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Novel 2-[(benzylamino)methyl]pyrrolidine-3,4-diol derivatives as α-mannosidase inhibitors and with antitumor activities against hematological and solid malignancies

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ABSTRACT

Novel α-mannosidase inhibitors of the type (2*R*,3*R*,4*S*)-2-({[(1*R*)-2-hydroxy-1-arylethyl]amino}methyl) pyrrolidine-3,4-diol have been prepared and assayed for their anticancer activities. Compound **30** with the aryl group = 4-trifluoromethylbiphenyl inhibits the proliferation of primary cells and cell lines of different origins, irrespective of Bcl-2 expression levels, inducing a G2/Mcell cycle arrest and by modification of genes involved in cell cycle progression and survival.

1. Introduction

Glycosylation is one of the most important post-translational modifications that proteins undergo. Starting from a basic protein scaffold, glycosylation leads to proteins endowed with varied biological properties and thereby allows amplifying the information contained in a genome.[1]

Consistently, aberrant glycosylation leads to the abnormalities observed in numerous hereditary diseases and appears to also have a role in acquired conditions such as autoimmunity and cancer.[2] The development of new therapeutics that target the glycosylation pathway is therefore a major goal for the treatment of such conditions and it can now take advantage of a fairly well detailed knowledge of the enzymes and substrates involved in this process.[3] The majority of these enzymes are transmembrane proteins that function in the endoplasmic reticulum (ER) and Golgi apparatus in an ordered manner.[4,5] Improvement of the metastatic phenotype through inhibition of the machinery of complex carbohydrate formation would provide an important weapon in the arsenal of cancer treatment.[6,7]

A key enzyme involved in this process is α-mannosidase deputed to removing mannose residues from the maturing oligosaccharide by hydrolyzing the mannosyl glycosidic bond.[8] Two classes of processing α-mannosidases, each one with distinct biochemical properties, catalytic mechanism and amino acid sequences have been described.[9–12] Since the aberrant glycosylation of glycoproteins and glycolipids is one of the molecular changes involved in malignant transformation[13,14] the specific inhibition of α-mannosidases has been proposed as an anticancer strategy to prevent tumor progression and diffusion.[15–19] Kino et al. were the first to report that the subcutaneous administration of swainsonine, a natural inhibitor of Golgi α-mannosidase II containing a 4-amino-4-deoxy-mannofuranoside moiety,[20] completely inhibited formation and growth of lung metastases in a mouse sarcoma model.[21] Afterwards clinical trials have demonstrated that swainsonine

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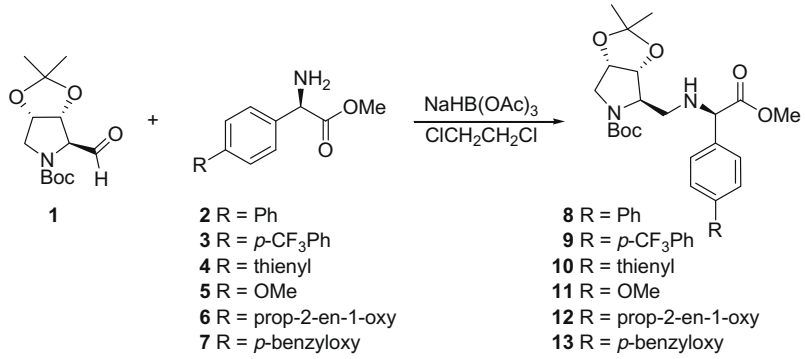


Scheme 1. Reductive aminations.

induces clinical responses in patients with solid and haematological malignancies.[22] However the toxicity observed, possibly resulting from the undesired co-inhibition of lysosomal α-mannosidase,[23,24] prevented further experimentations and resulted in the search for new and more selective α-mannosidases inhibitors. Some analogues of swainsonine as well as simpler derivatives have shown interesting inhibitory properties.[24,25]

In order to facilitate the discovery of new specific α-mannosidase inhibitors, we have developed a new synthesis protocol leading to the generation of a new family of compounds with selective and competitive inhibitory activity for α-mannosidase from jack bean.[26–28] The novel α-mannosidase inhibitors contain in their chemical structure a pyrrolidine ring functionalized to allow their internalization by cells. The introduction of a lipophilic ester moiety led to derivatives that inhibit the growth of human cancer cells lines more efficiently than swainsonine and the other analogues.[29,30]

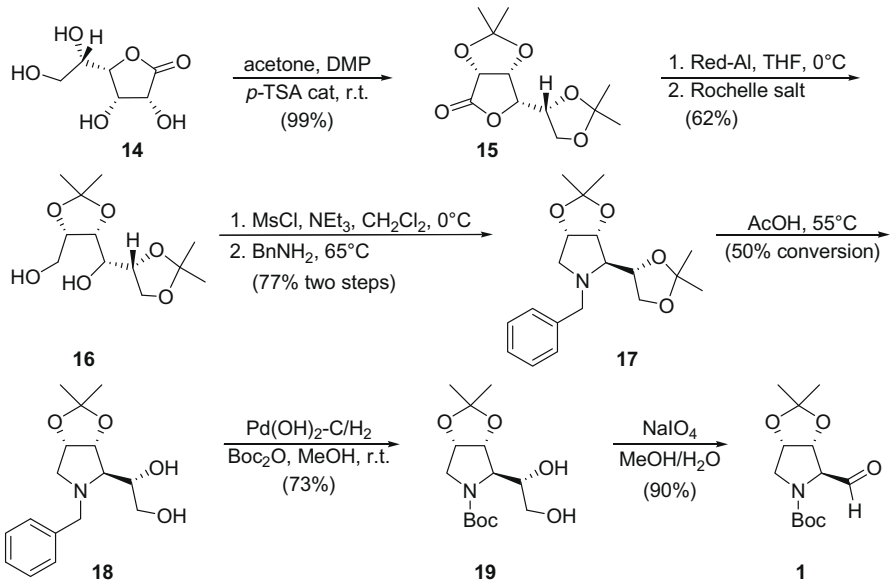
In the present work, we report on the identification, synthesis and biological evaluation of novel α-mannosidase inhibitors with dihydroxypyrrolidine structure characterized by potent and specific anticancer activity in cell lines of different histology and in primary leukemic cells. The choice of biaryl appendices was inspired by reports showing that these groups might improve therapeutic properties of compounds containing them.[31–36]

2. Results and discussion

2.1. Chemical synthesis

The synthetic pathway for the preparation of the new inhibitors was centred on the coupling of two fragments, carbaldehyde **1** and *p*-substituted phenylglycine methyl esters **2**–**7** (Scheme 1), via reductive amination.

The preparation of the starting material, *tert*-butyl(3a*R*,4*R*,6a*S*)-4-formyl-2,2-dimethyltetrahydro-5*H*-[1,3]dioxolo-[4,5-*c*]pyrrole-5-carboxylate (**1**), was performed following a modified procedure (Scheme 2) based on Fleet's synthesis.[37] The modifications allowed us to perform the synthesis faster and in better overall yield, thanks to faster work-up and purification of the intermediates. The first step was the protection of the two diols present in D-gulonic acid γ-lactone **14** (Scheme 2) as acetonides, in the presence of acetone, dimethoxypropane and *p*-toluenesulfonic acid as catalyst. In that case, after neutralization with sodium carbonate, the solvent was evaporated and the product recovered by adding ethyl acetate and extracting with water, instead of filtering the salts on a Celite pad and purify by flash chromatography, as reported in the literature. In the second step lithium aluminium hydride was substituted with Red-Al as reducing agent: in that way the reaction was cleaner and the work-up was performed by simple extraction



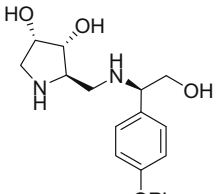
Scheme 2. Synthesis of 2,5-dideoxy-2,5-imino-L-arabinose derivative **1**.

after stirring with saturated aqueous Rochelle's salt, instead of filtration on silica gel.

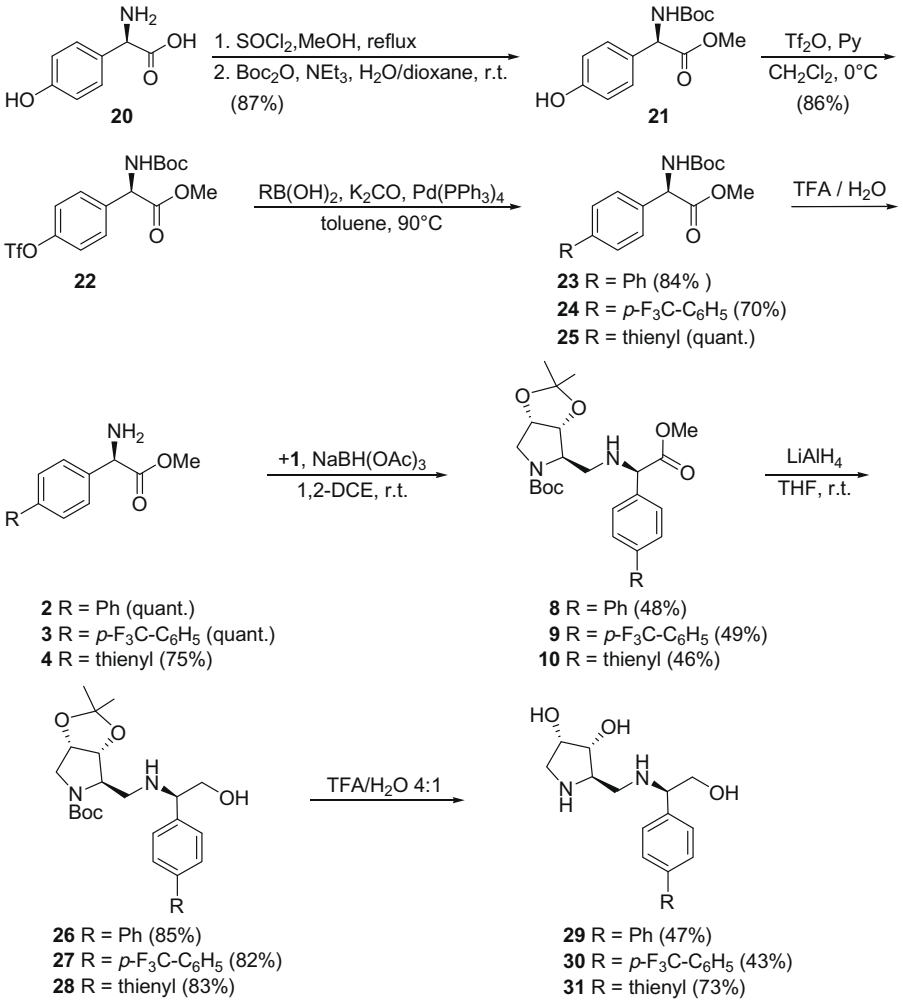
Dimesylate of diol **16** (Scheme 2) was prepared by reaction with methanesulfonyl chloride in CH_2Cl_2 using trietylamine as base. This procedure allowed to isolate the dimesylate by simple extraction. On the contrary, in the Fleet's method, pyridine was used as solvent and its elimination required evaporation in vacuo before extraction and flash chromatography on silica gel. Formation of intermediates **17–19** followed the Fleet's procedure, except for the purification of **17** where the excess of benzylamine was evaporated as its azeotrope with *p*-xylene. The targeted aldehyde **1** was obtained then by oxidation of diol **19** using sodium periodate. For the synthesis of the biarylglycinol moieties, commercially available 4-hydroxy-D-phenylglycine (**20**) (Scheme 3) was used as starting material. After protection of both amino and carboxylic groups, the intermediate **21** (Scheme 3) reacted with triflic anhydride to generate the corresponding triflate **22** (Scheme 3) in good yield. Suzuki cross-coupling[38,39] with phenylboronic acid, 4-(trifluoromethyl)phenylboronic acid and thienylboronic acid in toluene using tetrakis(triphenylphosphine)palladium as catalyst led to the desired *N*-*tert*-butyloxycarbonyl biphenylglycine methyl ester **23**, *N*-*tert*-butyloxycarbonyl-4-(trifluoromethyl)biphenylglycine methylester **24** and *N*-*tert*-butyloxycarbonyl-4-(thienyl)phenylglycine methyl ester **25** (Scheme 3) in 84%, 70% and 84% yield, respectively. *tert*-Butylcarbamate was cleaved and the resulting amines **2–4** were submitted to reductive aminations with the pyrrolidinecarbaldehyde **1**. The corresponding products **8–10** were treated with $LiAlH_4$ to convert the esters into the corresponding primary alcohols **26–28**. Finally, reaction with trifluoroacetic acid/water 4:1 released the completely deprotected diamines **29–31** (Scheme 3).

For the synthesis of the ether series we used simple Williamson's etherification.[40] Reaction of protected 4-hydroxy-D-phenylglycine (**21**) with allyl and benzyl bromide in the presence of potassium carbonate as base, led, after Boc deprotection with trifluoroacetic acid, to the corresponding ethers **6** and **7** (Scheme 4) in good yields. Methyl ether **5** (Scheme 4) was prepared starting from unprotected 4-hydroxy-D-phenylglycine (**20**) through reaction of both carboxylic and phenol groups with MeI at 0 °C leading to the desired 4-methoxy-D-phenylglycine methyl ester **5** (Scheme 4), after treatment with trifluoroacetic acid.

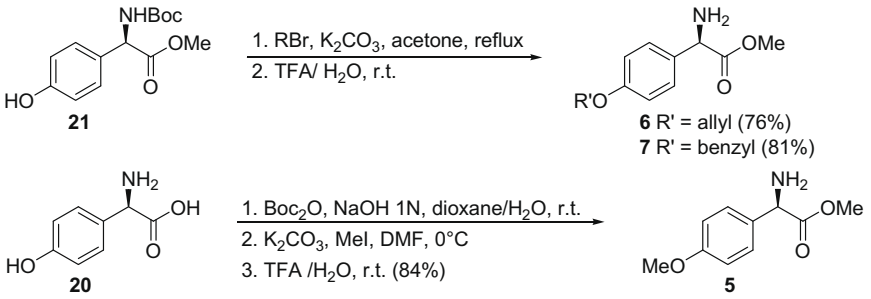
Amines **5–7** were used in the reductive aminations with aldehyde **1**. Reduction of the ester groups into primary alcohols ($LiAlH_4$ in THF) and subsequent deprotection of the diol and amine of the pyrrolidines with trifluoroacetic acid led to the final products **32–34**.



32 R' = allyl
33 R' = benzyl
34 R' = Me



Scheme 3. Synthesis of new 2-[(arylmethylamino)methyl]pyrrolidine-3,4-diols.



Scheme 4. Synthesis of ethers of 4-hydroxy-D-phenylglycine.

2.2. Bioassays

Compounds **29**–**34** were assayed for their inhibitory activities toward 13 commercially available glycosidases[41,42] (Table 1). Under enzyme optimal pH and at 1 mM concentration they did not inhibit α-L-fucosidase from bovine kidney, α-galactosidase from coffee beans, β-galactosidases from *Escherichia coli*, bovine liver and *Aspergillus orizae*, α-glucosidase from rice, amyloglucosidase from *Aspergillus niger*, β-mannosidase from snail and β-*N*-acetylglucosaminidases from jack beans and from bovine kidney. Pyrrolidines **29**–**34** showed weak inhibition of α-glucosidase from yeast (24%, <10%, <10%, 41%, 45%, 41%, respectively), of β-glucosidase from almonds (20%, 50%, 36%, 72%, 40%, 62%, respectively) and of β-xylosidase from *Aspergillus niger* (50%, 52%, 50%, 28%, 45%, 28%, respectively, at 1 mM). However, **29**–**34** were found to be potent inhibitors of α-mannosidase from jack bean, a good model of mammalian Golgi α-mannosidase II,[43] with >98% inhibition at 1 mM. Kinetics measurements gave for **29**: K_i = 0.9 μM, IC_{50} = 1.4 μM; for **30**: K_i = 2.7 μM, IC_{50} = 5 μM; for **31**: K_i = 0.8 μM, IC_{50} = 1.1 μM; for **32**: K_i = 0.5 μM, IC_{50} = 0.9 μM; for **33**: K_i = 0.6 μM, IC_{50} = 1.4 μM and for **34**: K_i = 0.5 μM, IC_{50} = 0.8 μM.

Dihydroxypyrrolidine derivatives **29**–**34** were then evaluated for their capacity to inhibit cell growth on nine established human tumor cell lines of different histology (multiple myeloma, T-cell leukemia, glioblastoma, prostate cancer, lung cancer, and breast cancer). Cells were exposed to increasing concentrations of **29**–**34** for 72 h and viability was subsequently detected by colorimetric assay. Compound **30** (with the 4-(4-$CF_3C_6H_4$)-benzylamino group) showed potent cytotoxic activity in all the cell lines assayed for concentrations <200 μM (Table 2 and Fig. 1 part A and B). Compound **29** and, to a lesser extent, **32** and **34** also exhibited growth inhibition effects although with IC_{50}s >200 μM (data not shown). Compounds **31** and **33** did not show antiproliferative activity.

Table 2
Evaluation of the ability of compound **30** to inhibit cell growth on nine human tumor cell lines of different histology

Cell lines	Histology	IC_{50} (μM) Compound **30**
U266	Multiple myeloma	50
Jurkat	T cell Leukemia	75
U87	Glioblastoma	80
PC3	Prostate adenocarcinoma	132
A549	Lung cancer	152
MCF7	Breast cancer	77
MDA-MB-231	Breast cancer	66
BT474	Breast cancer	93
SKBR3	Breast cancer	48

Table 1
Calculation of the percentage of inhibition (1 mM) and kinetics measurement (IC_{50} and K_i, given in μM) for products **29**, **30**, **31**, **32**, **33** and **34** (optimal pH)

Enzyme (pH)	Compound Percentage of inhibition					
	29	**30**	**31**	**32**	**33**	**34**
α-L-Fucosidase EC 3.2.1.51 1-bovine kidney (6)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
α-Galactosidase EC 3.2.1.22 2-coffee beans (6)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β-Galactosidase EC 3.2.1.23 5-escherichia coli (7)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
8-Aspergillus orizae (4)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
α-Glucosidase EC 3.2.1.20 10-yeast (7)	24	n.i.	n.i.	41	45	41
11-Rice (4)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Amyloglucosidase EC 3.2.1.3 13-aspergillus niger (5)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β-Glucosidase EC 3.2.1.21 15-almonds (5)	20	50	36	72	40	62
α-Mannosidase EC 3.2.1.24 16-jack beans (5)	**99** IC_{50} = 1.4 K_i = 0.9	**98** IC_{50} = 5 K_i = 2.7	**97** IC_{50} = 1.1 K_i = 0.8	**99** IC_{50} = 0.9 K_i = 0.5	**99** IC_{50} = 1.4 K_i = 0.6	**98** IC_{50} = 0.8 K_i = 0.5
β-Mannosidase EC 3.2.1.25 18-snail (4)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
β-Xylosidase EC 3.2.1.37 19-aspergillus niger (5)	50	52	50	28	45	28
β-*N*-Acetylglucosaminidase EC 3.2.1.30 21-jack beans (5)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
22-BOVINE kidney (4)	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.

n.i. = no inhibition (inhibition lower than 10% at 1 mM).

The cytotoxic activity of these drugs is typically detected after 6–12 h of incubating and is initially characterized by extensive intracellular vacuolization. In line with our previous findings with this class of compounds[26,27] these data confirm that the dihydroxypyrrolidine moiety is pivotal for their tumoricidal activity. The side chains of these small molecules are likely to affect drug potency by conferring variable membrane-permeability. However, additional effects of these pharmacophoric moieties cannot, in principle, be excluded. Experiments conducted in A549 cells showed that cell death induced by **30** and related analogues is neither affected by the antioxidant *N*-acetyl-cysteine, nor by the pan-caspase inhibitor zVAD-fmk (data not shown) suggesting that cell killing by these compounds is unlikely to involve reactive oxygen species or caspase activity. Since pyrrolidine **30** exhibited the most potent inhibitory activity on cancer cell growth (Fig. 2) further experiments were conducted on this compound. Initially, in order to provide clues to its mechanism of action, we performed cell cycle analysis and monitored the changes in gene expression profile in breast cancer cell lines treated with this novel compound. Cell cycle analysis showed that the **30** increases the percentage of cells in G2/M phase in a time and dose-dependent manner (Fig. 3). Increasing **30** concentration from 12.5 to 200 μM led to an increase of the cell fraction with G2/M DNA content from 12.5% to 41.9% after a 12-h treatment, while S-phase nuclei decrease from 30% to 16%. These data indicate that the α-mannosidase inhibitor **30** induces cell cycle arrest and accumulation of cells in G2/M phase. Meanwhile, the effect on gene expression was investigated by TLDA using as a framework a panel of 90 genes selected for their relevance in cancer biology and metastasis. As shown in Figure 4, treatment with this compound consistently downregulated

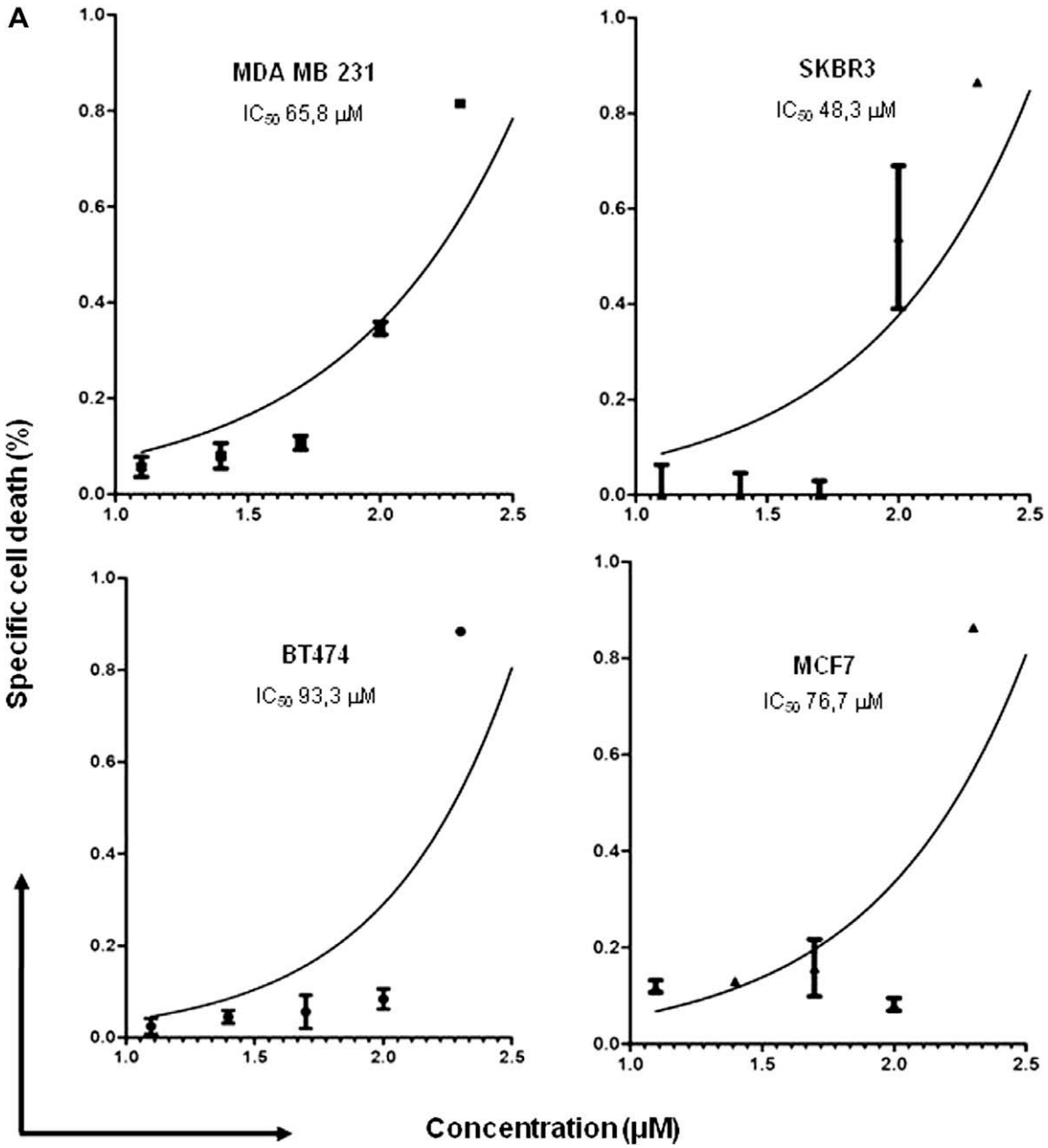


Figure 1. The novel dihydroxypyrrolidine derivatives provide an antiproliferative activity in 8 human cancer cell lines. (part A–B). 5×10^4 cells/well were incubated in 96-well plates, allowed to adhere for 24 h and then incubated with novel compound at concentration ranging between 10^{-2} and 400 μM for 72 h. Thereafter, viability was determined by a commercially available colorimetric assay (CellTiter 96 Aqueous1). Results are shown as means of triplicate wells with SD. IC_{50s} were estimated using GraphPad prism 4.

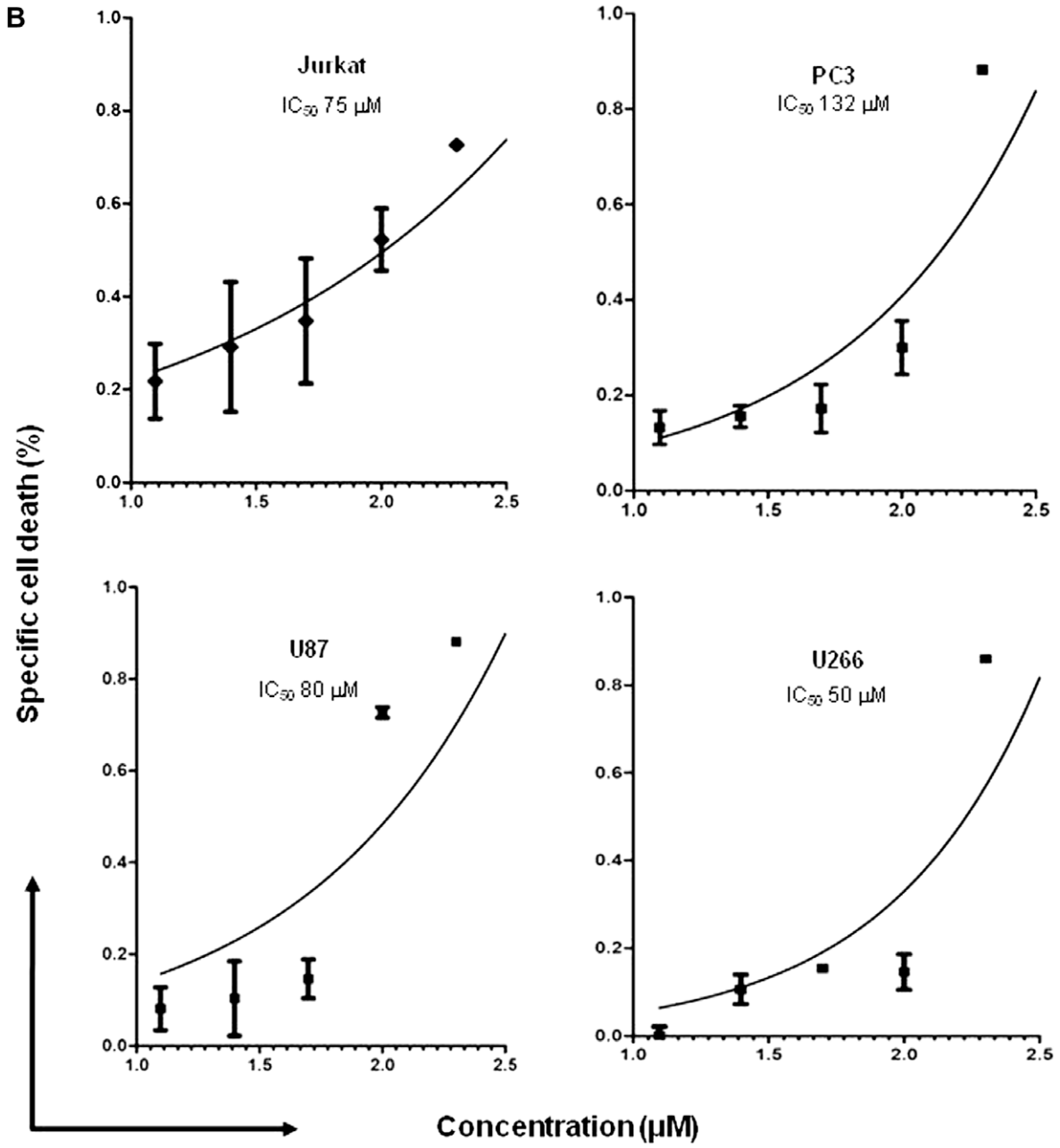


Fig. 1 (*continued*)

CDC25, ID1, cyclin E1, and hTERT in SKBR3 as well as in MCF7 cells. Furthermore, it induced upregulation of CDKN1C and CDKN1A, which inhibit several cyclin/Cdk complexes and act as negative regulators of cell proliferation. Thus, although restricted to a limited set of genes, the analysis performed by TLDA detected molecular sequelae of **30** that could possibly underlie the effects of this compound on cell cycle and cell viability.

B-cell leukemia/lymphoma-2 (Bcl2) is an antiapoptotic protein frequently upregulated in hematological and solid malignancies, where it confers resistance to therapy and a poor prognosis.[44–46] Therefore, to investigate the effect of Bcl-2 overexpression on the cytotoxic activity of the dihydroxypyrrolidine drugs, we used Jurkat T cells engineered to overexpress this protein and the respective control cells.[45,47–50] As shown in Figure 5 (A–C), while almost completely preventing cell death in response to doxorubicin and etoposide, Bcl2 failed to affect the cytotoxic effect of **30**. These data suggest that this compound is unlikely to act via the mitochondrial apoptotic pathway and that it could therefore possibly be of use in Bcl2-expressing cancers. Finally, the in vivo cytotoxic activity was evaluated on primary tumors cells, collected by patients with B-cell chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM), with increasing concentrations ranging from 50 to 200 μM for 24 h and subsequently quantifying apoptotic cells by staining with annexin V (AV) and propidium iodide (PI) and flow cytometric analysis. As expected, the percentage of annexin V-positive cells increased in a time- and dose-dependent manner (Fig. 6).

3. Conclusion

Despite the existence of numerous therapeutic options for the treatment of human cancers the results are still disappointing

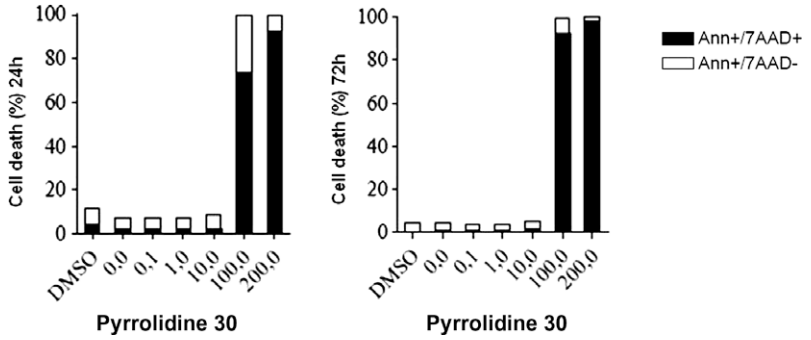


Figure 2. Pyrrolidine **30** induces dose-dependent cell death in Jurkat cells. 5×10^3 Jurkat cells/well were incubated for 24 h and 72 h in the presence of the indicated concentrations of pyrrolidine **30**. Thereafter, cell viability was assessed by flow cytometry using annexin V and 7AAD double staining. The percentage of early apoptotic cells (annexin V+7AAD−) are shown as white columns and that of late apoptotic cells (annexin V+7AAD+) are shown as solid black columns. Data are derived from at least three independent experiments.

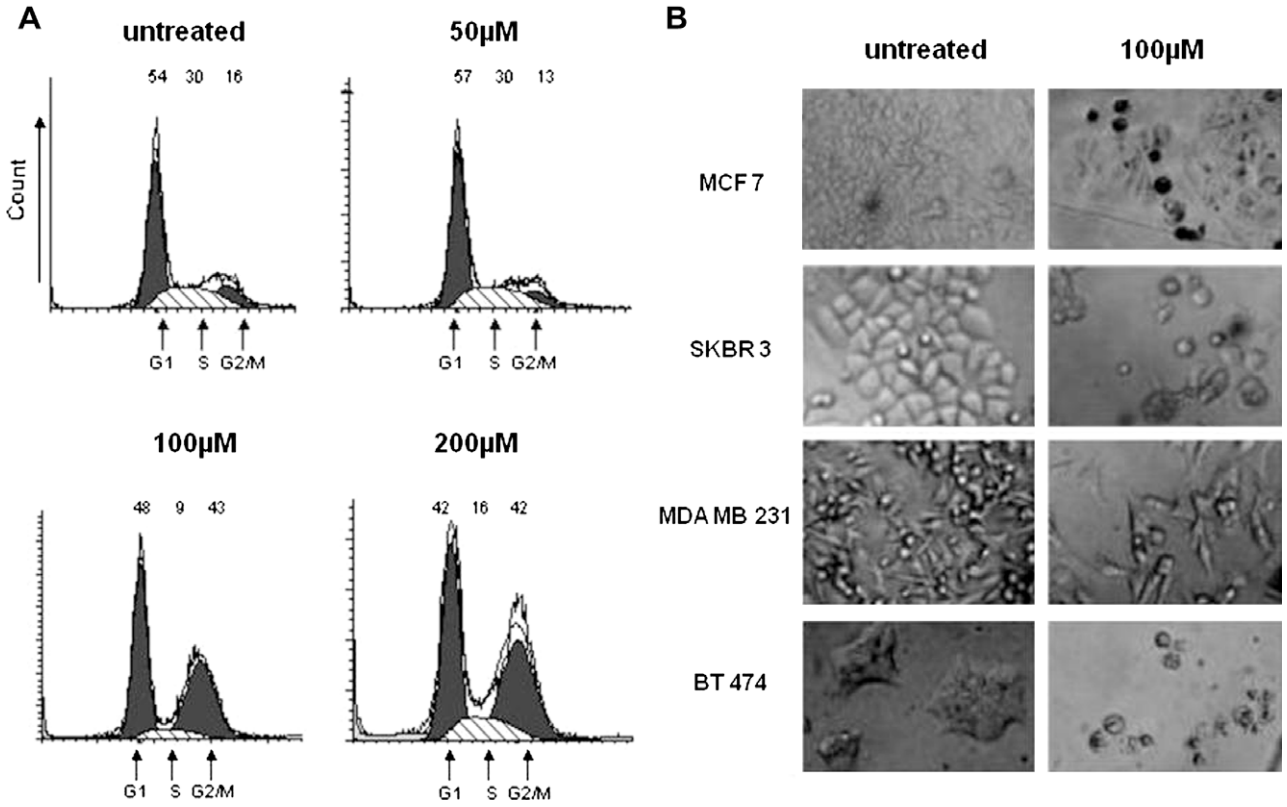


Figure 3. Effects of pyrrolidine **30** on cell cycle progression. A: 10^5 SKBR3 cells/well were seeded in 24-well plates. 24 h later cells were treated with increasing concentration of pyrrolidine **30** from 50 to 200 μM. Cell cycle was analyzed after 48 h by propidium iodide staining of isolated cell nuclei and flow cytometry. Representative graphs show the percentage of cells in G1, S and G2/M cell cycle phases. B: MCF7, SKBR3, MDA-MB-231 and BT474 cells exposed to novel drug for 72 h were imaged using a 40X magnification.

and thus mandate the search for new compounds to improve the clinical outcome. Interfering with the glycosylation pathway has recently emerged as a promising therapeutic strategy to produce antitumour effects in numerous types of malignancies.[13]

In a preliminary report, we found that pyrrolidine compounds with α-mannosidase inhibitory capacity had cytostatic activity in melanoma and glioblastoma cells. Here, we extend these observations using more potent analogues that effectively prevent growth in cancer cell lines of different histology, including primary leukemic cells. Probably, due to the pharmacophoric properties and the lipophilic character of the biphenyl moiety compound **30** proved to be more active toward the cancer cells tested than the simpler analogues **29**, **31**, **32**–**34** and those described earlier.[28–30]

Our data show that the new functionalized pyrrolidines affect the expression of genes involved in cell cycle progression thereby arresting cells in the G2/M phase of cell cycle and causing the upregulation of cell cycle inhibitors CDKN1A and CDKN1C, as well as the downregulation of CDC25A cyclin E1, and of MYBL2 which, conversely, promote cells cycle progression. These experiments establish the new α-mannosidase inhibitors as effective cell cycle modulators. Additionally the cell death via dihydroxypyrrolidine **30** happens irrespective of Bcl-2 overexpression, a condition that normally inhibits apoptosis by preventing mitochondrial pore formation and cytochrome c release into the cytosol.[45] The latter property of this novel compound appears of special importance as Bcl2 is frequently overexpressed in cancer, mainly in B-CLL, con-

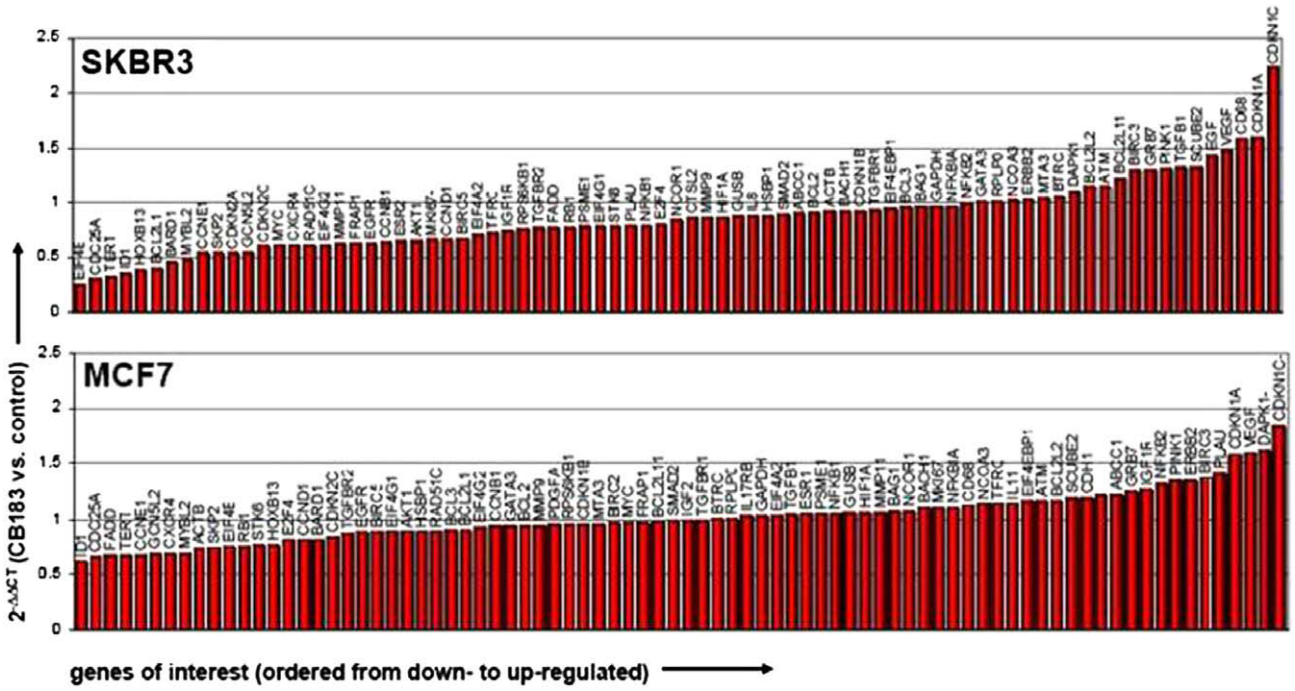


Figure 4. Effects of pyrrolidine **30** on gene expression profile in Human breast cancer cell lines (SKBR3 and MCF7). 2×10^5 SKBR3 and MCF7 cells/well were seeded in 6-well plates and treated with 50 μM for 12 h. Thereafter, RNA was extracted and used for cDNA synthesis and TLDA. Results were calculated using RPLP0 as an housekeeping gene and changes in gene expression were determined using cells treated with vehicle DMSO alone as a control.

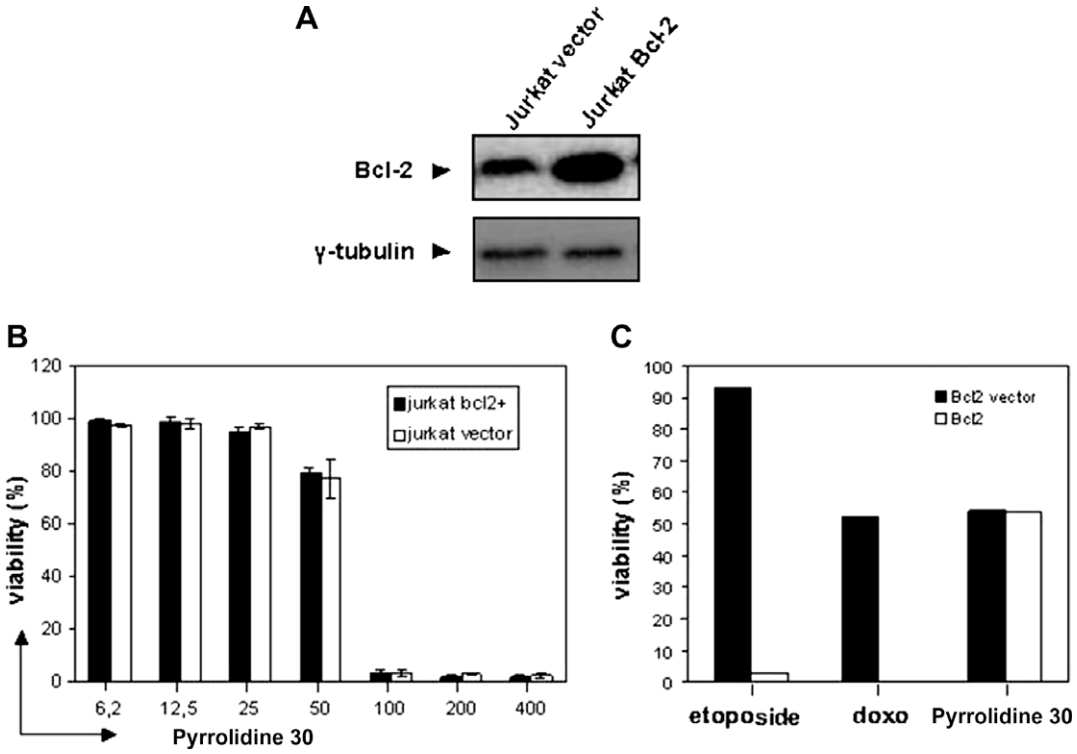


Figure 5. Effect of Bcl-2 overexpression on cell death in response to etoposide, doxorubicin and pyrrolidine **30**. A, B, Bcl-2-overexpressing and vector control Jurkat cells were treated for 72 h with increasing concentrations of pyrrolidine **30**. Dead cells were enumerated by flow cytometric analysis of propidium iodide-stained cells. Viability was calculated using the following formula: 100 − [(drug − induced death − spontaneous death)/(100 − spontaneous death) × 100]. Means of triplicate wells with SD are shown. C, 4×10^4 Jurkat cells engineered to overexpress Bcl-2 and the respective vector control cells were seeded in 96-well plates and treated for 72 h with 40 μM etoposide, 2 μM doxorubicin or 50 μM pyrrolidine **30**. Thereafter, cells were harvested and dead cells were quantified by flow cytometry after staining with PI. Results are presented as means of triplicates with SD.

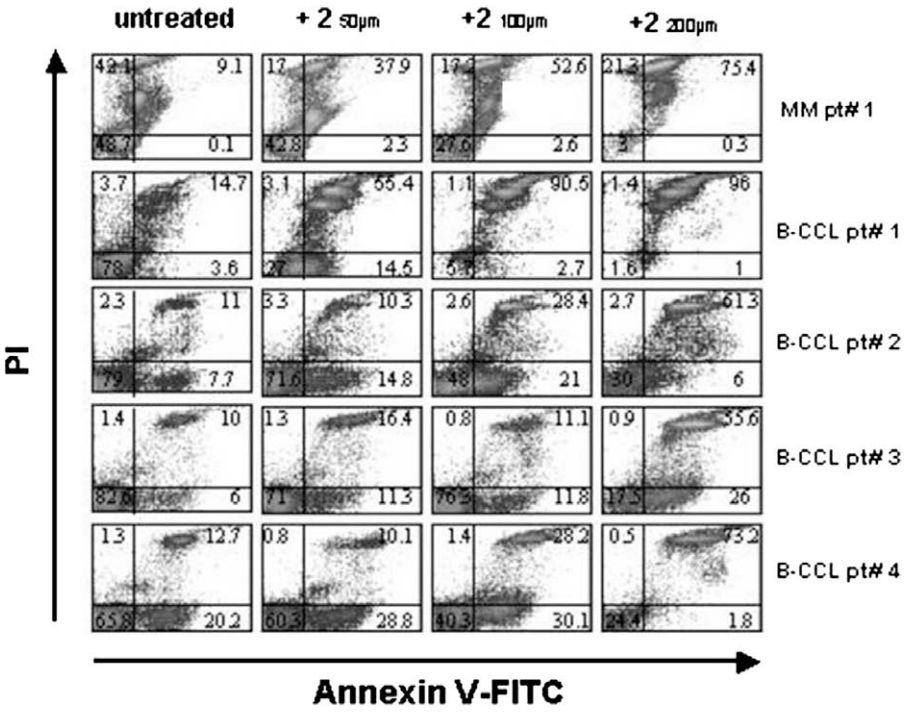


Figure 6. Pyrrolidine **30** efficiently kills primary B-CLL and multiple myeloma cells. A, primary B-CLL cells (>60% CD19+ cells) were isolated from PB samples, while primary multiple myeloma (MM) cells were obtained from a bone marrow aspirate in a patient at disease diagnosis. 10^6 cells/well were seeded in 24-well plates and cultured with or without pyrrolidine **30** at the indicated concentrations. 24 h later cells were harvested, washed, stained with FITC-conjugated Annexin-V and PI, and analyzed by flow-cytometry.

ferring unfavorable growth conditions and to cancer therapeutics.[48] In summary, the novel α-mannosidase inhibitors reported display promising anticancer activity through cell cycle modulation. Additionally and significantly, their cytotoxic potential is not affected by obstruction of the intrinsic mitochondrial pathway. More studies are required to define their effectiveness and safety in in vivo tumor models and to elucidate their own mechanism of action.[51]

4. Experimental data

4.1. Synthesis of 3,4-dihydroxypyrrolidine derivatives

All commercially available reagents (Fluka, Aldrich, Acros) were used without further purification. Technical solvents were used for extraction without any purification. For reactions requiring anhyd conditions, dry solvents were bought (Fluka) or filtered prior to use (Innovative Technology). In absence of any particular notification, experiments were carried out under argon atmosphere. Reactions were monitored by thin layer chromatography (Merck silica gel $60F_{254}$ plates). Revelation was carried out by UV light (254 nm) and $KMnO_4$ [MnO_4 (3 g), K_2CO_3 (20 g, AcOH (0.25 ml) and WATER (300 ml)] or Pancaldi [$(NH_4)_6Mo_4$ (21 g), $Ce(SO_4)_2$ (1 g), H_2SO_4 (31 ml) and WATER (470 ml)] reagents. Purifications were performed by flash chromatography (Fluka silica gel 60, 230–400 mesh, 0.04–0.063 mm). ^1H NMR spectra were recorded on Bruker ARX-400 and DPX-400 spectrometers at 400 MHz. The signals of residual solvents were used as reference (MeO-d_4: 3.32 ppm, $CDCl_3$: 7.27 ppm). Coupling constants are given in Hz. When necessary, the ^1H-signal assignments were confirmed by COSY-45 or by analogy with spectra of similar compounds. ^{13}C NMR spectra were recorded on Bruker ARX-400 and DPX-400 spectrometers at 101 MHz. The signals of residual solvents were used as reference (MeO-d_4: 49 ppm, $CDCl_3$: 77 ppm). When necessary, the ^{13}C-signals assignments were confirmed by HSQC spectra. IR spectra were recorded on Perkin Elmer Paragon 1000 FT-IR spectrometer or on Perkin Elmer Spectrum One FT-IR spectrometer. UV spectra were recorded on a Perkin Elmer Lambda 10 UV/vis spectrometer. Optical rotations were recorded at 25 °C on a Jasco P-1020 polarimeter. *Mass spectra*: GC-IE/CI/MS experiments were preformed with a 1200 L instrument (Varian) coupled to a GC CP-3800 (Varian). ESI-TOF-MS experiments were performed on a Q-Tof Ultima mass spectrometer (Waters) fitted with a standard Z-spray ion source and operated in the positive ionization mode. MALDI-TOF-MS experiments were performed with an AXIMA CFR-Plus instrument (Shimadzu) operated in the positive reflectron ionization mode.

(3a*R*,4*R*,6a*S*)-5-Benzyl-4-[(7*S*)-10,1-dimethyl-9,11-dioxolan-7-yl]-2,2-dimethyltetrahydro-3a*H*-[1,3]dioxolo[4,5-*c*]pyrrole (**17**). D-Gulonolactone (**14**) (5 g, 28.07 mmol) was dissolved in acetone/DMP (5/1 vol: vol, 150 ml). *p*-Toluenesulfonic acid was added until pH 3 and the solution was then stirred at 25 °C until the starting material was consumed (control by TLC, AcOEt/petrol ether 4:1). The solution was neutralized with solid Na_2CO_3 and, after solvent evaporation in vacuo, the residue was poured into water and extracted with EtOAc (3 × 50 ml). The combined organic extracts were washed with brine (50 ml) and dried with $MgSO_4$. After solvent evaporation in vacuo, pure 2,3:5,6-di-*O*-isopropylidene-D-gulono-1,4-lactone (**15**) was obtained (7.210 g, 27.91 mmol, 99% yield) as a light yellow solid. This product (7.21 g, 27.9 mmol) was added portionwise to a Red-Al solution in toluene/THF (16 ml of a 3.5 M solution in toluene diluted in 29 ml of anhyd THF) at 0 °C. The solution was stirred at 25 °C for 3 h and then methanol was added until the excess of Red-Al was consumed. The solution was poured into a satd aq soln. of sodium potassium tartrate (50 ml) and the mixture was stirred for 2 h at 25 °C. The organic phase was collected and the aqueous phase extracted with EtOAc (3 × 30 ml). The combined organic extracts were washed successively with a satd aq soln of $NaHCO_3$ (30 ml) and brine (30 ml), then dried ($MgSO_4$). After solvent evaporation in vacuo, 5,6-di-*O*-isopropylidene-D-gulitol (**16**) was obtained as a white solid (4.56 g, 17.4 mmol, 62%

yield) and used without any further purification. **16** (4.56 g, 17.4 mmol) was dissolved in dry CH_2Cl_2 (100 ml), Et_3N (9 ml, 6.57 g, 64.9 mmol, 3.7 equiv) was added and the solution was cooled to 0 °C with an ice bath. $MeSO_2Cl$ (5 ml, 7.37 g, 51.0 mmol, 3 equiv) was added dropwise and the mixture was stirred at 0 °C for 2 h (reaction monitored by TLC: EtOAc/petroleum ether 3:1). The solution was washed with cold water (30 ml), then with 1 M aq HCl (50 ml) and finally with brine (30 ml), dried with $MgSO_4$ and the solvent was evaporated in vacuo.

The crude mesylate was dissolved in benzylamine (120 ml) and the solution was stirred at 70 °C for 2 days. CH_3Cl was added and the solution was washed successively with 1 M aq HCl (2 × 70 ml) then with water (50 ml) and brine (50 ml). The solvent was evaporated in vacuo and pure **17** (3.93 g, 13.4 mmol, 77% two steps) was obtained as white solid after flash column chromatography on silica gel (petroleum ether/EtOAc 3:1). ^{1}H NMR (400 MHz, $CDCl_3$, Ph = phenyl): δ 7.36–7.32 (m, 5H Ph) 4.73–4.69 (m, 2H, H-C(6a) and H-C(3a)) 4.18 (m, 1H, *H*HC(8)) 4.08–4.01 (m, 2H, N-*H*HC-Ph and H*H*C(8)) 3.84 (d, ^2J = 13.2, N-H*H*C-Ph) 3.69 (dd, ^3J = 7.9, ^3J = 7.2, H-C(7)) 3.12 (dd, ^2J = 11.6, ^3J = 4.7, 1H, *H*HC(6)) 3.02 (br d, ^3J = 4.0, 1H, H-C(4)) 2.82 (dd, ^2J = 11.6, ^3J = 2.0, 1H, H-C(6)) 1.57 (s, 3H, H_3C(1')) 1.42 (s, 3H, H_3C(1")) 1.34 (s, 6H, H_3C(1"') and $H_3C(1^{IV})$). Analytical and spectroscopic data are in accord with those reported in the literature.[34]

N-Benzyl-1,4-dideoxy-5,6-*O*-isopropylidene-1,4-imino-D-allitol (**18**). *N*-Benzyl-1,4-dideoxy-2,3:5,6-di-*O*-isopropylidene-1,4-imino-D-allitol (**17**) (3.93 g, 13.4 mmol) was dissolved in AcOH (60 ml of 80% vol/vol in water) and the solution was stirred at 60 °C overnight. The reaction was stopped at about 50% of conversion (monitored by TLC: pure EtOAc), in order to avoid the deprotection of the secondary acetate (the starting material was recovered). After evaporation of the solvent in vacuo, the product was purified by flash chromatography using pure EtOAc as eluent yielding 29% of *N*-benzyl-1,4-dideoxy-5,6-*O*-isopropylidene-1,4-imino-D-allitol (**18**: 1.005 g, 3.4 mmol) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$, Ph = phenyl): δ 7.33 (m, 5H, H Ph) 4.76 (dd, ^3J = 6.7, ^3J = 4.0, 1H, H-C(3a)) 4.64 (m, 1H, H-C(7)) 4.15 (m, 1H, *H*HC-Ph + AcOEt) 3.95 (m, 1H, H-C(6a)) 3.81 (dd, ^2J = 11.5, ^2J = 6.3, 1H, *H*HC(8)) 3.70 (dd, ^2J = 11.5, ^3J = 5.3, 1H, H*H*C(8)) 3.62 (d, ^2J = 12.9, 1H, H*H*C-Ph) 3.32 (dd, ^2J = 11.2, ^2J = 5.9, 1H *H*HC(6)) 3.25 (br s, 2H, 2 × H–O) 2.92 (m, 1H, H-C(4)) 2.70 (dd, ^2J = 11.2, ^2J = 3.8, 1H, H*H*C(4)) 1.56 (s, 3H, H_3C(1')) 1.34 (s, 3H, H_3C(1")). Analytical and spectroscopic data are in accord with those reported in the literature.[37]

Methyl 4-phenyl-D-phenylglycinate (**2**). Thionyl chloride (7.5 ml, 12.23 g, 102.8 mmol, 2.8 equiv) was added dropwise to anhyd MeOH (72 ml) at 0 °C. 4-Hydroxy-D-phenylglycine (**20**) (6.054 g, 36.2 mmol) was added slowly and the solution was stirred and heated under reflux until consumption of the starting material (monitored by TLC, CH_3CN/NH_3 5:1). After solvent evaporation in vacuo the crude was dissolved in dioxane/water (1:1, 130 ml), di-*tert*-butyldicarbonate (7.81 g, 41.9 mmol, 1.1 equiv) and then, slowly, Et_3N (until pH 8.5) were added and the solution was stirred at 20 °C overnight. EtOAc (50 ml) was added and the two phases were separated. The organic layer was washed with satd aq soln of NH_4Cl (30 ml), then with satd aq soln of $NaHCO_3$ (30 ml) and finally with brine, dried over $MgSO_4$ and the solvent was evaporated in vacuo. Pure methyl *N*-*tert*-buthoxycarbonyl 4-hydroxy-D-phenylglycinate (**21**: 8.815 g, 31.3 mmol, 87% yield) was obtained after flash column chromatography on silica gel (petroleum ether/EtOAc 2:1). **21** (1.01 g, 3.6 mmol) was dissolved in CH_2Cl_2 (5 ml), pyridine (1.35 ml, 1.26 g, 4.4 equiv) was added and the solution was cooled to 0 °C. $(MeSO_2)_2O$ (0.7 ml, 1.179 g, 4.2 mmol, 1.2 equiv) was added dropwise (the temperature should not be higher than 5 °C) and the solution was stirred at 0 °C for 1 h (reaction monitored by TLC, petroleum ether/EtOAc 3:1). The solution was diluted with CH_2Cl_2 (20 ml), water (20 ml) was added and the two layers were separated. The organic phase was washed with aqueous NaOH (0.5 N, 2 × 20 ml), with 10% aq soln of citric acid (2 × 20 ml) and finally with water (20 ml), dried over $MgSO_4$ and the solvent was evaporated in vacuo. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 3:1) led to pure methyl *N*-*tert*-butoxycarbonyl-4-[[(trifluoromethyl)sulfonyl]oxy]-D-phenylglycinate (**22**: 1.301 g, 3.1 mmol, 86% yield) as pale yellow solid. **22** (0.155 g, 0.4 mmol) was dissolved in anhyd toluene (3.0 ml), phenylboronic acid (0.089 g, 0.7 mmol, 1.9 equiv) and K_2CO_3 (0.076 g, 0.6 mmol, 1.5 equiv) were added and the mixture was degassed under Ar. $(Ph_3P)_4Pd$ (0.014 g, 3% mol) was dissolved in degassed toluene (0.5 ml) under nitrogen (glove box) and the solution was added to the reaction mixture. The suspension was stirred at 90 °C overnight. EtOAc (4 ml) was added and the mixture was washed with satd aq soln of $NaHCO_3$ (3 ml), then with water (3 ml), with 10% aq soln of citric acid (3 ml), again with water (3 ml) and finally with brine (3 ml). The solution was dried with $MgSO_4$ and the solvent was evaporated in vacuo. After flash chromatography on silica gel (petroleum ether/EtOAc 6:1), pure methyl *N*-*tert*-butoxycarbonyl-4-phenyl-D-phenylglycinate (**23**: 0.107 g, 0.3 mmol, 84% yield) was recovered as white foam. Spectral data were the same as those reported for this compound.[52] ^{1}HNMR (400 MHz, $CDCl_3$): δ 7.60–7.57 (m, 4H, H (bph)) 7.46–7.43 (m, 4H, H (bph)) 7.38–7.34 (m, 1H, H-C(4') (bph)) 5.60 (bd, ^3J = 7.0, 1H, H–N–Boc) 5.38 (d, ^3J = 7.0, 1 H, H-C(2)) 3.76 (s, 3H, OCH_3) 1.45 (s, 9H, $(CH_3)_3$ *t*-butoxy). CI-MS: 182, 225, 242, 286, 303, 342. **23** (0.107 g, 0.30 mmol) was dissolved in trifluoroacetic acid (80% aq, 4 ml) at 0 °C. The solution was stirred at 20 °C for 3 h. The solvent was evaporated in vacuo and pure **2** was obtained quantitatively after flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5). Spectral data were the same as those reported for this compound.[53] ^{1}HNMR (400 MHz, $CDCl_3$, bhp = biphenyl): δ 7.59–7.57 (m, 4H, H (bhp)) 7.49–7.44 (m, 4H, H (bhp)) 7.40–7.36 (m, 1H, H-C(4') (bhp)) 4.86 (br s, 1H, H-C(2)) 3.76 (s, 3H, OCH_3).

Methyl 4-(4-trifluorophenyl)-D-phenylglycinate (**3**). Methyl *N*-*tert*-butoxycarbonyl-4-[[(trifluoromethyl)sulfonyl]oxy]-D-phenylglycinate (**22**: 0.205 g, 0.5 mmol) was dissolved in anh. toluene (4.0 ml), 4-(trifluoromethyl)phenylboronic acid (0.194 g, 1.0 mmol, 2.0 equiv) and K_2CO_3 (0.111 g, 0.8 mmol, 1.6 equiv) were added and the mixture was degassed under Ar. $(Ph_3P)_4Pd$ (0.060 g, 10% mol) was dissolved in degassed toluene (1.0 ml) under nitrogen (gloves box) and the solution was added to the reaction mixture. The suspension was stirred at 90 °C overnight. EtOAc (4 ml) was added and the mixture was washed successively with satd aq soln of $NaHCO_3$ (3 ml), water (3 ml), 10% aq soln of citric acid (3 ml), water (3 ml) and finally with brine (3 ml). The solution was dried ($MgSO_4$) and the solvent was evaporated in vacuo. After flash chromatography on silica gel (petroleum ether/EtOAc 7:1), pure methyl *N*-*tert*-butoxycarbonyl-4-(4-trifluorophenyl)-D-phenylglycinate (**24**: 0.143 g, 0.4 mmol, 70% yield) was recovered as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (m, 4H, arom. H) 7.59 (d, ^3J = 8.2, 2H, arom. H) 7.48 (d, ^3J = 8.2, 2H, arom. H) 5.66 (bd, ^3J = 7.0, 1H, H–N–Boc) 5.39 (d, ^3J = 7.0, 1H, H-C(2)) 3.76 (s, 3H, OCH_3) 1.45 (s, 9H, $(CH_3)_3$ *t*-butoxy). *N*-*tert*-Butoxycarbonyl-4-(4-trifluorophenyl)-D-phenylglycinate (0.1431 g, 0.35 mmol) was dissolved in trifluoroacetic acid (80% aq, 3.5 ml) at 0 °C. The solution was stirred at 0 °C for 1 h and then at 20 °C for 3 h (reaction monitored by TLC, petroleum ether/EtOAc 5:1). The solvent was evaporated in vacuo and pure **3** was obtained quantitatively after flash chromatography on silica gel (CH_2Cl_2/MeOH from 95:5 to 90:10). $[\alpha]^{25}_{589} = -47$; $[\alpha]^{25}_{577} = -19$; $[\alpha]^{25}_{435} = -18$; $[\alpha]^{25}_{405} = -26$ (*c* 0.175, MeOH).

IR(pure): 3377, 3040, 2959, 2919, 2850, 1734 (C=O), 1614, 1324, 1115, 821. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.69 (m, 4H, arom. H) 7.60 (d, ^3J = 8.1, 2H, arom. H) 7.49 (d, ^3J = 8.2, 2H, arom. H) 4.71 (br s, 1H, H-C(2)) 3.75 (s, 3H, OCH_3). ^{1}H NMR (400 MHz,

MeOH-d_4): δ 7.80 (d, ^3J = 7.7, 2H, arom. H) 7.73 (m, 4H arom. H) 7.56 (d, ^3J = 7.6, 2H, arom. H) 3.75 (s, 3H, OCH_3). ^{13}C NMR (101 MHz, MeOH-d_4): δ 173.05 (s, C(1) (C(O)OCH_3)) 145.25 (s, C(1)arom) 141.43 (s, C(4)) 137.77 (s, C(1')) 130.64 (q, $^2J_{CF}$ = 32.3, C(4')) 129.31 (dd, ^1J = 159.9, ^2J = 6.0, 2C, arom. C) 128.89 (dd, ^1J = 159.9, ^2J = 6.6, 2C, arom. C) 128.59 (dd, ^1J = 161.2, ^2J = 6.3, 2C, arom. C) 126.82 (dq, $^1J_{CH}$ = 109.1, $^3J_{CF}$ = 3.8, C(3') and C(5')) 125.74 (q, $^1J_{CF}$ = 269.5, CF_3) 58.30 (d, ^1J = 142.3, C(2)) 53.36 (q, ^1J = 147.1, OCH_3). HR-ESI-TOF-MS: calcd for $C_{16}H_{14}F_3NO_2$: 310.1049, found 310.1039 ([M+H]$^+$).

Methyl 4-(3-thienyl)-D-phenylglycinate (**4**). Methyl *N-tert*-butoxycarbonyl-4-[[(trifluoromethyl)sulfonyl]oxy]-D-phenylglycinate (**22**: 0.255 g, 0.6 mmol) was dissolved in anhyd toluene (6.0 ml), 3-thiopheneboronic acid (0.153 g, 1.2 mmol, 2.0 equiv) and K_2CO_3 (0.125 g, 0.9 mmol, 1.5 equiv) were added and the mixture was degassed under Ar. $(Ph_3P)_4Pd$ (0.140 g, 20% mol) was dissolved in degassed toluene (1.0 ml) under nitrogen (gloves box) and the solution was added to the reaction mixture. The suspension was stirred at 90 °C for 6 h (reaction monitored by TLC: petroleum ether/EtOAc 7:1). EtOAc (4 ml) was added and the mixture was washed successively with satd aq soln of $NaHCO_3$ (3 ml), water (3 ml), 10% aq soln of citric acid (3 ml), water (3 ml) and finally with brine (3 ml). The solution was dried ($MgSO_4$) and the solvent was evaporated in vacuo. After flash chromatography on silica gel (petroleum ether/EtOAc 7:1), pure methyl *N-tert*-butoxycarbonyl-4-(3-thienyl)-D-phenylglycinate (**25**) was obtained quantitatively, white foam. ^{1}H NMR (400 MHz, $CDCl_3$, thi = thienyl): δ 7.60 (d, ^3J = 8.2, 2H, arom. H) 7.54 (m, 1H thi) 7.41 (m, 4H, arom. H) 5.57 (br s, 1H, H–N) 5.36 (d, ^3J = 7.1, 1H, H-C(2)) 3.76 (s, 3H, CH_3) 1.46 (s, 9H, $(CH_3)_3$ *t*-butyl). **25** (0.20 g, 0.6 mmol) was dissolved in trifluoroacetic acid (80% aq, 4.3 ml) at 0 °C. The solution was stirred at 0 °C for 1 h and then at 20 °C for 3 h (reaction monitored by TLC, petroleum ether/ EtOAc 5:1). The solvent was evaporated in vacuo and the pure **4** (0.111 g, 0.5 mmol, 75% yield) was obtained after flash chromatography on silica gel (CH_2Cl_2/MeOH 95:5). $[\alpha]^{25}_{589} = -51$; $[\alpha]^{25}_{577} = -23$; $[\alpha]^{25}_{435} = -25$; $[\alpha]^{25}_{405} = -36$ (*c* 0.167, MeOH).

IR(pure): 3378, 3039, 2959, 2919, 2853, 1735 (C=O), 1610, 1324, 1111, 821. ^{1}H NMR (400 MHz, MeOH-d_4, Ph = phenyl, thi = thienyl): δ 7.62 (d, ^3J = 7.8, 2H Ph) 7.46–7.38 (m, 5H, 2H Ph and 3H thi) 5.31 (s, 1H, H-C(2)) 3.75 (s, 3H, CH_3). ^{13}C NMR (101 MHz, MeOH-d_4, Ph = phenyl, thi = thienyl): δ 174.15 (s, C=O) 142.68 (s, arom. C) 137.62 (s, arom. C) 137.54 (s, arom. C) 128.89 (d, ^1J = 185.6, C thi) 127.77 (d, ^1J = 168.1, C Ph) 127.51 (d, ^1J = 181.7, C thi) 127.03 (d, ^1J = 111.3, C Ph) 121.82 (d, ^1J = 187.4, C thi) 58.70 (d, ^1J = 142.4, C(2)) 53.10 (q, ^1J = 147.8, CH_3). HR-ESI-TOF-MS: calcd for $C_{13}H_{13}NO_2S$: 248.0745, found 248.0746 ([M+H]$^+$).

(2*R*,3*R*,4*S*)-2{{[(1*R*)-1-[1,1'-Biphenyl]-4-yl-2-hydroxyethyl]amino}-methyl}pyrrolidine-3,4-diol (**29**). *N*-Benzyl-1,4-dideoxy-5,6-*O*-isopropylidene-1,4-imino-D-allitol (**18**: 1.006 g, 3.4 mmol) was dissolved in dry methanol (35 ml), Boc_2O (2.378 g, 12.8 mmol, 2 equiv) was added and then $Pd(OH)_2$–C as catalyst under Ar. The mixture was stirred under H_2 atmosphere for 3 h. The catalyst was filtered off on a Celite pad and the product was purified by flash chromatography (diethyl ether/petroleum ether 4:1 to diethyl ether 100%) giving *N-tert*-butyloxycarbonyl-1,4-dideoxy-5,6-*O*-isopropylidene-1,4-imino-D-allitol (**19**: 0.760 g, 2.5 mmol, 73% yield) as white sol $NaIO_4$ (0.5289 g, 2.5 mmol, 2.8 equiv) was added to a solution of **19** (0.261 g, 0.9 mmol) in methanol/water (6.7 ml, 4:1) at 0 °C and the solution was stirred at 0 °C for 1 h. The solution was poured into water and EtOAc was added. The two phases were separated and the aqueous phase was extracted twice with EtOAc. The combined organic phases were washed with brine, dried with $MgSO_4$ and the solvent was evaporated in vacuo giving pure *tert*-butyl(3a*R*,4*R*,6a*S*)-4-formyl-2,2-dimethyltetrahydro-5*H*-[1,3]dioxolo-[4,5-*c*]pyr role-5-carboxylate (**1**: 0.209 g, 0.8 mmol, 90% yield) that was used without any further purification. $NaBH(OAc)_3$ (0.134 g, 0.6 mmol) was added portionwise to a stirred solution of aldehyde (**1**: 0.118 g, 0.4 mmol) and amine **2** (0.102 g, 0.4 mmol) in 1,2-dichloroethane (4.5 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was poured into a satd aq soln of $NaHCO_3$ (5 ml per mmol). The organic phase was collected and the aqueous phase extracted with EtOAc (10 ml per mmol, three times). The combined organic extracts were washed with brine (10 ml per mmol, once) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1) gave pure **8** as light yellow oil (0.094 g, 0.2 mmol, 49% yield). Ester **8** was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.017 g, 0.4 mmol) in anhyd THF (5 ml). The solution was stirred at room temperature for 3 h, then WATER was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, the obtained amino alcohol **26** (0.077 g, 0.2 mmol, 85% yield) was deprotected without any further purification. Compound **26** was dissolved in trifluoroacetic acid (80% aq, 1.7 ml) at 0 °C. The solution was stirred at 0 °C for 1 h and then at 20 °C for 3 h (reaction monitored by TLC, petroleum ether/EtOAc 5:1). The solvent was evaporated in vacuo and pure **29** (0.027 g, 0.1 mmol, 47% yield) was collected as a white solid after flash chromatography on silica gel (CH_3CN/NH_4OH 8:1). Melting point: 151–156 °C (dec).

$[\alpha]^{25}_{589} = -12$; $[\alpha]^{25}_{577} = -14$; $[\alpha]^{25}_{435} = -28$; $[\alpha]^{25}_{405} = -360$ (*c* 0.271, MeOH). UV(CH_3CN): 257 (22605) 208 (32249) IR (pure): 3294, 2873, 2445, 1487, 1407, 1109, 1029, 835, 761, 687. ^{1}H NMR (400 MHz, MeOH-d_4, bph = biphenyl): δ 7.58 (m, 4H, H (bph)) 7.43 (m, 4H, H (bph)) 7.32 (m, 1H, H-C(4') (bph)) 4.05 (m, 1H, H-C(4)) 3.82 (dd, ^3J = 8.3, ^3J = 4.4, 1H, H-C(3)) 3.69 (m, 2H, CHCH_2OH) 3.61 (dd, ^3J = 10.7, ^3J = 8.7, 1H C*H*CH_2OH) 3.18 (dd, ^2J = 12.2, ^3J = 5.1, 1H, *H*H-C(5)) 3.12 (m, 1H, H-C(2)) 2.88 (dd, ^2J = 12.2, ^3J = 3.2, 1H, H*H*-C(5)) 2.73 (dd, ^2J = 12.1, ^3J = 5.0, 1H, H*H*-C(6)) 2.61 (dd, ^2J = 12.1, ^3J = 7.4, 1H, *H*H-C(6)). ^{13}C NMR (101 MHz, MeOH-d_4, bph = biphenyl): δ 142.19 (s, C (bph)) 141.88 (s, C (bph)) 141.12 (s, C (bph)) 129.89 (m, 2*C*H (bph)) 129.29 (m, 2*C*H (bph)) 128.35 (br s, C(4') (bph)) 128.20 (d, ^1J = 166.0, 2*C*H (bph)) 127.92 (d, ^1J = 160.0, 2*C*H (bph)) 76.59 (d, ^1J = 141.1, C(3)) 72.40 (d, ^1J = 150.1, C(4)) 67.77 (t, ^1J = 140.7, CH*C*H_2OH) 66.49 (d, ^1J = 134.3, *C*HCH$_2$OH) 62.91 (d, ^1J = 140.7, C(2)) 52.07 (t, ^1J = 140.7, C(5)) 50.45 (t, ^1J = 130.9, C(6)).HR-MALDI-TOF-MS: calcd for $C_{19}H_{24}N_2O_3$ 329.1865, found 329.1868 ([M+H]$^+$).

(2*R*,3*R*,4*S*)-2-{{{(1*R*)-2-Hydroxy-1-[4'-(trifluoromethyl)-[1,1'-biphenyl]-4-l]ethyl}amino} methyl}pyrrolidine-3,4-diol (**30**). $NaBH(OAc)_3$ (0.112 g, 0.5 mmol) was added portionwise to a stirred solution of aldehyde **1** (0.124 g, 0.45 mmol) and amine **3** (0.159 g, 0.4 mmol) in 1,2-dichloroethane (4 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was poured into a satd aq soln of $NaHCO_3$ (5 ml per mmol). The organic phase was collected and the aqueous phase extracted with EtOAc (10 ml per mmol, three times). The combined organic extracts were washed with brine (10 ml per mmol, once) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1 to 2:1) gave pure **9** as light yellow oil (0.094 g, 0.2 mmol, 49% yield). Ester **9** was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.017 g, 0.4 mmol) in anhyd THF (4 ml). The solution was stirred at room temperature for 3 h, then water was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, amino alcohol **27** was obtained (0.077 g, 0.1 mmol, 82% yield). It was deprotected without any further purification. **27** was dissolved in trifluoroacetic acid (80% aq, 1.5 ml) at 0 °C. The solution was stirred at 0 °C for 1 h and then at 20 °C for 3 h (reaction monitored by TLC, petroleum ether/EtOAc 5:1). The solvent was evaporated in vacuo and pure **30**

(0.0241 g, 0.06 mmol, 43% yield) was collected as a white foam after flash chromatography on silica gel (CH_3CN/NH_4OH 8:1). $[\alpha]_{589}^{25} = -4$; $[\alpha]_{577}^{25} = -3$; $[\alpha]_{435}^{25} = -8$; $[\alpha]_{405}^{25} = -11$ (*c* 0.241, MeOH). UV (CH_3CN): 260 (15449); 202 (29536). IR (solid): 3043, 1670, 1327, 1120, 824, 720. ^{1}H NMR (400 MHz, MeOH-d_4, bph = biphenyl): δ 7.77 (m, 2H bph) 7.69 (m, 4H bph) 7.49 (m, 2H bph) 4.20 (m, 1H, H-C(4)) 3.93 (dd, ^3J = 8.0, ^3J = 4.0, 1H, H-C(3)) 3.88 (dd, ^2J = 7.6, ^3J = 4.8, 1H C*H*CH_2OH) 3.67 (m, 2H, C*H*CH_2OH and 1H CHCH_2OH) 3.43 (m, 2H, H-C(2) and H*H*-C(5)) 3.27 (m, 1H, *H*H-C(5) + MeOH) 2.94 (bd, ^2J = 11.7, 1H, H*H*-C(6)) 2.81 (dd, ^2J = 11.7, ^3J = 8.8, 1H, *H*H-C(6)). ^{13}C NMR (101 MHz, MeOH-d_4, bph = biphenyl): δ 145.73 (s, C bph) 141.29 (s, C bph) 140.43 (s, C bph) 130.52 (q, ^2J = 32, C(4') bph) 129.44 (d, ^1J = 158.9, 2*C*H bph) 128.63 (dd, ^1J = 159, ^3J = 64, 2*C*H bph) 128.50 (dd, ^1J = 161, ^3J = 63, 2 *C*H bph) 126.80 (d, ^1J = 166.3, 2*C*H bph) 125.81 (q, ^1J = 270, *C*F_3) 74.75 (d, ^1J = 143.7, C(3)) 70.92 (d, ^1J = 152.4, C(4)) 68.39 (t, ^1J = 109, *C*HCH_2OH) 66.22 (d, ^1J = 136.3, *C*HCH_2OH) 63.33 (d, ^1J = 145.6, C(2)) 50.71 (t, ^1J = 148.9, C(5)) 47.29 (t, ^1J = 124.0, C(6)). HR-MALDI-TOF-MS: calcd for $C_{20}H_{23}F_3N_2O_3$: 397.1739, found 397.1744 ([M+H]$^+$).

(2*R*,3*R*,4*S*)-2{{{(1R)-2-Hydroxy-1-[4-(3-thienyl)phenyl]ethyl}-amino}methyl}pyrrolidine-3,4-diol (**31**). $NaBH(OAc)_3$ (0.138 g, 0.7 mmol, 1.7 equiv) was added portionwise to a stirred solution of aldehyde **1** (0.119 g, 0.4 mmol) and amine **3** (0.110 g, 0.4 mmol) in 1,2-dichloroethane (4.5 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was poured into a satd aq soln of $NaHCO_3$ (5 ml). The organic phase was collected and the aqueous phase extracted with EtOAc (3 × 5 ml). The combined organic extracts were washed with brine (3 ml) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1 to 2:1) gave pure **10** as light yellow oil (0.089 g, 0.2 mmol, 46% yield). Ester **10** was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.015 g, 0.4 mmol) in anhyd THF (4.5 ml). The solution was stirred at room temperature for 3 h, then water was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, the obtained amino alcohol **28** (0.071 g, 0.2 mmol, 83% yield) was deprotected without any further purification. Compound **28** was dissolved in trifluoroacetic acid (80% aq, 1.5 ml) at 0 °C. The solution was stirred at 0 °C for 1 h and then at 20 °C for 3 h (reaction monitored by TLC, petroleum ether/EtOAc 5:1). The solvent was evaporated in vacuo and pure **31** (0.038 g, 0.1 mmol, 73% yield) was collected as a white foam after flash chromatography on silica gel (CH_3CN/NH_4OH 8:1). $[\alpha]_{589}^{25} = +15$; $[\alpha]_{577}^{25} = +15$; $[\alpha]_{435}^{25} = +18$; $[\alpha]_{405}^{25} = +36$ (*c* 0.062, MeOH). UV (CH_3CN): 265 (17169) 228 (17173). IR (pure): 3242, 2916, 2849, 1467, 1237, 1029, 779, 719. ^{1}H NMR (400 MHz, MeOH-*d*, Ph = phenyl and thi = thienyl): δ 7.57 (d, ^3J = 8.1, 2H Ph) 7.54 (m, 1H thi) 7.38 (m, 2H thi) 7.27 (d, ^3J = 8.0, 2H Ph) 4.24 (m, 1H, H-C(4)) 3.94 (dd, ^3J = 7.7, ^3J = 4.1, 1H H-C(3)) 3.81 (dd, ^3J = 8.1, ^3J = 4.3, 1H, C*H*CH_2OH) 3.71 (dd, ^2J = 10.8, ^3J = 4.3 1H, CHC*H*OH) 3.64 (dd, ^2J = 10.8, ^3J = 8.4, 1H, CHC*H*HOH) 3.47–3.49 (m, 2H, H-C(2) and H*H*-C(5)) 3.23 (dd, ^2J = 12.5, ^3J = 2.0, 1H, *H*H-C(5)) 2.93 (dd, ^2J = 13.3, ^3J = 4.3, 1H, H*H*-C(6)) 2.75 (dd, ^2J = 13.3, ^3J = 8.8, 1H, *H*H-C(6)). ^{13}C NMR (101 MHz, MeOH-*d*, Ph = phenyl and thi = thienyl): δ 141.35 (s, 1 arom. *C*) 136.77 (s, 1 arom. *C*) 133.79 (s, 1 arom. *C*) 130.44 (d, ^1J = 132.6, 2*C*H Ph) 128.36 (d, ^1J = 154.3, *C*H thi) 127.49 (d, ^1J = 170.6, 2*C*H Ph) 126.93 (d, ^1J = 194,9 *C*H thi) 123.06 (d, ^1J = 184.1, *C*H, thi) 74.99 (d, ^1J = 148.1, C(3)) 70.23 (d, ^1J = 151.8, C(4)) 65.19 (d, ^1J = 196.3, *C*HCH_2OH) 64.21 (t, ^1J = 148.1, CH*C*H_2OH) 59.38 (d, ^1J = 137.0, C(2)) 51.36 (t, ^1J = 144.4, C(5)) 46.03 (t, ^1J = 85.2, C(6)). HR-MALDI-TOF-MS: calcd for $C_{17}H_{22}N_2O_3S$: 335.1429, found 335.1434 ([M+H]$^+$).

Methyl 4-(prop-2-enyloxy)-D-phenylglycinate (**6**). Methyl *N*-*tert*-butoxycarbonyl 4-hydroxy-D-phenylglycinate **21** (0.154 g, 0.6 mmol) was dissolved in acetone (5 ml), K_2CO_3 (0.152, 1.1 mmol, 2 equiv) was added followed by allyl bromide (0.092 ml, 0.1286 g, 1.1 mmol, 2 equiv) and the solution was stirred at 25 °C overnight. The solvent was evaporated in vacuo and the crude was dissolved in EtOAc (5 ml). Water (5 ml) was added and the two layers were separated. The aqueous phase was extracted with EtOAc (3 × 3 ml), the collected organic phases were washed with brine (5 ml), dried on $MgSO_4$ and the solvent was evaporated in vacuo, giving methyl *N*-*tert*-butoxycarbonyl-4-(prop-2-enyloxy)-D-phenylglycinate as a colorless oil (0.136 g, 0.4 mmol, 76% yield). $[\alpha]_{589}^{25} = -91$; $[\alpha]_{577}^{25} = -99$; $[\alpha]_{435}^{25} = -212$; $[\alpha]_{405}^{25} = -260$ (*c* 0.1375, MeOH) IR (pure): 3375, 2977, 1745, 1713, 1611, 1586, 1510, 1457, 1437, 1391, 1366, 1342, 1304, 1245, 1166, 1054, 1025, 997, 926, 834, 781, 642, 592, 559, 552, 545, 538. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.28 (d, ^3J = 8.6, 2H Ph) 6.90 (d, ^3J = 8.6, 2H Ph) 6.05 (m, 1H, H-C(2) allyl) 5.51 (bd, ^3J = 6.3, 1H, H–N) 5.41 (dd, 1H, ^2J = 17.2, ^3J = 1.4, *H*H-C(3) allyl) 5.31–5.26 (m, 2H, H*H*-C(3) allyl and H-C(2)) 4.53 (d, ^3J = 5.3, 2H, H_2-C(1) allyl) 3.72 (s, 3H, OCH_3) 1.44 (s, 9H, $(CH_3)_3$ *t*-butoxy). ^{13}C NMR (101 MHz, MeOH-d_4): δ 171.83 (s, C(1) C(O)OCH_3) 158.72 (s, C(1) Boc) 154.83 (s, Ph) 133.0 (d, ^1J = 155.5, Ph) 129.11 (s, Ph) 128.34 (d, ^1J = 157.4, Ph) 117.77 (t, ^1J = 156.6, C(3) allyl) 115.06 (d, ^1J = 158.8, C(2) allyl) 79.95 (s, C *t*-butoxy) 68.78 (t, ^1J = 142.9, C(1) allyl) 56.94 (d, ^1J = 141.7, C(2)) 52.62 (q, ^1J = 147.0, O-CH_3) 28.26 (q, ^1J = 126.6, 3 × CH_3 *t*-butoxy). HR-ESI-TOF-MS: calcd for $C_{12}H_{15}NO_3$: 322.1649, found 322.1638 ([M+H]$^+$). Methyl *N*-*tert*-butoxycarbonyl-4-(prop-2-enyloxy)-D-phenylglycinate (0.136 g, 0.4 mmol) was dissolved in trifluoroacetic acid (80% aq, 4.2 ml) at 0 °C and then stirred at 25 °C for 4 h. The solvent was evaporated in vacuo, the crude was dissolved in CH_2Cl_2 (2 ml) and neutralized with 25% aq. NH_3 (0.3 ml), the solvent was evaporated in vacuo and the crude methyl 4-(prop-2-enyloxy)-D-phenylglycinate **6** was used in the subsequent reaction without any further purification.

Methyl 4-(phenylmethoxy)-D-phenylglycinate (**7**). Methyl *N*-*tert*-butoxycarbonyl-4-hydroxy-D-phenylglycinate **21** (0.316 g, 1.1 mmol) was dissolved in acetone (5.5 ml), K_2CO_3 (0.458, 3.3 mmol, 3 equiv) was added followed by benzyl bromide (0.26 ml, 0.375 g, 2.2 mmol, 2 equiv) and the solution was stirred at 25 °C for 8 h (reaction monitored by TLC: petroleum ether/EtOAc 4:1). The solvent was evaporated in vacuo and the crude was dissolved in EtOAc (5 ml). Water (5 ml) was added and the two layers were separated. The aqueous phase was extracted with EtOAc (3 × 5 ml), the collected organic phases were washed with brine (5 ml), dried on $MgSO_4$ and the solvent was evaporated in vacuo, giving pure methyl *N*-*tert*-butoxycarbonyl-4-(phenylmethoxy)-D-phenylglycinate as a colorless oil (0.325 g, 0.9 mmol, 81% yield). ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.36 (d, ^3J = 8.4, 2H Ph) 7.33 (t, ^3J = 7.3, 2H Ph) 7.31 (m, 3H Ph) 7.0 (d, ^3J = 8.6, 2H Ph) 5.16 (s, 1H, H-C(2)) 5.10 (s, 2H, H_2C-Ph) 3.71 (s, 3H, CH_3). Methyl *N*-*tert*-butoxycarbonyl-4-(phenylmethoxy)-D-phenylglycinate (0.104 g, 0.3 mmol) was dissolved in trifluoroacetic acid (80% aq, 2.8 ml) at 0 °C and then stirred at 25 °C for 3 h. The solvent was evaporated in vacuo, the crude was dissolved in CH_2Cl_2 (2 ml) and neutralized with 25% aq NH_3 (0.3 ml), the solvent was evaporated in vacuo and the pure **7** (0.0744 g, 0.3 mmol, quantitative) was obtained as a white foam after flash column chromatography on silica gel (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, $CDCl_3$, Ph = phenyl): δ 7.42–7.29 (m, 7H Ph) 6.96 (d, ^3J = 8.7, 2H Ph) 5.07 (s, 2H, H_2C-Ph) 4.58 (s, 1H, H-C(2)) 3.71 (s, 3H, CH_3). Analytical and spectroscopical data are in accord with those reported in literature.[54]

Methyl 4-methoxyphenyl-D-glycinate (**5**). 1 N aq NaOH (60 ml) was added to a solution of 4-hydroxy-D-phenylglycine **20** (9.95 g, 59 mmol) in dioxane/water (1:1, 120 ml). A solution of di-*tert*-butyl dicarbonate (14.33 g, 77 mmol, 1.3 equiv) in dioxane (60 ml) was added and the pale yellow solution was stirred at 20 °C overnight. The dioxane was evaporated in vacuo and the aqueous phase

was washed with diethyl ether (25 ml). $KHSO_4$ was added until pH 2.5 and the aqueous layer was extracted with EtOAc (3 × 30 ml), dried ($MgSO_4$) and the solvent was evaporated in vacuo. Pure *N-tert*-butoxycarbonyl-4-hydroxy-D-phenylglycine was recovered as white solid after recristallization from EtOAc (13.36 g, 50 mmol, 85% yield). *N-tert*-Butoxycarbonyl-4-hydroxy-D-phenylglycine (13.36 g, 50 mmol) was dissolved in DMF (450 ml) at 0 °C. K_2CO_3 (34.51 g, 250 mmol, 5 equiv) and then, dropwise, MeI (12.5 ml, 28.5 g, 201 mmol, 4 equiv) were added and the solution was stirred at 0 °C for 2 h (monitored by TLC CH_2Cl_2/MeOH 7:3). Satd aq soln of $NaHCO_3$ (500 ml) was added and the solution was extracted with diethyl ether (3 × 100 ml). The collected organic layers were washed with brine, dried over $MgSO_4$ and the solvent was evaporated in vacuo. Pure methyl *N-tert*-butoxycarbonyl-4-methoxyphenyl-D-glycinate was recovered as white foam (7.24 g, 24.4 mmol, 49% yield) after column chromatography on silica gel (pentane/EtOAc 3:1). ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.27 (d, ^3J = 8.4, 2H, Ph) 6.90 (d, ^3J = 8.4, 2H, Ph) 5.13 (s, 1H, H-C(2)) 3.78 (s, 3H, *p*-OCH_3) 3.69 (s, 3H, CH_3-OC(1)) 1.44 (s, 9H, $(CH_3)_3$ *t*-butoxy). Methyl *N-tert*-butoxycarbonyl-4-methoxyphenyl-D-glycinate (0.253 g, 0.9 mmol) was dissolved in trifluoroacetic acid (80% aq, 8.5 ml) at 0 °C and the mixture was stirred for 2 h at 20 °C. The solvent was evaporated in vacuo and pure methyl 4-methoxy-D-phenylglycinate **5** was obtained quantitatively after flash column chromatography on silica gel (CH_2Cl_2/MeOH from 95:5 to 9:1). Analytical and spectroscopic data were in accord to those reported in the literature.[54]

(2*R*,3*R*,4*S*)-2{{{(1*R*)-2-Hydroxy-1-[4-(prop-2-enyloxy)phenyl]-ethyl}amino}methyl} pyrrolidine-3,4-diol (**32**). $NaBH(OAc)_3$ (0.141 g, 0.7 mmol) was added portionwise to a stirred solution of aldehyde **1** (0.129 g, 0.5 mmol) and amine **6** (0.169 g, 0.4 mmol) in 1,2-dichloroethane (5 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was poured into a satd aq soln of $NaHCO_3$ (3 ml). The organic phase was collected and the aqueous phase extracted with EtOAc (3 × 5 ml). The combined organic extracts were washed with brine (5 ml) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1 to 2:1) gave pure **12** as light yellow oil (0.085 g, 0.2 mmol, 43% yield). *tert-Butyl (3aR,4R*,6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-methoxy-2-oxo-1-[4-(prop-2-enyloxy)phenyl]ethyl}amino}methyl}-tetrahydro-3a*H*-[1,3]dioxolo[4,5-*c*]pyrrole-5-carboxylate (**12**: 0.085 g, 0.2 mmol) was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.014, 0.4 mmol) in anhyd THF (4 ml). The solution was stirred at room temperature for 3 h, then water was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, *tert-butyl(3aR,4R*,6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-hydroxy-1-[4-(prop-2-enyloxy)phenyl]ethyl}amino}methyl}-tetrahydro-3a*H*-[1,3]dioxolo[4,5-*c*]pyrrole-5-carboxylate was obtained (0.081 g, 0.2 mmol, quantitatively). It was deprotected without any further purification. *tert-Butyl(3aR,4R*,6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-hydroxy-1-[4-(prop-2-enyloxy)phenyl]ethyl}amino}methyl}-tetrahydro-3a*H*-[1,3]dioxolo[4,5-]pyrrole-5-carboxylate (0.081 g, 0.2 mmol) was dissolved in trifluoroacetic acid (80% aq, 2.8 ml) at 0 °C and then stirred at 25 °C for 3 h. The solvent was evaporated in vacuo, the crude was dissolved in CH_2Cl_2 (2 ml) and neutralized with 25% aq NH_3 (0.3 ml), the solvent was evaporated in vacuo and the pure **32** 0.0241 g, 0.08 mmol, 44% yield) was obtained as a white solid after flash column chromatography on silica gel (CH_3CN/NH_4OH 8:1). Melting point: 160–164° C (dec) $[\alpha]_{589}^{25} = +6$; $[\alpha]_{577}^{25} = +22$; $[\alpha]_{435}^{25} = +21$ (0.241 g/100 ml, MeOH). IR (pure): 3419, 1384, 1243, 1014, 839,697. ^{1}H NMR (400 MHz, MeOH-d_4, all = allyl): δ 7.25 (d, ^3J = 8.6, 2H, Ar-H) 6.89 (d, ^3J = 8.6, 2H, Ar-H) 6.04 (m, 1H, CH_2 = C*H*CH_2O) 5.38 (d, ^2J = 17.8, 1H, H_{trans}-C(3) all) 5.22 (d, ^2J = 10.0, 1H, H_{cis}-C(3) all) 4.52 (d, ^3J = 5.12, 2H, H_2C(1) all) 3.69 (dd, ^3J = 8.4, ^3J = 4.4, 1H, H-C(3)) 4.01 (m, 1H, H-C(4)) 3.58 (m, 3H C*H*CH_2OH and CHC*H*$_2$OH) 3.12 (dd, ^2J = 12.3, ^3J = 5.2, 1H, H*H*-C(5)) 3.03 (m, 1H, H-C(2)) 2.81 (dd, ^2J = 12.3, ^3J = 3.4, 1H, *H*H-C(5)) 2.63 (dd, ^2J = 12.0, ^3J = 5.2, 1H, H*H*-C(6)) 2.52 (dd, ^2J = 12.0, ^3J = 7.6, 1H, *H*H-C(6)). ^{13}C NMR (101 MHz, MeOH-d_4, Ph = phenyl, all = allyl): δ 159.57 (s, C Ph) 134.98 (s, C Ph) 133.99 (d, ^1J = 21.3, C(2) all) 129.79 (d, ^1J = 156.0, 2 *C*H Ph) 117.40 (t, ^1J = 158.1, C(3) all) 115.83 (d, ^1J = 158.0, 2 *C*H Ph) 76.91 (d, ^1J = 141.9, C(3)) 72.58 (d, ^1J = 148.0, C(4)) 69.79 (t, ^1J = 144.7, C(1) all) 67.81 (t, ^1J = 141.2, CHCH_2OH) 66.04 (d, ^1J = 132.8, *C*HCH_2OH) 62.81 (d, ^1J = 137.9, C(2)) 52.26 (t, ^1J = 138.9, C(5)) 50.88 (t, ^1J = 138.3, C(6)). HR-ESI-TOF-MS: calcd for $C_{16}H_{24}N_2O_4$ 309.1814, found 309.1818 $[M+H]^+$ and 331.1044 $[M+Na]^+$.

(2*R*,3*R*,4*S*)-2{{(1*R*)-2-Hydroxy-1-{4-[(phenylmethoxy)phenyl]-hydroxyethyl}amino}methyl} pyrrolidine-3,4-diol (**33**). $NaBH(OAc)_3$ (0.094 g, 0.4 mmol) was added portionwise to a stirred solution of aldehyde **1** (0.133 g, 0.5 mmol) and amine **7** (0.075 g, 0.3 mmol) in 1,2-dichloroethane (3.5 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was poured into a satd aq soln of $NaHCO_3$ (5 ml). The organic phase was collected and the aqueous phase extracted with EtOAc (3 × 5 ml). The combined organic extracts were washed with brine (5 ml) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1 to 2:1) gave pure **13** as colorless oil (0.065 g, 0.1 mmol, 41% yield). This compound was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.012, 0.3 mmol) in anhyd THF (3.2 ml). The solution was stirred at room temperature for 3 h, then water was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, *tert*-butyl(3a*R*,4*R*,6a*S*)-2,2-dimethyl-4{{{(1R)-2-hydroxy-1-[4-(phenylmethoxy)phenyl]ethyl}amino}methyl}-tetrahydro-3a*H*-[1, 3]dioxolo[4,5-*c*]pyrrole-5-carboxylate was obtained (0.060 g, 0.1 mmol, quant.). It was deprotected without any further purification. *tert*-Butyl(3a*R*,4*R*,6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-hydroxy-1-[4-(phenylmethoxy)phenyl]ethyl}amino}methyl}-tetrahydro-3a*H*-[1, 3]dioxolo[4,5-*c*]pyrrole-5-carboxylate (0.0598 g, 0.12 mmol) was dissolved in trifluoroacetic acid (80% aq, 1.3 ml) at 0 °C and then stirred at 25 °C for 3 h. The solvent was evaporated in vacuo, the crude was dissolved in CH_2Cl_2 (2 ml) and neutralized with 25% aq NH_3 (0.3 ml), the solvent was evaporated in vacuo and the pure **33** (0.017 g, 0.05 mmol, 42% yield) was obtained as a pale yellow foam after flash column chromatography on silica gel (CH_3CN/NH_4OH 6:1). $[\alpha]_{589}^{25} = -6$; $[\alpha]_{577}^{25} = -6$; $[\alpha]_{435}^{25} = -13$; $[\alpha]_{405}^{25} = -16$ (*c* 0.342, MeOH). UV (CH_3CN): 275 (1754); 226 (23510). IR (solid (KBr), cm^{-1}): 3420, 2923, 1384, 1244, 1014, 839, 744, 697. ^{1}H NMR (400 MHz, MeOH-*d*): δ 7.44 (m, 2Ph) 7.38 (m, 2H Ph) 7.32 (m, 1H Ph) 7.29 (d, ^3J = 8.5, 2H Ph) 7.00 (d, ^3J = 8.5, 2H Ph) 5.09 (s, 2H, H_2-C-Ph) 4.10 (m, 1H, H-C(4)) 3.73 (m, 2H, C*H*CH_2OH and H-C(3)) 3.62 (dd, ^2J = 10.8, ^3J = 4.4, 1H, CHCH_2OH) 3.56 (dd, ^2J = 10.8, ^3J = 8.8, 1H CHCH_2OH) 3.24 (dd, ^2J = 12.2, ^3J = 4.5, 1H, H*H*-C(5)) 3.18 (m, 1H, H-C(2)) 2.96 (d, ^2J = 12.2, 1H, *H*H-C(5)) 2.74 (dd, ^2J = 12.3, ^3J = 4.7, 1H, H*H*-C(6)) 2.61 (dd, ^2J = 12.3, ^3J = 7.9, 1H, *H*H-C(6)). ^{13}C NMR (100 MHz, MeOH-*d*): δ 158.82 (s, C Ph) 137.75 (s, C Ph) 132.90 (s, C Ph) 128.71 (d, ^1J = 159.9 2*C*H Ph) 128.51 (dd, ^1J = 159.9, ^2J = 7.0, 2*C*H Ph) 127.86 (d, ^1J = 86.3, 2*C*H Ph) 127.49 (d, ^1J = 157.1, *C*H Ph) 115.11 (d, ^1J = 158.2, 2*C*H Ph) 74.40 (d, ^1J = 143.3, C(3)) 70.58 (d, ^1J = 150.8, C(4)) 69.96 (t, ^1J = 142.0, CHCH_2OH) 66.59 (t, ^1J = 141.0, H_2C-Ph) 65.00 (d, ^1J = 132.8, *C*HCH_2OH) 61.81 (d, ^1J = 142.5, C(2)) 51.11 (t, ^1J = 142.3, C(5)) 48.38 (t, ^1J = 134.5, C(6)). HR-ESI-TOF-MS: calcd for $C_{20}H_{26}N_2O_3$: 359.1971, found 359.1969 ($[M+H]^+$).

(2*R*,3*R*,4*S*)-2{{[(1*R*)-2-Hydroxy-1-(4-methoxyphenyl)ethyl]amino}-methyl}pyrrolidine-3,4-diol (**34**). $NaBH(OAc)_3$ (0.169 g, 0.8 mmol) was added portionwise to a stirred solution of aldehyde **1** (0.168 g, 0.6 mmol) and amine **5** (0.166 g, 0.9 mmol) in 1,2-dichloroethane (6 ml) at room temperature. After complete disappearance of reagents (reaction monitored by TLC), the solution was

poured into a satd aq soln of $NaHCO_3$ (5 ml). The organic phase was collected and the aqueous phase extracted with EtOAc (3 × 5 ml). The combined organic extracts were washed with brine (5 ml) and dried ($MgSO_4$). Solvent evaporation in vacuo and flash chromatography (light petroleum/EtOAc 4:1 to 2:1) gave pure **11** as as a yellow oil (0.144 g, 0.3 mmol, 52% yield). This product was added portionwise to a cooled suspension (0 °C) of $LiAlH_4$ (0.038, 1.0 mmol) in anhyd THF (8 ml). The solution was stirred at room temperature for 3 h, then water was added dropwise (0.5 ml) and the mixture was filtered on silica gel conditioned with EtOAc. After solvent evaporation in vacuo, *tert*-butyl (3a*R*, 4*R*, 6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-hydroxy-1-[4-(methoxyphenyl)phenyl]-ethyl} amino}methyl}-tetrahydro-3a*H*-[1,3]dioxolo[4,5-*c*]pyrrole-5-carboxylate so obtained (0.070 g, 0.2 mmol, 53% yield) was deprotected without any further purification. *Tert*-butyl(3a*R*,4*R*,6a*S*)-2,2-dimethyl-4{{{(1*R*)-2-hydroxy-1-[4-(methoxyphenyl)phenyl]ethyl} amino}methyl}-tetrahydro-3a*H*-[1,3]dioxolo[4,5-*c*]pyrrole-5-carboxylate (0.070 g, 0.2 mmol) was dissolved in trifluoroacetic acid (80% aq, 1.7 ml) at 0 °C and then stirred at 25 °C for 3 h. The solvent was evaporated in vacuo, the crude was dissolved in dichloromethane (2 ml) and neutralized with 25% aq NH_3 (0.3 ml), the solvent was evaporated in vacuo and the pure **34** (0.0370 g, 0.13 mmol, 76% yield) was obtained as a white foam after flash column chromatography on silica gel (CH_3CN/NH_4OH 6:1). $[\alpha]_{589}^{25} = -19$; $[\alpha]_{577}^{25} = -13$; $[\alpha]_{435}^{25} = -29$; $[\alpha]_{405}^{25} = -35$ (*c* 0.185, MeOH) UV (CH_3CN): 277 (1447). IR (pure): 3298, 3270, 2874, 2833, 1512, 1245, 1114, 1025, 807. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.29 (d, ^3J = 8.8, 2H Ph) 6.93 (d, ^3J = 8.8, 2H Ph) 4.05 (m, 1H, H-C(4)) 3.80 (s, 3H, H_3-CO) 3.75 (dd, ^3J = 9.0, ^3J = 4.6, 1H, H-C(3)) 3.62 (m, 3H, C*H*CH_2OH and 2H CHC*H*$_2$OH) 3.14 (dd, ^2J = 12.0, ^3J = 5.2, 1H, H*H*-C(5)) 3.07 (m, 1H, H-C(2)) 2.84 (dd, ^2J = 12.2, ^3J = 3.2, 1H, *H*H-C(5)) 2.66 (dd, ^2J = 11.8, ^3J = 4.6, 1H, H*H*-C(6)) 2.53 (dd, ^2J = 11.8, ^3J = 7.8, 1H, *H*H-C(6)). ^{13}C-NMR (101 MHz, MeOH-d_4): δ 160.38 (s, C Ph) 133.52 (s, C Ph) 129.85 (d, ^1J = 157.4, 2 *C*H Ph) 115.01 (d, ^1J = 158.1, 2*C*H Ph) 76.95 (d, ^1J = 143.5, C(3)) 72.50 (d, ^1J = 149.1, C(4)) 67.54 (t, ^1J = 140.5, CHCH_2OH) 65.77 (d, ^1J = 133.9, *C*HCH_2OH) 62.40 (d, ^1J = 137.3, C(2)) 55.86 (q, ^1J = 142.7, *C*H_3OH) 51.95 (t, ^1J = 139.7, C(5)) 51.00 (t, ^1J = 133.9, C(6)). HR-ESI-TOF-MS: calcd for $C_{14}H_{22}N_2O_4$: 283.1658, found 283.1658 ($[M+H]^+$) and 305.1393 ($[M+Na]^+$).

4.2. Cells lines

U266, A549, MCF7, SKBR3, MDA-MB-231, BT474, U87 and PC3 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Jurkat T cells engineered to stably overexpress Bcl2 and the respective vector control cells were a gift of Dr. Claus Belka (Department of Radiation Oncology, University of Tuebingen, Tuebingen, Germany). Adherent cell lines were grown in 10-cm culture dishes in McCoy's medium (Sigma Chemical Co., USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FCS, GIBCO, Grand Island, NY), L-glutamine and antibiotics. U266 and Jurkat cells were maintained in RPMI-1640 medium (Sigma Chemical Co., USA) supplemented with 10% (v/v) fetal bovine serum, L-glutamine and antibiotics. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The dihydroxypyrrolidine derivative was weighted and dissolved in DMSO to prepare a 100 mM stock solution.

4.3. B-CLL and multiple myeloma cell isolation

Peripheral blood (PB) samples from B-chronic lymphocytic leukemia (B-CLL) patients, and bone marrow (BM) samples from multiple myeloma patients were obtained from routine diagnostic samples after informed consent of patients and permission by the local ethics committee. PB and BM samples were subjected to Ficoll (Biochrom) density gradient centrifugation. The mononuclear cell fraction was collected, washed with phosphate-buffered saline (PBS) and immediately seeded in RPMI 1640-based culture medium. B-CLL cells, as detected by CD19 and CD23 expression, were >60% in all of the samples used for this study. Similarly, the purity of multiple myeloma cells was detected by staining with an anti-CD138 antibody and flow cytometry.

4.4. Viability assays

5 × 103 cells/well were plated in 200 μl medium in 96-well plates. 48 h later, the dihydroxypyrrolidine derivative was added to the wells at concentrations ranging between 1 and 400 μM, such that the vehicle DMSO never exceeded 0.4%. Viability was determined 72 h later using CellTiter 96 Aqueous1 (Promega Italia, Milan, Italy) according to the manufacturer's instructions. Incubation times with CellTiter96 Aqueous1 ranged between 2 and 4 h. Plates were read with a spectrophotometer (Labsystems iEMS Reader MF) at 490 nm wave length. IC_{50}s were estimated using GraphPad Prism4. For apoptosis detection using Annexin V and propidium iodide, 2 × 106 cells/well were plated in a 24-well plate and stimulated with different concentrations of novel compound. 24 h later, cells were harvested, washed with PBS and stained with FITC-conjugated Annexin V (Becton Dickinson, BD Italia, Milan, Italy) and propidium iodide (2 μg/ml) according to the manufacturer's instructions. FACS analysis was done on a FACS Calibur (Becton Dickinson) by acquiring 20000 events.

4.5. Cell cycle analysis

For cell cycle analysis, cells were resuspended in a buffer containing 0.1% sodium citrate, 0.1% TritonX, and 50 μg/ml propidium iodide. Cell cycle analysis was performed on a FACS Calibur using ModFit LT software.

4.6. Microscopy

Cells were imaged using the 40X magnification of a Zeiss AXIOVERT200 microscope, camera Qlympus C-4040ZOOM. The image files were downloaded using the software Olympus CAMEDIA Master 2.5.

5.6 RNA extraction and Taqman low-density arrays (TLDA).

Total RNA was isolated from cell lysates using QIAGEN RNeasy Mini anion-exchange spin columns (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. Total RNA (1 μg) was subjected to a 20-μL cDNA synthesis reaction using SuperScript RTII (Invitrogen, Karlsruhe, Germany). Oligo(dT) was used as primer. PCR amplification was performed using 2 μL cDNA TLDA were done for a panel of 90 genes that were previously selected for their relevance in cancer biology and metastasis. For these experiments, SKBR3 and MCF7 cells were plated in 6-well plates and treated for 12 h with 50 μM of novel drug. Thereafter, cells were washed and used for RNA extraction. cDNA was generated using random hexamers and used for multiple quantitative PCR (Q-PCR) as described previously.[55] TLDA were performed on a 7900HT Fast Real Time PCR System using primer collections assembled by Agilent. Results were plotted as mRNA fold induction in drug-treated versus vehicle-treated cells (calcd as $2^{-\Delta\Delta CT}$).[56] Q-PCRs were performed in duplicate for each gene. RPLP0 was used as an housekeeping gene to normalized the results.

4.7. Immunoblotting

Bcl2 expression in Jurkat cells was evaluated by immunoblotting as previously described.[47]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.009.

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