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Original article

Differential effect of heterocyclic D-ribofuranoside derivatives on human prostate cancer cell viability and cell cycle progression

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ABSTRACT

New D-ribofuranoside derivatives containing two five membered heterocycles, isoxazole and triazole or two triazole rings, were synthesized. The final products as well as the synthetic precursors were physically and spectroscopically characterized. These new diheterocyclic derivatives together with other D-riboside compounds were assessed for their impact on PC3 cell line viability. We found that exposure of prostate cancer cells to some of these compounds caused a significant inhibition of cell growth and a G_0/G_1 cell cycle arrest, which was concomitant with alterations in the expression of proteins involved in cell cycle progression. Furthermore, the inhibitory activity was improved in di-heterocycles when the carbohydrate moiety was protected with a cyclopentylidene group compared to the isopropylidene analogues.

1. Introduction

The development of potent and effective antitumoral agents has become one of the most intensely studied topics of contemporary medicinal chemistry. In this context, we have been interested in exploiting the synthesis and biological properties of five membered heterocyclic rings linked to carbohydrate moieties. In a previous work, we have reported the synthesis of some 1,2,4-triazole D-ribose derivatives (Fig. 1) and their antiproliferative activity, *in vitro*, against a BW 5147 lymphoma cell line [1].

Among these structures, the synthesized compounds containing a 1,2,4-triazole ring linked by sulfur to the carbohydrate moiety (compounds **1**, **4**, **6** and **8**) displayed a moderate antiproliferative activity against this cell line, but compounds **1** and **6** showed a strong inhibitory behavior in the range of measured concentrations. The structures with 3-thiobenzyl-5-substituted-1,2,4-triazole ring (compounds **2**, **3**, **5**, **7** and **9**) led to compounds with a biphasic behavior, meanwhile the deprotected compounds (**4**, **5**, **8** and **9**) showed a reduction in the antiproliferative activity [1].

It is well known that a large number of compounds with important biological activities contain a 1,2,3-triazole ring, for example, β-lapachone based 1,2,3-triazole derivatives were highly active ($IC_{50} < 2$ μM) for HL-60 and MDA-MB435 cancer cell lines [2]. Moreover, series of 1,2,3-triazole 1,5-disubstituted analogs of combretastatin A-4 exhibited potent cytotoxic activity in the nanomolar range in several cancer cell lines as well as a moderate tubulin inhibitory activity in the low micromolar range [3,4].

The histone deacetylase inhibitors (HDAC) have been revealed as a promising new class of anticancer agents that act through a variety of mechanisms [5–7]. The novel HDAC inhibitor MHY219, a suberoylanilide hydroxamic acid-like (SAHA-like), showed an important antiproliferative effect in prostate cancer cells [8]. However, compounds, which were several folds more potent than SAHA, were obtained when the amide group in SAHA-like inhibitors is replaced by a triazole ring [9].

On the other hand, the isoxazole ring is known for its medicinal importance and form the basis of several drugs, such as zonisamide (anti-convulsant) [10], valdecoxib (COX-2 inhibitor) [11] and leflunomide (a disease-modifying antirheumatic drug) [12]. In addition, an isoxazole derivative from curcumin has been reported as a good antioxidant and COX inhibitory agent [13] and showed greater activity against both MCF-7 and MCF-7R cell lines than its natural product precursor [14]. Promising properties as a heat

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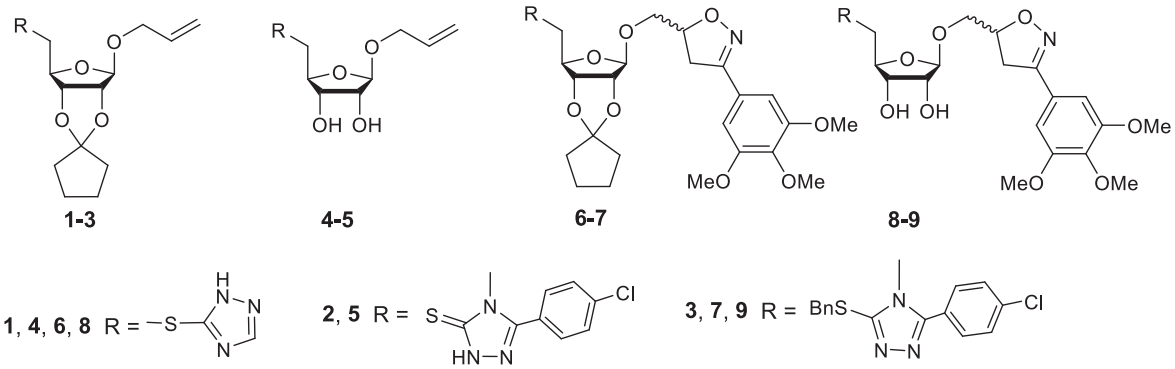


Fig. 1. Chemical structures of 1,2,4-triazole D-ribose derivatives.

shock protein 90 (HSP90) inhibitor have been reported for a diaryl resorcinylic isoxazole amide derivative, with antiproliferative potency quite similar to the clinical drug 17-AAG [15]. HSP90 is a molecular chaperone required for the folding and post-translational stability and function of several signal transducing proteins referred as "client" proteins [16]. Many known oncoproteins are client proteins of HSP90, thus targeting HSP90 has emerged as an interesting avenue for cancer therapeutics. In particular, these inhibitors have appeared in the prostate cancer (PCa) scene [17], since several HSP90 client proteins are implicated in the pathogenesis of PCa.

In the present work, we report the synthesis of new lipophylic diheteroaromatic D-ribose derivatives having an isoxazole or a 1,2,3-triazole ring at the anomeric carbohydrate position using two different protective groups (cyclopentylidene or isopropylidene). These diheterocyclic derivatives together with the most promising molecules (**1**, **6**), that we previously described [1], were screened against the PC3 cells (human PCa cell line) and evaluated the impairment of cell growth and cell cycle progression.

2. Materials and methods

2.1. Chemistry

The syntheses were carried out using reagents as purchased, without further purification. Solvents were reagent grade and, in most cases, dried and distillated before use according to standard procedures. Analytical TLC was conducted on Silica Gel 60G (Merck) on precoated plates and visualization was made by UV light and ethanol/sulfuric acid (10:1) or cerium molybdate followed by heating. Column-chromatographic separations were performed on Silica Gel (240–400 mesh, Merck). Elemental analysis was performed on an Exeter Analytical CE-440 elemental analyzer. Optical rotations were recorded at 20 °C on a Perkin Elmer 343 polarimeter, and melting points were uncorrected. ^{1}H, ^{13}C NMR spectra were recorded on a Bruker AC-200 spectrometer, operating at 200, 50 MHz respectively; or a Bruker AMX-500 spectrometer, operating at 500, 125 MHz respectively. Assignments of the ^{1}H and ^{13}C NMR spectra were confirmed with the aid of two dimensional techniques ^{1}H, ^{13}C (COSY, HSQC, HMBC). Chemical shifts (δ) are reported in parts per million downfield from tetramethyl silane as internal standard. Compounds **1**, **6** and **8** were synthesized as we previously reported [1].

2.1.1. General procedure for the synthesis of propargyl derivatives (10, 11)

Powered D-ribose (6.05 g, 40.30 mmol) and anhydrous cuprous sulfate (12.9 g) were suspended in a mixture of cyclopentanone (110 mL) or acetone (96 mL) and propargyl alcohol (28 mL) containing a catalytic amount of H_2SO_4 (0.3 mL). The resulting mixture was stirred at 40 °C for 48 h and then neutralized with $NaHCO_3$, filtered, and the solvents were evaporated. The crude product was extracted with ethyl acetate and washed with brine, dried (Na_2SO_4), and the solvent was evaporated to give the crude product, which was purified by flash chromatography (cyclohexane:acetone) to obtain compound **10**, **11**.

2.1.1.1. Propargyl 2,3-O-cyclopentylidene-β-D-ribofuranoside (10). Compound **10** was obtained as a colorless oil (22.77 mmol, 57%); $[\alpha]_D^{20}$ −100.2 (*c* 1.1, chloroform). IR υ_{max} 3474 (υ O–H), 3284 (υ C_{sp}–H), 2957 (υ C_{sp3}–H), 2125 (υ C_{sp}–C_{sp}), cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 5.26 (s, 1H, H-1), 4.74 (dd, 1H, $J_{3,2}$ 6.0 Hz, $J_{3,4}$ 0.5 Hz, H-3), 4.57 (d, 1H, $J_{2,3}$ 6.0 Hz, H-2), 4.42 (t, 1H, $J_{4,5}$ 3.6 Hz, H-4), 4.27 (d, 2H, *J* 2.3 Hz, C$\underline{H}_2$-C≡CH), 3.70 (ddd, 1H, $J_{5b,5a}$ 12.3 Hz, $J_{5b,OH}$ 4.4 Hz, $J_{5b,4}$ 3.2 Hz, H-5b), 3.61 (ddd, 1H, $J_{5a,5b}$ 12.6 Hz, $J_{5a,OH}$ 8.6 Hz, $J_{5a,4}$ 4.2 Hz, H-5a), 2.86 (dd, 1H, $J_{OH,5a}$ 8.9 Hz, $J_{OH,5b}$ 4.6 Hz, OH), 2.48 (t, 1H, *J* 2.4 Hz, ≡CH), 1.95–1.63 (m, 8H, cyclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 122.0 (quaternary carbon of cyclopentylidene ring), 107.5 (C-1), 88.4, 85.7, 81.3 (C-2, C-3, C-4), 78.5 (CH$_2$–$\underline{C}$≡CH), 75.4 (≡CH), 64.0 (C-5), 55.3 ($\underline{C}$H$_2$-C≡), 35.8, 23.7, 23.2 (cyclopentylidene carbons). EIMS *m/z* 254 $[M]^{+\bullet}$ (16), 253 (11), 225 (100), 199 (75), 169 (93). Anal. Calcd for $C_{13}H_{18}O_5$: C, 61.40; H, 7.14. Found: C, 61.54; H, 7.29.

2.1.1.2. Propargyl 2,3-O-isopropylidene-β-D-ribofuranoside (11). Compound **11** was obtained as a colorless oil (21.36 mmol, 53%); $[\alpha]_D^{20}$ −112.0 (*c* 1.0, chloroform). IR υ_{max} 3466 (υ O–H), 3284 (υ C_{sp}–H), 2941 (υ C_{sp3}–H), 2118 (υ C_{sp}–C_{sp}), 1076 (υ C_{sp3}–O) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 5.25 (s, 1H, H-1), 4.80 (d, 1H, $J_{3,2}$ 6.0 Hz, H-3), 4.63 (d, 1H, $J_{2,3}$ 5.9 Hz, H-2), 4.41 (t, 1H, $J_{4,5}$ 3.5 Hz, H-4), 4.27 (d, 2H, *J* 2.4 Hz, C$\underline{H}_2$-C≡CH), 3.70 (dd, 1H, $J_{5b,5a}$ 12.4 Hz, $J_{5b,4}$ 3.2 Hz, H-5b), 3.61 (dd, 1H, $J_{5a,5b}$ 12.6 Hz, $J_{5a,4}$ 4.2 Hz, H-5a), 2.48 (t, 1H, *J* 2.4 Hz, ≡CH), 1.47 (s, 3H, CH_3), 1.30 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 112.4 (quaternary carbon of isopropylidene group), 107.8 (C-1), 88.7, 86.0, 81.5 (C-2, C-3, C-4), 78.5 (CH$_2$-$\underline{C}$≡CH), 75.5 (≡CH), 64.0 (C-5), 55.3 ($\underline{C}$H$_2$-C≡), 26.5, 24.9 (CH_3). EIMS *m/z* 228 $[M]^{+\bullet}$ (16), 227 (29), 173 (100), 169 (50), 137 (40), 131 (71). Anal. Calcd for $C_{11}H_{16}O_5$: C, 57.88; H, 7.07. Found: C, 58.23; H, 7.22.

2.1.2. General procedures for the synthesis of tosyl derivatives (12, 13)

To a solution of propargyl derivative **10** or **11** (21.40 mmol) dissolved in anhydrous pyridine (10 mL), tosyl chloride (26.2 mmol) was added with continuous stirring. The mixture was kept at room temperature during overnight. Then, the reaction mixture was dissolved in methylene chloride and extracted with water, hydrogen chloride (5%), sodium bicarbonate (5%) and finally washed with water. The organic layer was dried with Na_2SO_4 (anhydrous) and the solvent was evaporated. The crude product

was purified by column chromatography (cyclohexane:acetone) affording compound **12** or **13**.

2.1.2.1. Propargyl 2,3-O-cyclopentylidene-5-tosyl-β-D-ribofuranoside (12). Compound **12** was obtained as a colorless oil (16.40 mmol, 77%); $[\alpha]_D^{20}$ –68.1 (*c* 1.1, chloroform). IR υ_{max} 3286 (υC_{sp}–H), 2964 (υC_{sp3}–H), 2121 (υC_{sp}–C_{sp}), 1599 (υC_{sp2}–C_{sp2}), 1362 (υ S=O), 1182 (υ S=O) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.78 (d, 2H, *J* 8.2 Hz, aromatic protons), 7.34 (d, 2H, *J* 7.9 Hz, aromatic protons), 5.25 (s, 1H, H-1), 4.52 (s, 2H, H-2, H-3), 4.34 (t, 1H, $J_{4,5}$ 7.1 Hz, H-4), 4.07 (d, 2H, *J* 2.4 Hz, C<u>H</u>$_2$-C≡CH), 3.99 (d, 2H, $J_{5,4}$ 7.1 Hz, H-5), 2.44 (s, 3H, CH_3), 2.40 (t, 1H, *J* 2.5 Hz, ≡CH), 1.90–1.62 (m, 8H, cyclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 145.2, 132.7, 130.1 and 128.1 (aromatic carbons), 122.5 (quaternary carbon of cyclopentylidene ring), 106.3 (C-1), 84.8 (C-2), 83.7 (C-4), 81.1 (C-3), 78.5 (CH_2-<u>C</u>≡CH), 75.1 (≡CH), 69.3 (C-5), 54.4 (<u>C</u>H$_2$-C≡), 35.8, 35.7, 23.6, 23.2 (cyclopentylidene carbons), 21.8 (CH_3). EIMS *m/z* 408 $[M]^{+\bullet}$ (19), 379 (100), 353 (50), 155 (18), 91 (37). Anal. Calcd for $C_{20}H_{24}O_7S$: C, 58.81; H, 5.92. Found: C, 58.71; H, 6.16.

2.1.2.2. Propargyl 2,3-O-isopropylidene-5-tosyl-β-D-ribofuranoside (13). Compound **13** (16.50 mmol, 77%) was obtained as a white solid; mp: 47–48 °C, $[\alpha]_D^{20}$ –73.3 (*c* 1.1, chloroform). IR υ_{max} 3284 (υC_{sp}–H), 2942 (υC_{sp3}–H), 2120 (υC_{sp}–C_{sp}), 1595 (υC_{sp2}–C_{sp2}), 1362 (υ S=O), 1178 (υ S=O) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.80 (d, 2H, *J* 8.5 Hz, aromatic protons), 7.35 (d, 2H, *J* 8.0 Hz, aromatic protons), 5.26 (s, 1H, H-1), 4.60 (s, 2H, H-2, H-3), 4.34 (t, 1H, $J_{4,5}$ 7.2 Hz, H-4), 4.09 (d, 2H, *J* 2.6 Hz, C<u>H</u>$_2$-C≡CH), 4.01 (d, 2H, $J_{5,4}$ 7.3 Hz, H-5), 2.45 (s, 3H, CH_3), 2.41 (t, 1H, *J* 2.6 Hz, ≡CH), 1.45 (s, 3H, isopropylidene protons), 1.28 (s, 3H, isopropylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 145.3, 132.8, 130.1 and 128.1 (aromatic carbons), 113.0 (quaternary carbon of isopropylidene group), 106.6 (C-1), 85.1 (C-2), 84.1 (C-4), 81.4 (C-3), 78.5 (CH_2-<u>C</u>≡CH), 75.1 (≡CH), 69.3 (C-5), 54.5 (<u>C</u>H$_2$-C≡), 26.4, 25.0 (CH_3 of isopropylidene group), 21.8 (CH_3 of tosyl group). EIMS *m/z* 172 (68), 108 (46), 107 (70), 91 (100). Anal. Calcd for $C_{18}H_{22}O_7S$: C, 56.53; H, 5.80. Found: C, 56.64; H, 6.02.

2.1.3. General procedure for the synthesis of isoxazol derivatives (14, 15)

To a solution of 3,4,5-trimethoxy benzaldoxime (8.00 mmol) in ethanol:water 1:1, chloramine-T (16.00 mmol) was added in small portions. This solution was slowly added in portions to compound **12** or **13** (4.00 mmol) dissolved in ethanol:water 1:1 and the reaction mixture was heated at 40 °C for 4 h. Then, the solution was evaporated and the residue was purified.

2.1.3.1. (3″-(3,4,5-Trimethoxyphenyl)-isoxazol-5″-yl)methyl 2,3-O-cyclopentylidene-5-tosyl-β-D-ribofuranoside (14). Compound **14** was purified by flash column chromatography using toluene:ethyl acetate as eluent and was obtained as a white solid (2.68 mmol, 67%); mp: 31–33 °C, $[\alpha]_D^{20}$ –27.1 (*c* 1.2, chloroform). IR υ_{max} 2938 (υC_{sp3}–H), 1588 (υC_{sp2}–C_{sp2}), 1468 (υC_{sp2}–C_{sp2}), 1361 (υ S=O), 1180 (υ S=O), 1122 (υ C–O), 978 (υ S–C–O), 816 (δ C–H) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.76 (d, 2H, *J* 8.6 Hz, aromatic protons), 7.31 (d, 2H, *J* 8.6 Hz, aromatic protons), 7.05 (s, 2H, trimethoxyphenyl protons), 6.60 (s, 1H, H-4″ isoxazolyl ring), 5.19 (s, 1H, H-1), 4.69 (d, 1H, $J_{1'b,1'a}$ 13.4 Hz, H-1′b), 4.58 (s, 2H, H-2, H-3), 4.57 (d, 1H, $J_{1'a,1'b}$ 13.4 Hz, H-1′a), 4.40 (t, 1H, $J_{4,5}$ 6.9 Hz, H-4), 4.05 (d, 2H, $J_{5,4}$ 6.6 Hz, H-5), 3.92 (s, 6H, OCH_3), 3.89 (s, 3H, OCH_3), 2.41 (s, 3H, CH_3), 1.91-1.65 (m, 8H, ciclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.5, 162.5 (C-5″, C-3″ isoxazolyl ring), 153.7, 139.8, 124.3, 104.2 (trimethoxyphenyl carbons), 145.4, 132.5, 130.1, 128.0 (tosyl carbons), 122.6 (quaternary carbon of ciclopentylidene ring), 107.6 (C-1), 101.9 (C-4″ isoxazolyl ring), 84.9 (C-2), 84.0 (C-4), 81.0 (C-3), 69.4 (C-5), 61.0 (OCH_3), 59.8 (C-1′), 56.4 (OCH_3), 35.8, 35.8, 23.6, 23.2 (ciclopentylidene carbons), 21.8 (CH_3). EIMS *m/z* 617 $[M]^{+\bullet}$ (7), 361 (42), 249 (19), 248 (18), 234 (24), 172 (61), 91 (100). Anal. Calcd for $C_{30}H_{35}NO_{11}S$: C, 58.34; H, 5.71; N, 2.27. Found: C, 58.29; H, 6.11; N, 2.60.

2.1.3.2. (3″-(3,4,5-Trimethoxyphenyl)-isoxazol-5″-yl)methyl 2,3-O-isopropylidene-5-tosyl-β-D-ribofuranoside (15). Compound **15** was purified by flash column chromatography using cyclohexane:acetone as eluent and was obtained as a white solid (2.56 mmol, 64%); mp: 30 °C, $[\alpha]_D^{20}$ –40.0 (*c* 1.1, chloroform). IR υ_{max} 2940 (υC_{sp3}–H), 1591 (υC_{sp2}–C_{sp2}), 1467 (υC_{sp2}–C_{sp2}), 1366 (υ S=O), 1183 (υ S=O), 1124 (υ C–O), 982 (υ S–C–O), 814 (δ C–H) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 7.73 (d, 2H, *J* 8.2 Hz, aromatic protons), 7.27 (d, 2H, *J* 7.9 Hz, aromatic protons), 7.02 (s, 2H, trimethoxyphenyl protons), 6.58 (s, 1H, H-4″ isoxazolyl ring), 5.15 (s, 1H, H-1), 4.66 (d, 1H, $J_{1'b,1'a}$ 13.4 Hz, H-1′b), 4.61 (s, 2H, H-2, H-3), 4.54 (d, 1H, $J_{1'a,1'b}$ 13.6 Hz, H-1′a), 4.35 (t, 1H, $J_{4,5}$ 6.9 Hz, H-4), 4.02 (d, 2H, $J_{5,4}$ 7.0 Hz, H-5), 3.88 (s, 6H, OCH_3), 3.85 (s, 3H, OCH_3), 2.37 (s, 3H, CH_3 of tosyl group), 1.41 (s, 3H, CH_3 of isopropylidene group), 1.24 (s, 3H, CH_3 of isopropylidene group); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.4, 162.3 (C-5″, C-3″ isoxazolyl ring), 153.6, 139.7, 124.2, 104.1 (trimethoxyphenyl carbons), 145.2, 132.5, 130.0, 127.9 (tosyl carbons), 112.9 (quaternary carbon of isopropylidene group), 107.7 (C-1), 101.7 (C-4″ isoxazolyl ring), 85.0 (C-2), 84.2 (C-4), 81.1 (C-3), 69.3 (C-5), 60.9 (OCH_3), 59.7 (C-1'), 56.2 (OCH_3), 26.3, 24.8 (CH_3 of isopropylidene group), 21.5 (CH_3 of tosyl group). EIMS *m/z* 591 $[M]^{+\bullet}$ (100), 576 (11), 293 (32), 248 (66), 155 (44), 91 (85). Anal. Calcd for $C_{28}H_{33}NO_{11}S$: C, 56.84; H, 5.62; N, 2.37. Found: C, 57.04; H, 6.01; N, 2.56.

2.1.4. General procedure for the synthesis of triazole derivatives (16, 17)

Compound **12** or **13** (4.00 mmol), sodium ascorbate (0.61 mmol), and $CuSO_4{\cdot}5H_2O$ (0.48 mmol) were suspended in 10 mL of *t*-BuOH/H_2O (1:1). Then 5-azido-1,2,3-trimethoxybenzene (5.00 mmol) was added after 15 min, and the mixture was stirred at 40 °C for 24 h. The reaction mixture was extracted with CH_2Cl_2, and the combined organic phases were washed with water, dried over Na_2SO_4 (anhydrous), and evaporated to dryness. The crude product was purified by column chromatography affording the triazole derivative.

2.1.4.1. (1″-(3,4,5-Trimethoxyphenyl)-triazol-4″-yl)methyl 2,3-O-cyclopentylidene-5-tosyl-β-D-ribofuranoside (16). Compound **16** was purified by column chromatography using toluene:ethyl acetate as eluent and was obtained as a white solid (3.58 mmol, 89%); mp: 49 °C, $[\alpha]_D^{20}$ –40.8 (*c* 1.0, chloroform). IR υ_{max} 2946 (υC_{sp3}–H), 1603 (υC_{sp2}–C_{sp2}), 1470 (υC_{sp2}–C_{sp2}), 1360 (υ S=O), 1183 (υ S=O), 1126 (υ C–O), 976 (υ S–C–O), 820 (δ C–H) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 8.13 (s, 1H, H-5″ of triazolyl group), 7.75 (d, 2H, *J* 8.1 Hz, tosyl protons), 7.33 (d, 2H, *J* 8.1 Hz, tosyl protons), 7.02 (s, 2H, trimethoxyphenyl protons), 5.24 (s, 1H, H-1), 4.86 (d, 1H, $J_{1'b,1'a}$ 11.9 Hz, H-1′b), 4.66 (d, 1H, $J_{1'a,1'b}$ 11.9 Hz, H-1′a), 4.58 (d, 1H, $J_{2,3}$ 6.1 Hz, H-2), 4.54 (d, 1H, $J_{3,2}$ 6.1 Hz, $J_{3,4}$ 0.7 Hz, H-3), 4.38 (dt, 1H, $J_{4,5}$ 6.6 Hz, $J_{4,3}$ 0.7 Hz, H-4), 4.12 (dd, 1H, $J_{5b,5a}$ 10.1 Hz, $J_{5b,4}$ 6.3 Hz, H-5b), 4.05 (dd, 1H, $J_{5a,5b}$ 9.7 Hz, $J_{5a,4}$ 6.7 Hz, H-5a), 3.92 (s, 6H, OCH_3), 3.88 (s, 6H, OCH_3), 2.43 (s, 3H, CH_3), 1.92-1.65 (m, 8H, cyclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 154.0, 138.3, 133.0, 98.4 (trimethoxyphenyl carbons), 145.5, 132.5, 130.2, 128.0 (tosyl carbons), 144.5 (C-4″ triazolyl ring), 122.6 (quaternary carbon of cyclopentylidene ring), 121.8 (C-5″ triazolyl ring), 107.5 (C-1), 85.0 (C-2), 83.8 (C-4), 81.0 (C-3), 69.7 (C-5), 61.2 (OCH_3), 60.7 (C-1′), 56.6 (OCH_3), 35.9, 35.8, 23.7, 23.2 (cyclopentylidene carbons), 21.8 (CH_3). EIMS *m/z* 617 $[M]^{+\bullet}$ (9), 589 (2), 220 (40), 186 (24), 172 (25), 91 (100). Anal. Calcd for $C_{29}H_{35}N_3O_{10}S$: C, 56.39; H, 5.71; N, 6.80. Found: C, 56.79; H, 6.06; N, 6.98.

2.1.4.2. (1″-(3,4,5-Trimethoxyphenyl)-triazol-4″-yl)methyl 2,3-O-isopropylidene-5-tosyl-β-D-ribofuranoside (17). Compound **17** was purified by column chromatography using cyclohexane:acetone as eluent and was obtained as a white solid (3.68 mmol, 92%); mp: 40–41 °C, $[\alpha]_D^{20}$ –38.7 (*c* 1.0, chloroform). IR υ_{max} 2942 (υ C_{sp3}-H), 1603 (υ C_{sp2}–C_{sp2}), 1467 (υ C_{sp2}–C_{sp2}), 1365 (υ S=O), 1178 (υ S=O), 1124 (υ C–O), 978 (υ S–C–O), 818 (δ C–H) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 8.13 (s, 1H, H-5″ of triazolyl group), 7.74 (d, 2H, *J* 8.4 Hz, tosyl protons), 7.32 (d, 2H, *J* 8.1 Hz, tosyl protons), 7.01 (s, 2H, trimethoxyphenyl protons), 5.22 (s, 1H, H-1), 4.85 (d, 1H, $J_{1'b,1'a}$ 11.9 Hz, H-1′b), 4.65 (d, 1H, $J_{1'a,1'b}$ 11.7 Hz, H-1′a), 4.64 (d, 1H, $J_{2,3}$ 6.1 Hz, H-2), 4.59 (dd, 1H, $J_{3,2}$ 6.1 Hz, $J_{3,4}$ 0.9 Hz, H-3), 4.35 (dt, 1H, $J_{4,5}$ 6.6 Hz, $J_{4,3}$ 0.7 Hz, H-4), 4.11 (dd, 1H, $J_{5b,5a}$ 9.9 Hz, $J_{5b,4}$ 6.6 Hz, H-5b), 4.04 (dd, 1H, $J_{5a,5b}$ 10.1 Hz, $J_{5a,4}$ 6.8 Hz, H-5a), 3.91 (s, 6H, OCH_3), 3.87 (s, 3H, OCH_3), 2.42 (s, 3H, CH_3 of tosyl group), 1.44 (s, 3H, CH_3 of isopropylidene group), 1.26 (s, 3H, CH_3 of isopropylidene group); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 154.0, 138.3, 133.0, 98.4 (trimethoxyphenyl carbons), 145.5, 132.5, 130.2, 128.0 (tosyl carbons), 144.5 (C-4″ triazolyl ring), 121.7 (C-5″ triazolyl ring), 113.0 (quaternary carbon of isopropylidene group), 107.7 (C-1), 85.2 (C-4), 84.1 (C-2), 81.2 (C-3), 69.7 (C-5), 61.2 (OCH_3), 60.7 (C-1′), 56.6 (OCH_3), 26.5, 25.0 (CH_3 of isopropylidene group), 21.8 (CH_3 of tosyl group). EIMS *m/z* 591 $[M]^{+\bullet}$ (2), 576 (4), 563 (6), 548 (3), 220 (67), 172 (53), 91 (100). Anal. Calcd for $C_{27}H_{33}N_3O_{10}S$: C, 54.81; H, 5.62; N, 7.10. Found: C, 54.53; H, 5.93; N, 6.98.

2.1.5. General procedure for the synthesis of dihetherocyclic derivatives (18, 19, 20, 21)

To a solution of sodium ethoxide in ethanol, prepared adding metallic sodium (approximately 30 mg, 1.30 mmol) to ethanol (15 mL), was added 1,2,4-triazolyl-5-thiol (120.4 mg, 1.19 mmol). The reaction mixture was heated at 50 °C during 10 minutes and then the solvent was evaporated at reduced pressure. The resulting solid was added to a solution of compound **14**, **15**, **16** or **17** (0.39 mmol) in DMF (2 mL) and heated overnight under argon atmosphere at 50 °C. The solution was evaporated and the residue was dissolved in dichloromethane and washed with brine, dried with sodium sulfate, filtered and evaporated. The crude product was purified by flash column chromatography (cyclohexane:acetone).

2.1.5.1. (3″-(3,4,5-Trimethoxyphenyl)-isoxazol-5″-yl)methyl 5-deoxy-5-S-(1,2,4-triazol-3-yl)-2,3-O-cyclopentylidene-β-D-ribofuranoside (18). Compound **18** was obtained as a crystalline solid (0.27 mmol, 70%); mp: 141–142 °C, $[\alpha]_D^{20}$ –42.2 (*c* 1.0, chloroform). IR υ_{max} 3281 (υ N–H), 2938 (υ C_{sp3}–H), 1587 (υ C_{sp2}–C_{sp2}), 1467 (υ C_{sp2}–C_{sp2}), 1125 (υ C–O) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 8.20 (s, 1H, H-3 of triazolyl group), 6.99 (s, 2H, trimethoxyphenyl protons), 6.55 (s, 1H, H-4″ isoxazolyl ring), 5.22 (s, 1H, H-1), 4.83 (d, 1H, $J_{1'b,1'a}$ 13.2 Hz, H-1'b), 4.71 (d, 1H, $J_{3,2}$ 6.6 Hz, H-3), 4.67 (d, 1H, $J_{2,3}$ 5.9 Hz, H-2), 4.66 (d, 1H, $J_{1'a,1'b}$ 13.6 Hz, H-1'a), 4.53 (t, 1H, $J_{4,5}$ 7.7 Hz, H-4), 3.90 (s, 6H, OCH_3), 3.87 (s, 3H, OCH_3), 3.42 (dd, 1H, $J_{5b,5a}$ 13.6 Hz, $J_{5b,4}$ 7.3 Hz, H-5b), 3.28 (dd, 1H, $J_{5a,5b}$ 13.6 Hz, $J_{5a,4}$ 8.4 Hz, H-5a), 1.90-1.63 (m, 8H, cyclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.7 (C-5″ isoxazolyl ring), 162.4 (C-3″ isoxazolyl ring), 153.6, 139.7, 124.3, 104.2 (trimethoxyphenyl carbons), 146.5 (C-5 triazolyl ring), 122.4 (quaternary carbon of cyclopentylidene ring), 107.9 (C-1), 101.9 (C-4″ isoxazolyl ring), 85.6 (C-4), 85.2 (C-2), 83.1 (C-3), 61.1 (OCH_3), 59.8 (C-1′), 56.4 (OCH_3), 35.9, 35.8, 23.7, 23.2 (cyclopentylidene carbons), 35.7 (C-5). EIMS *m/z* 546 $[M]^{+\bullet}$ (2), 281 (18), 252 (100), 209 (41), 194 (28), 101 (15). Anal. Calcd for $C_{25}H_{30}N_4O_8S$: C, 54.93; H, 5.53; N, 10.25. Found: C, 55.33; H, 5.86; N, 10.07.

2.1.5.2. (3″-(3,4,5-Trimethoxyphenyl)-isoxazol-5″-yl)methyl 5-deoxy-5-S-(1,2,4-triazol-3-yl)-2,3-O-isopropylidene-β-D-ribofuranoside (19). Compound **19** was obtained as a crystalline solid (0.26 mmol, 67%); mp: 127–128 °C, $[\alpha]_D^{20}$ –40.8 (*c* 0.7, chloroform). IR υ_{max} 3282 (υ N–H), 2934 (υ C_{sp3}–H), 1591 (υ C_{sp2}–C_{sp2}), 1468 (υ C_{sp2}–C_{sp2}), 1126 (υ C–O) cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ: 8.16 (s, 1H, H-3 of triazolyl group), 7.00 (s, 2H, trimethoxyphenyl protons), 6.56 (s, 1H, H-4″ isoxazolyl ring), 5.22 (s, 1H, H-1), 4.85 (d, 1H, $J_{1'b,1'a}$ 13.4 Hz, H-1′b), 4.79 (d, 1H, $J_{3,2}$ 6.2 Hz, H-3), 4.73 (d, 1H, $J_{2,3}$ 6.0 Hz, H-2), 4.67 (d, 1H, $J_{1'a,1'b}$ 13.4 Hz, H-1′a), 4.52 (t, 1H, $J_{4,5}$ 7.7 Hz, H-4), 3.91 (s, 6H, OCH_3), 3.88 (s, 3H, OCH_3), 3.43 (dd, 1H, $J_{5b,5a}$ 13.5 Hz, $J_{5b,4}$ 7.2 Hz, H-5b), 3.28 (dd, 1H, $J_{5a,5b}$ 13.7 Hz, $J_{5a,4}$ 8.2 Hz, H-5a), 1.45 (s, 3H, CH_3), 1.30 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 168.7 (C-5″ isoxazolyl ring), 162.4 (C-3″ isoxazolyl ring), 153.7, 139.8, 124.3, 104.3 (trimethoxyphenyl carbons), 146.7 (C-5 triazolyl ring), 112.9 (quaternary carbon of isopropylidene group), 108.2 (C-1), 101.9 (C-4″ isoxazolyl ring), 85.9 (C-4), 85.5 (C-2), 83.3 (C-3), 61.1 (OCH_3), 59.9 (C-1′), 56.5 (OCH_3), 35.7 (C-5), 26.5, 25.0 (CH_3). EIMS *m/z* 520 $[M]^{+\bullet}$ (100), 505 (14), 293 (73), 256 (39), 248 (80), 101 (42). Anal. Calcd for $C_{23}H_{28}N_4O_8S$: C, 53.07; H, 5.42; N, 10.76. Found: C, 53.29; H, 5.74, N, 10.36.

2.1.5.3. (1″-(3,4,5-Trimethoxyphenyl)-triazol-4″-yl)methyl 5-deoxy-5-S-(1,2,4-triazol-3-yl)-2,3-O-cyclopentylidene-β-D-ribofuranoside (20). Compound **20** was obtained as a crystalline solid (0.24 mmol, 61%); mp: 145–147 °C, $[\alpha]_D^{20}$ –28.8 (*c* 1.0, chloroform). IR υ_{max} 3279 (υ N–H), 2930 (υ C_{sp3}–H), 1603 (υ C_{sp2}–C_{sp2}), 1468 (υ C_{sp2}–C_{sp2}), 1120 (υ C–O) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.09 (s, 1H, triazolyl proton), 8.02 (s, 1H, triazolyl proton), 6.95 (s, 2H, trimethoxyphenyl protons), 5.28 (s, 1H, H-1), 5.07 (d, 1H, $J_{1'b,1'a}$ 11.9 Hz, H-1′b), 4.71 (d, 1H, $J_{1'a,1'b}$ 11.9 Hz, H-1′a), 4.68 (d, 1H, $J_{3,2}$ 6.3 Hz, H-3), 4.65 (d, 1H, $J_{2,3}$ 6.0 Hz, H-2), 4.53 (t, 1H, $J_{4,5}$ 7.6 Hz, H-4), 3.93 (s, 6H, OCH_3), 3.90 (s, 3H, OCH_3), 3.48 (dd, 1H, $J_{5b,5a}$ 12.9 Hz, $J_{5b,4}$ 8.2 Hz, H-5b), 3.36 (dd, 1H, $J_{5a,5b}$ 13.0 Hz, $J_{5a,4}$ 7.2 Hz, H-5a), 1.93–1.66 (m, 8H, cyclopentylidene protons); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 154.0, 138.5, 132.8, 98.8 (trimethoxyphenyl carbons), 147.4 (C-5 1,2,4-triazolyl rings), 144.4 (C-4″ 1,2,3-triazolyl ring), 122.4 (quaternary carbon of cyclopentylidene ring), 121.9 (C-5″ 1,2,3-triazolyl ring), 107.7 (C-1), 85.4 (C-4), 85.2 (C-2), 83.2 (C-3), 61.2 (OCH_3), 60.4 (C-1′), 56.6 (OCH_3), 36.3 (C-5), 35.9, 35.8, 23.7, 23.2 (cyclopentylidene carbons). EIMS *m/z* 445 (4), 220 (100), 189 (22), 101 (26). Anal. Calcd for $C_{24}H_{30}N_6O_7S$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.79; H, 5.70; N, 14.98.

2.1.5.4. (1″-(3,4,5-Trimethoxyphenyl)-triazol-4″-yl)methyl 5-deoxy-5-S-(1,2,4-triazol-3-yl)-2,3-O-isopropylidene-β-D-ribofuranoside (21). Compound **21** was obtained as a crystalline solid (0.25 mmol, 63%); mp: 159–161 °C, $[\alpha]_D^{20}$ –25.5 (*c* 1.0, chloroform). IR υ_{max} 3290 (υ N–H), 2940 (υ C_{sp3}–H), 1603 (υ C_{sp2}–C_{sp2}), 1468 (υ C_{sp2}–C_{sp2}), 1128 (υ C–O) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.07 (s, 1H, triazolyl proton), 8.02 (s, 1H, triazolyl proton), 6.95 (s, 2H, trimethoxyphenyl protons), 5.28 (s, 1H, H-1), 5.09 (d, 1H, $J_{1'b,1'a}$ 11.8 Hz, H-1′b), 4.74 (d, 1H, $J_{3,2}$ 6.0 Hz, H-3), 4.72 (d, 1H, $J_{2,3}$ 6.9 Hz, H-2), 4.70 (d, 1H, $J_{1'a,1'b}$ 12.0 Hz, H-1′a), 4.51 (t, 1H, $J_{4,5}$ 7.6 Hz, H-4), 3.94 (s, 6H, OCH_3), 3.90 (s, 3H, OCH_3), 3.48 (dd, 1H, $J_{5b,5a}$ 12.9 Hz, $J_{5b,4}$ 8.2 Hz, H-5b), 3.35 (dd, 1H, $J_{5a,5b}$ 12.9 Hz, $J_{5a,4}$ 7.1 Hz, H-5a), 1.48 (s, 3H, CH_3), 1.31 (s, 3H, CH_3); ^{13}C NMR (50 MHz, $CDCl_3$) δ: 154.0, 138.6, 132.8, 98.9 (trimethoxyphenyl carbon), 147.6 (C-5 1,2,4-triazolyl rings), 144.5 (C-4″ 1,2,3-triazolyl ring), 121.9 (C-5″ 1,2,3-triazolyl ring), 112.8 (quaternary carbon of isopropylidene ring), 108.0 (C-1), 85.8 (C-4), 85.5 (C-2), 83.5 (C-3), 61.2 (OCH_3), 60.5 (C-1′), 56.6 (OCH_3), 36.5 (C-5), 26.5, 25.0 (CH_3). EIMS *m/z* 520 $[M]^{+\bullet}$ (2), 505 (10), 270 (24), 256 (45), 237 (65), 220 (100), 101 (39). Anal. Calcd for $C_{22}H_{28}N_6O_7S$: C, 50.08; H, 5.64; N, 15.03. Found: C, 50.25; H, 5.90; N, 15.22.

2.2. Pharmacological studies

2.2.1. Cell culture, treatments, reagents and antibodies

PC3 cells were obtained from the American Type Culture Collection (Manassas, VA) and were routinely cultured in RPMI

1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS).

For treatments, cells were incubated 24 h in RPMI media containing 10% FBS and then were exposed to the different compounds as specified in each case for 24 h.

Polyclonal and monoclonal anti-CycB1, anti-CDK4, anti-CDK2 and anti-β-Actin antibodies were from Cell Signaling, Technology (Beverly, MA). Anti-mouse and anti-rabbit secondary antibodies conjugated with HRP were from Amersham Ltd (UK).

2.2.2. Analyzed compounds

The compounds analyzed were **1**, **6**, **8**, **18**-**21**. All compounds were dissolved in DMSO.

2.2.3. Cell viability

Cell viability was assayed by MTS (Cell Titer 96 wells aqueous non-radioactive Cell proliferation assay, Promega) following the manufacture instructions. Each sample was done in triplicate in five independent experiments.

2.2.4. Cell cycle analysis

PC3 stable cell lines were treated with the heterocyclic D-riboside derivatives stained with propidium iodide (PI) and analyzed by FACS. After 24 h exposure of the PC3 cell line to the compounds, cells were harvested by trypsinization and gently pelleted by centrifugation at 3000 rpm for 3 min. Cells were resuspended in cold phosphate-buffered saline (PBS) and centrifuged again (3000 rpm for 3 min). Pellets were transferred dropwise to 1 mL of 70% (v/v) ethanol, allowed to fix for 2 h at 4 °C and kept on ice. The ethanol-suspended cells were collected, washed and resuspended in 1 mL PBS containing DNase-free RNase A (0.2 mg/mL), Triton X-100 (0.1% v/v), and propidium iodide (PI) (0.02 mg/mL). Each sample was then incubated at 37 °C for 15 min before cell cycle analysis with a BD flow cytometer and FlowJo 7.6.2 software.

2.2.5. Immunoblotting

Samples were incubated on ice for 20 min and then centrifuged at 12,000 rpm in a microcentrifuge for 3 min and the supernatant collected. Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit from SIGMA. Samples were then resolved by SDS–PAGE, transferred to a nitrocellulose membrane (Invitrogen). Membranes were blocked for 1 h in TBS-T (0.1% Tween-20 in 10 mM Tris–HCl, pH 7.4) with 5% (w/v) non-fat milk, and then incubated with the specific primary antibodies: CycB1 (1:500), CDK4 (1:500), CDK2 (1:250) or β-actin (1:5000) ON at 4 °C. After washes with TBS-T incubations with the appropriate secondary antibodies were performed. Specific protein bands were detected using ECL reagents (Amersham Ltd).

2.2.6. Statistical analysis

All results are given as mean ± S.E.M of "*n*" separate independent experiments unless stated otherwise. Student's *t*-tests were used to ascertain statistical significance with a threshold of $P < 0.05$.

3. Results and discussion

3.1. Chemistry

The new series of heterocyclic derivatives were synthesized as shown in Scheme 1. To obtain compounds **10**, **11**, the first step was carried out from D-ribose, propargyl alcohol and acetone or cyclopentanone using a modified methodology described by Busscher et al. [18]. Reaction of propargyl derivatives (**10**, **11**) with tosyl chloride in pyridine produced compounds **12**, **13**.

Then, isoxazole derivatives (**14**, **15**) were obtained with moderate yields by a 1,3-dipolar cycloaddition reaction using compound **12** or **13** as dipolarophile and 3,4,5-trimethoxyphenyl benzaldoxime as a dipole precursor [19]. On the other hand, compound **12** or **13** reacted with 3,4,5-trimethoxyphenylazide to obtain 1,2,3-triazole derivatives (**16** or **17**) via a "click reaction".

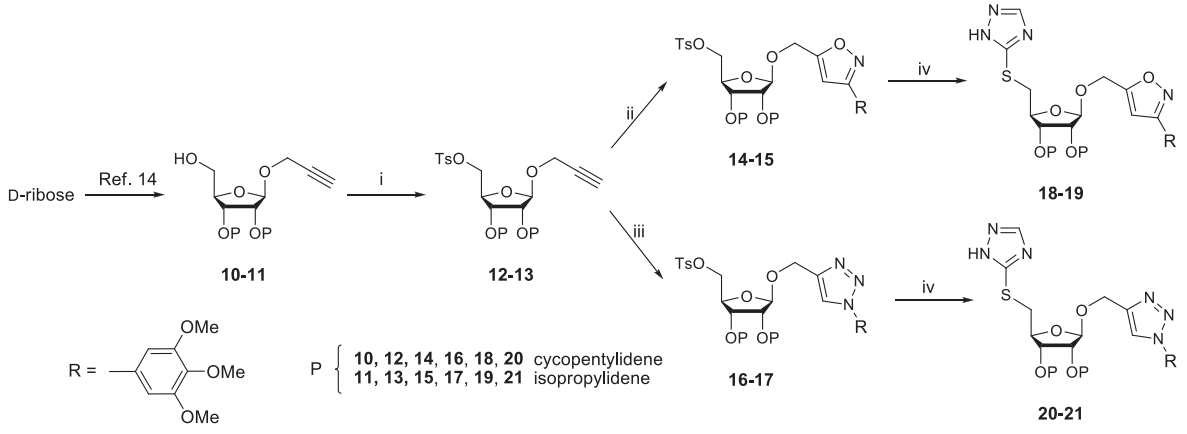
The diheterocyclic derivatives **18**-**21** were prepared from monoheterocyclic derivatives (**14**-**17**) by a nucleophilic displacement of tosyl group using sodium 1,2,4-triazolyl-5-thiolate.

All compounds were obtained in high to moderate yields and were totally characterized by physical and spectroscopic techniques as described in Section 2.

3.2. Cell viability assays

We assessed the pharmacologic effect of compounds **1**, **6**, **8**, **18**–**21** on cell growth and cell cycle progression in the prostate cancer cell line, PC3. Cells were treated with different concentrations of synthesized compounds (1, 2, 10, 50, 100, 200 and 400 μM) and incubated during 24 h. The inhibitory effect on cell growth and ClogP values are shown in Table 1 and Fig. 2.

While compounds **8**, **19** and **21** exerted almost no variation on PC3 cell growth (Fig. 2B), the exposure of prostate cancer cells to compounds **1**, **6**, **18** and **20** had an inhibitory effect on cell viability (Fig. 2A). It is remarkable that these compounds had a differential effect at different concentrations. Compound **1** produced 45.34% ($P < 0.05$) of inhibition at 400 μM; compound **6** produced 59.05% ($P < 0.05$) of inhibition at 200 μM; compound **18** produced 34.14%



Scheme 1. Reagents and conditions: (i) TsCl, pyridine; (ii) RHC═NOH, chloramine-T, $EtOH/H_2O$, 3 h; (iii) RN_3, sodium ascorbate, $CuSO_4{\cdot}5H_2O$, t-$BuOH/H_2O$, 40 °C, 24 h; (iv) triazolyl sodium tiolate, DMF, 50 °C, 18 h.

Table 1
Inhibitory effect of compounds **1**, **6**, **8**, **18**–**21** on PC3 cells viability.

	Cell viability								
	Concentration (uM)								
Compound	1	2	10	50	100	200	400	IC_{50}	ClogP
1	91.65 ± 7	84.93 ± 2.38	87.29 ± 2.29	77.1 ± 7.28	86.38 ± 2.07	72.55 ± 2.85	54.66 ± 3.19	400	0.78
6	100.16 ± 17.62	93.44 ± 4.2	85.8 ± 4.47	75.61 ± 3.32	75.48 ± 7.84	40.95 ± 5.63	13.77 ± 2.05	200	1.6
8	90.86 ± 4.89	97.12 ± 7.38	89.4 ± 5.54	83.17 ± 2.09	94.18 ± 4.06	83.41 ± 1.16	84.87 ± 3.61	> 400	−0.13
18	108.98 ± 6.36	113.73 ± 7.81	65.86 ± 3.40	67.39 ± 2.91	82.80 ± 5.24	102.43 ± 4.03	124.84 ± 7.89	NA	1.22
19	116.35 ± 5.45	103.73 ± 5.94	134.46 ± 13.61	114.54 ± 2.65	133.01 ± 11.16	101.36 ± 7.51	111.95 ± 2.08	> 400	1.09
20	85.64 ± 2.49	89.85 ± 2.01	80.84 ± 3.16	71.68 ± 1.46	59.71 ± 3.72	49.83 ± 6.21	43.24 ± 1.16	200	0.87
21	79.06 ± 4.62	89.85 ± 2.01	110.82 ± 4.73	111.52 ± 4.18	94.46 ± 4.94	98.08 ± 7.04	98.28 ± 4.32	> 400	0.84

Cell viability results were expressed as percentage (%) respect to the viability of untreated cells considered as 100%. Compound concentrations are expressed in mM. ClogP calculated using ChemBio3D Ultra 11.0. $^{*}P < 0.05$ significantly differences respect to basal accordingly to ANOVA + Dunnett's test. n/d: not determined.

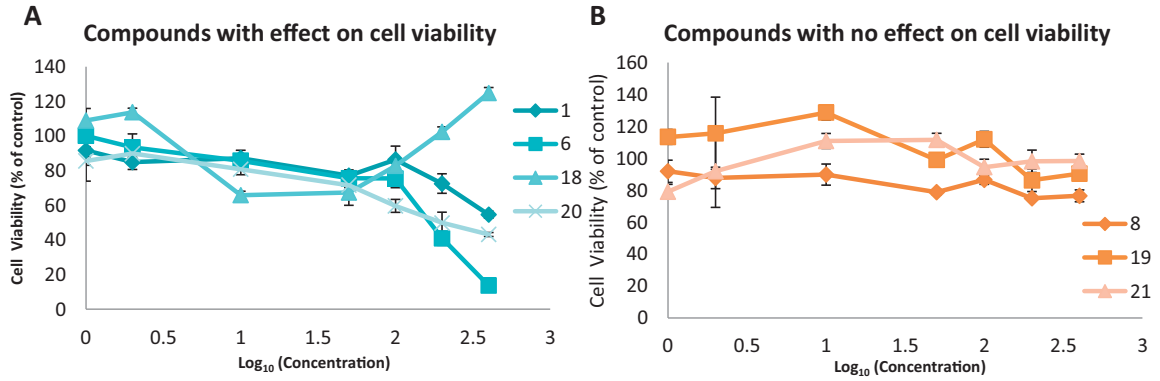


Fig. 2. Effect of compounds **1**, **6**, **8**, **18**–**21** on PC3 cells viability. Cell cultures were exposed to different concentrations (1–400 μM) for 24 h. Cell viability was determined by MTS and expressed as percentage of untreated control cells (vehicle) considered as 100%. All data are mean ± SD. *Significant difference, $P < 0.05$.

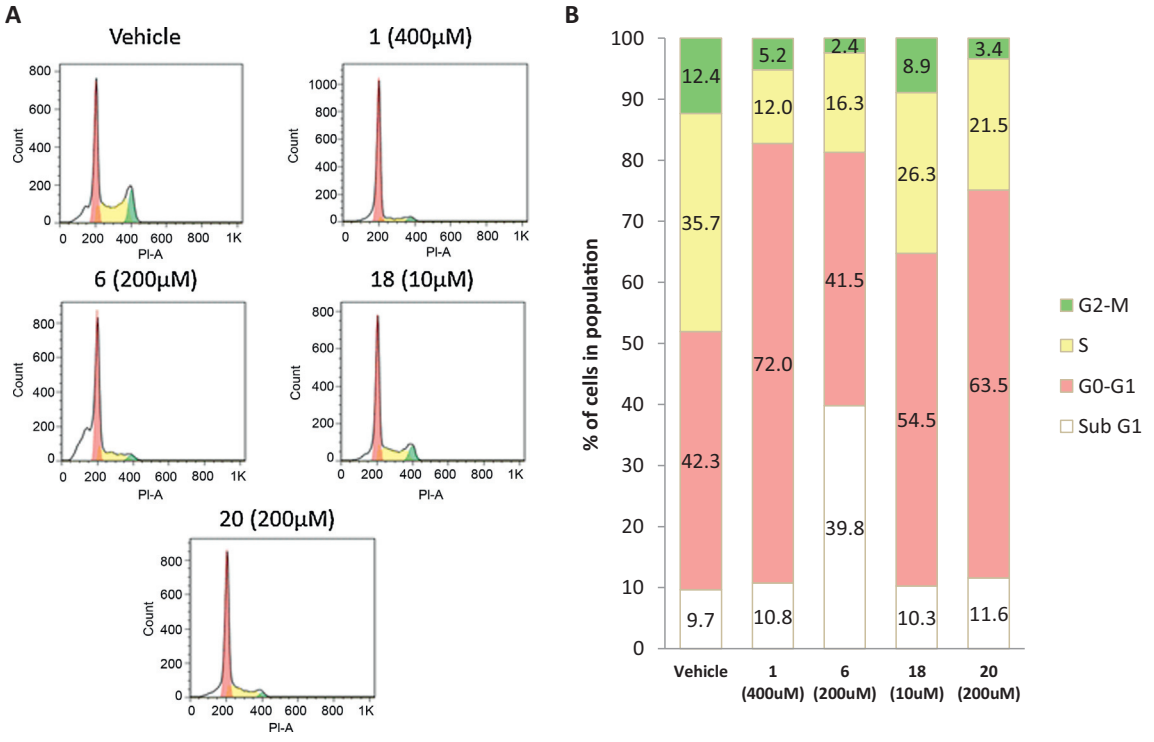


Fig. 3. Effect of compounds **1**, **6**, **18** and **20** on cell cycle progression in PC3 cells. Cells were treated with **1** (400 μM), **6** (200 μM), **18** (10 μM) and **20** (200 μM) for 24 h. After treatment, cells were collected, washed with PBS, digested with RNase, and then cellular DNA was stained with propidium iodide as detailed in Methods. Flow cytometric analysis was then performed for cell cycle distribution. A. Propidium iodide fluorescence pattern for cell cycle distribution with different treatments as specified. B. The percentage of cells in different phases of cell cycle was determined by FlowJo 7.6.2 cell cycle analysis software. The data represent a mean of five independent experiments ± SD. *Significant difference, $P < 0.05$.

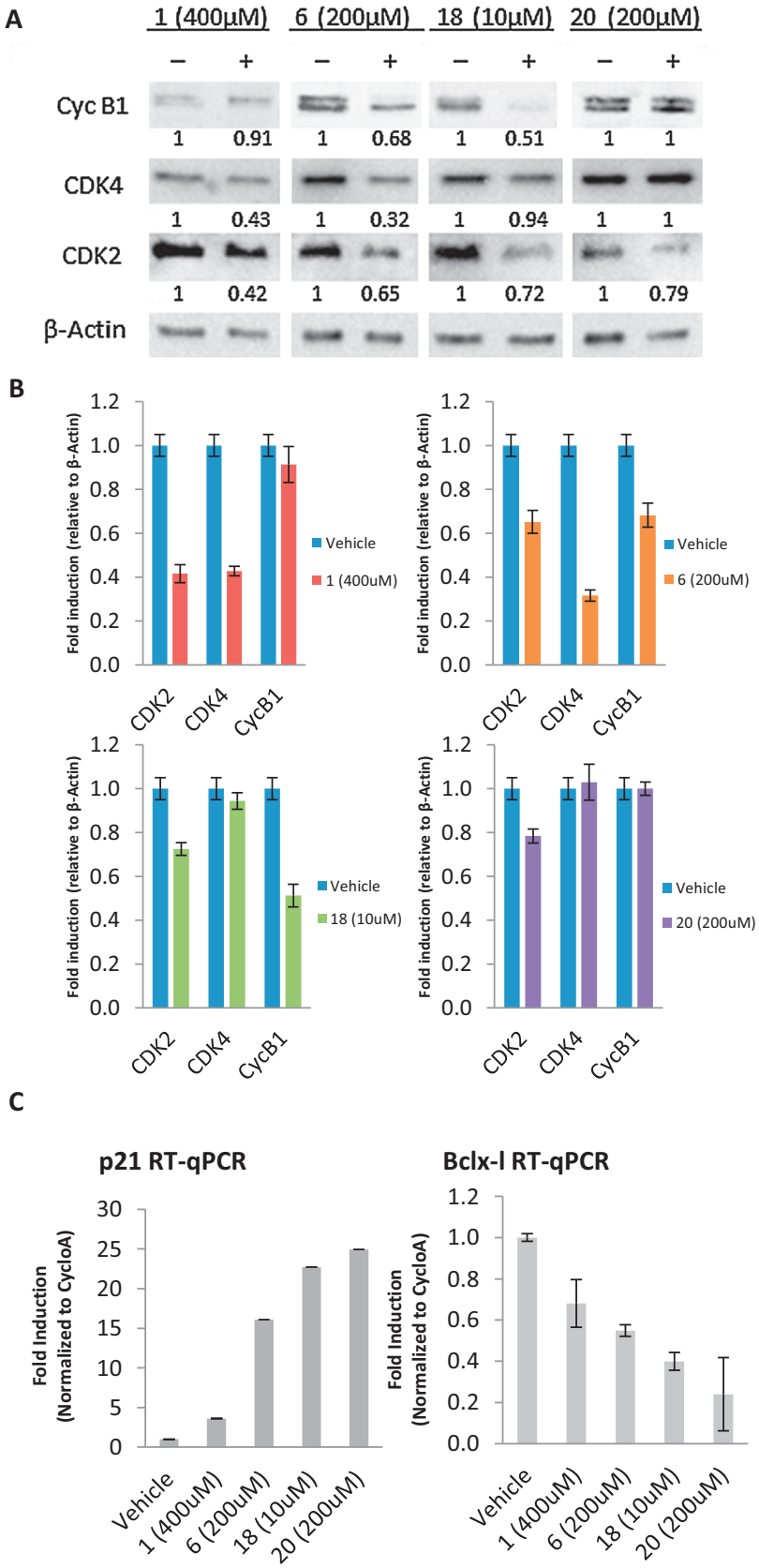


Fig. 4. Effect of Compounds **1**, **6**, **18** and **20** on the expression of regulators of cell cycle progression in PC3 cells. Cells were treated with **1** (400 μM), **6** (200 μM), **18** (10 μM) and **20** (200 μM) for 24 h. Western blot analysis showing differential expression of CycB1, CDK4, CDK2 and p21 β-Actin levels are shown to control for equal loading. Numbers under bands indicates quantitation (ImageJ software).

($P < 0.05$) of inhibition at 10 μM and compound **20** produced 50.17% ($P < 0.05$) of inhibition at 200 μM. It is note worthy to mention that the compounds with cyclopentylidene as protected groups, exhibited higher inhibitory effect than their isopropylidene analogues with lower ClogP values.

Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth and survival [20]. To analyze the effect of compounds **1**, **6**, **18** and **20** on cell cycle progression, we performed the cell cycle analysis using fluorescence-activated cell sorter analysis (FACS). Treatment of PC3 cells with **1**, **18** and **20** for 24 h produced an arrest of these cells in G_0/G_1 phase (72.0%, 54.5%, 63.5%, respectively; $P < 0.05$) (Fig. 3 A and B). An increase in G_0/G_1 cell population was mostly at the expense of the S phase cells with a minimal decrease in G_2/M cell population under compound **18** treatment, meanwhile exposure of cells to compounds **1** or **20** produced an increase in G_0/G_1 population, mostly at the expense of the S and G_2/M cell population (Fig. 3 A and B). Interestingly, when cells were treated with compound **6** a subG_1 peak was observed, indicating apoptosis (Fig. 3 A and B).

Throughout the different phases of the cell cycle progression, regulatory molecules, among them cyclins and cyclin-dependent kinases (CDK) and inhibitors, are critical in deciding cellular fate. Hence, our next step was to evaluate whether the alterations observed on cell cycle progression correlated with variations on the expression of Cyclin B1 (Cyc B1), CDK4 and CDK2 (Fig. 4 A and B). The expression of CycB1, CDK4 and CDK2 was diminished in PC3 cells treated with compounds **1** (400 μM), **6** (200 μM) and **18** (10 μM) for 24 h. Although compound **20** (200 μM) produced downregulation of CycB1 and CDK2, no alteration in CDK4 levels was detected. Accordingly, reverse transcription-quantitative PCR (RT-qPCR) revealed a significant induction of p21 mRNA in PC3 cell line, when cells were exposed to the same treatments (3.6-, 16.1-, 22.7-, 24.9-fold induction for **1**, **6**, **18** and **20** respectively; $P < 0.05$) (Fig. 4C), further indicating cellular arrest. Interestingly, mRNA levels of the B-cell lymphoma-extra large (Bcl-xl) isoform, member of the Bcl-2 family of proteins, were significantly reduced (32%, 45%, 60%, 76% for **1**, **6**, **18** and **20**, respectively; $P < 0.05$) (Fig. 4C).

Bcl-xl acts as a pro-survival protein, preventing the release of mitochondrial contents, leading to caspase activation. It is a well-established concept in the field of apoptosis that the cross talk of pro- and anti-survival Bcl-2 family of proteins, define whether the cell will undergo cell death.

Altogether these results show that exposure of prostate cancer cells to 5-deoxy-5-*S*-(1,2,4-triazol-3-yl)-2,3-*O*-cyclopentylidene-β-D-ribofuranoside derivatives correlates with differential alteration of the cell cycle and suggests that inhibition of deregulated cell cycle progression by these compounds could be one of the molecular events associated with selective anticancer efficacy of these D-riboside derivatives in prostate carcinogenesis.

4. Conclusions

In the present study, we showcase the synthesis and the physical and spectrocopical characterization of novel diheterocyclic D-ribofuranoside derivatives. These compounds together with some derivatives previous described [1] were evaluated for their biological actions in the prostate cancer PC3 cell line. Four of these compounds exhibited inhibitory activity, with IC_{50} values in the micromolar range, induced G_0/G_1 cell cycle arrest and apoptosis. Cyclopentylidene derivatives were more active than isopropylidene analogs, confirming that lipophilicity is a key parameter in the activity of such compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2014.08.010.

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