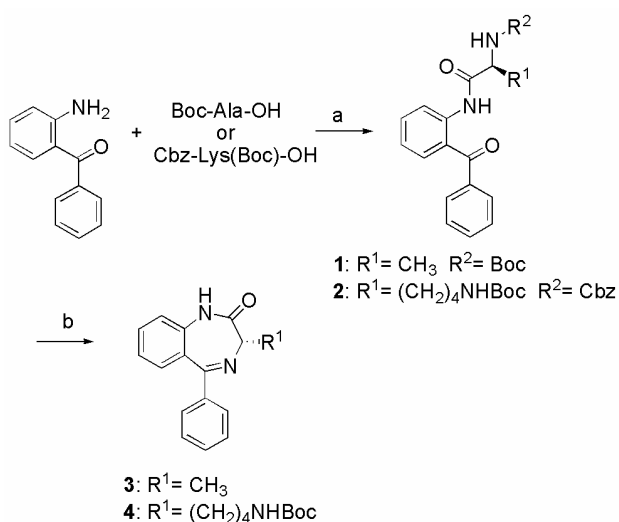


Figure 1. Structure of the 1,4-benzodiazepine compounds.

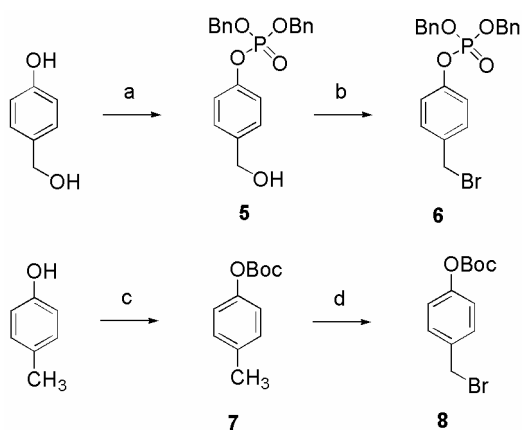
Keywords : Proliferation ; 1,4-benzodiazepines ; Cancer; Melanoma.
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Scheme 1. (a) TFFH, DIEA, CH₂Cl₂, rt; (b) i-20%TFA/CH₂Cl₂ for **3** or H₂/Pd-C, MeOH for **4**; ii- 5%AcOH in CH₂Cl₂, reflux.

The synthesis of the diazepine core is described in **Scheme 1**. Peptidic coupling of 2-aminobenzophenone with suitably protected alanine or lysine could be achieved by the *in situ* formation of acyl-fluoride⁹ compounds with the use of tetramethylfluoroformamidinium hexafluorophosphate (TFFH) to give **1** and **2** in 69-85% yields. Boc and Cbz protecting groups were completely removed respectively by treatment with 20% trifluoroacetic acid (TFA) in methylene chloride and by Pd-catalyzed hydrogenolytic cleavage.

Cyclization to obtain the diazepine core was achieved using 5% of acetic acid in methylene chloride to give **3** and **4** in quantitative yield.

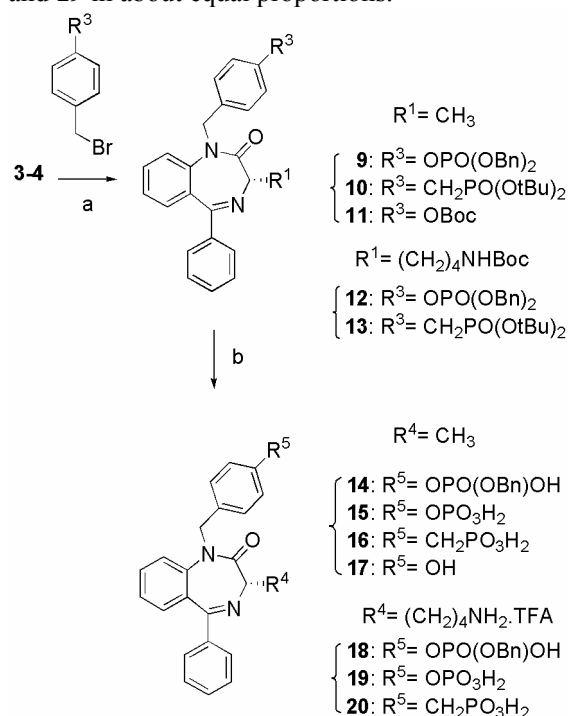


Scheme 2. (a) Dibenzylphosphite, DIEA, CCl₄, DMAP, CH₂Cl₂, -15°C, (b) CBr₄, PPh₃, CH₂Cl₂, rt; (c) Boc₂O, Na₂CO₃, THF, rt; (d) NBS, dibenzoylperoxyde, CCl₄, reflux.

The preparation of two brominated derivatives necessary for the N-alkylation of the core diazepine is described in **Scheme 2**. 4-hydroxybenzylalcohol was

treated with dibenzylphosphite¹⁰ to give dibenzylprotected phosphate derivative **5** in 70% yield. Transformation of the benzyl alcohol **5** to the bromide derivative **6** was achieved in 63% yield using triphenylphosphine and carbon tetrabromide.¹¹ Bromide **8** was obtained by Boc-protecting *p*-cresol followed by N-bromosuccinimide (NBS) bromination in 65% yield. The 4-(di-*tert*-butylphosphonomethyl)benzylbromide used to synthesize **10** and **13** was prepared as previously described.¹²

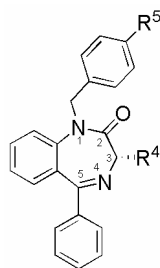
Alkylation of diazepines **3** and **4** (**Scheme 3**) with benzyl bromide derivatives in the presence of the mild base cesium carbonate¹³ afforded **9-13** in 80-90% yields. Final compounds **16**, **17** and **20** were obtained in quantitative yield, by treatment with 50% TFA in methylene chloride for 3h. Under such conditions, compounds **9** and **12** gave monobenzyl-protected compounds **14** and **18**, and the unprotected analogs **15** and **19** in about equal proportions.



Scheme 3. (a) Cs₂CO₃, CH₂Cl₂, rt; (b) 50%TFA/CH₂Cl₂, rt.

The lack of inhibitory effects of these benzodiazepines against STAT3 dimerization led us to investigate if alternatively, and in line with the findings reported in the literature¹⁴, they could inhibit Src kinase activity. However, no inhibitory effect was found in the case of the present compounds.

Nevertheless, in cell assays, some of the benzodiazepine derivatives demonstrated efficient antiproliferative activities, inhibiting cell growth with IC₅₀ in the micromolar range.

Table 1. Antiproliferative activity against tumor cell lines

Compound	R ⁴	R ⁵	IC ₅₀ ± SEM (μM) ^a		
			NIH 3T3/v-Src ^b	A2058 ^c	HeLa ^c
Ro 5-4864 ¹⁷	-	-	31.9 ± 4.4	≥ 100	≥ 100
3	CH ₃	N-1 unsubstituted	n.a. ^d	n.a	n.a
14	CH ₃		49.5 ± 3.2	61.5 ± 7.9	40.5 ± 0.5
15	CH ₃		36.6 ± 5.9	28.9 ± 1.5	21.3 ± 1.7
16	CH ₃		n.a	n.a	n.a
17	CH ₃		38.6 ± 3.5	27.2 ± 0.3	29.3 ± 0.6
19			≥ 80	33.8 ± 0.5	≥ 80
20			n.a	n.a	n.a

^a IC₅₀ values expressed with standard error.

^b Cell growth determined by counting cells with a particle counter.¹⁵

^c Cytotoxic activity determined by a WST-1 test.¹⁶

^d Showed no activity at 150 μM.

Cell growth inhibition was first evaluated on v-Src transformed mouse fibroblasts.¹⁵ The cytotoxic activity of all the compounds was then tested against two human tumor cell lines, A2058 and HeLa cells, using a WST-1 colorimetric test.¹⁶ Results are given in **Table 1**. In addition, the benzodiazepine Ro 5-4864 (4'-chlorodiazepam), whose antiproliferative activity has already been demonstrated against some tumor cell lines¹⁷⁻¹⁹, was also evaluated as the reference compound.

These data indicate that whilst the unsubstituted benzodiazepine **3** is inactive towards any of the three cell types, some of our substituted benzodiazepines are more efficient than reference compound Ro 5-4864, on both human tumor cell lines.

N-1 unsubstituted compound **3** showed no antiproliferative activity towards any of the three cell types, indicating that further substitutions are necessary for biological activity.

For the C-3 methyl series (R⁴ = CH₃), N-substitution with a *p*-phosphorylated benzyl moiety improved activity in each cell type with IC₅₀ in the micromolar range (**15**). Monobenzyl-protected phosphate derivative **14**, initially synthesized to improve cell

penetration, was less potent than the free phosphate analog **15** against the three cell types. Interestingly, phosphate replacement with phosphonate abrogated biological activity in each cell type. The higher pKa value of phosphonate derivative may be the cause for the loss of activity.²⁰ The presence of an oxygen atom at the *p*-position on the benzyl ring may also be important.

We therefore investigated if a hydroxyl group could be sufficient to retain activity. 4-hydroxylated compound **17** was accordingly synthesized and was found to be as efficient as the phosphate analogue **15**. These data indicate that the presence of an oxygen atom seems critical for antiproliferative activity. Moreover, they suggest that it may be possible to optimize potency with other *O*-substituted compounds. On the other hand, the most potent compounds (**14**, **15** and **17**) were also active on HeLa cells, indicating that they probably do not target the STAT3 pathway.

For the C-3 aminobutyl series (R⁴ = (CH₂)₄NH₂), the phosphonate derivative **20** was also inactive. These data confirmed the importance of a phenolic group or a *p*-phosphorylated benzyl moiety. Monobenzyl-protected phosphate derivative **18** could not be tested because of low solubility. Surprisingly,

introduction of a basic alkyl chain in C-3 position led to a compound (**19**) rather selective of melanoma cells, with only weak potency on the two other cell types.

In the literature, other benzodiazepines, such as Ro 5-4864, have been shown to have antiproliferative activity, most of the time *via* an undefined or uncertain mechanism.¹⁷⁻¹⁹ Whilst PBR (Peripheral Benzodiazepine Receptor) has been reported to be a possible target^{18,19}, benzodiazepine derivative Bz-423 has recently been found to induce antiproliferative activity independent of the PBR.²¹

Thus, to our knowledge, the present work reports one of the first SAR studies, regarding the effects of substitution on both N-1 and C-3 position, on antiproliferative properties of 1,4-benzodiazepines.

Although our compounds did not inhibit STAT3 dimerization in the Src-STAT3 pathway, they displayed interesting antiproliferative activities against different tumor cell lines. Benzodiazepines **14**, **15** and **17** were even more efficient than reference compound Ro 5-4864 on both human tumor cell lines.

Preliminary SAR helped us to identify critical motifs for activity and led to the discovery of a compound selective for a melanoma cell line, known for its resistance to chemotherapy.²²

In conclusion, these two series of benzodiazepines are promising leads for the development of anticancer agents. Further experiments will be done to identify their physiological target and improve the potency and the selectivity of both series of compounds.

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Supplementary data

Analytical data are available on-line.

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15. NIH 3T3/v-Src fibroblasts (10⁵/well) were seeded into 6-well dishes and incubated overnight at 37°C in 5% CO₂-95% air. After exposure to compounds (at concentrations between 0 and 150 μM) for 72h, cells were washed with PBS, then trypsinised and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The results are expressed as means of at least two independent experiments performed in duplicate. IC₅₀ was determined from a sigmoidal dose-response using GraphPad Prism (GraphPad Software, San Diego, CA).
16. The cytotoxic effect of compounds was determined using the Cell Proliferation Reagent WST-1 assay (Roche Diagnostics, Mannheim, Germany). This colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, leading to formazan formation. A2058 (4000 cells/well, 100μl) and HeLa cells (3000 cells/well, 100μl) were seeded in 96-well plates and incubated overnight at 37°C in 5% CO₂-95% air. After exposure to compounds (at concentrations between 0 and 150 μM) for 72h, cells were incubated with WST-1 (10μl) for 2h, and the absorbance of the samples against a background control was read at 450 nm using a microplate reader. Results are expressed as means of at least two independent experiments performed in triplicate. IC₅₀ was determined from a sigmoidal dose-response using GraphPad Prism (GraphPad Software, San Diego, CA).
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