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INHIBITION OF *TAQ* DNA POLYMERASE BY IRIDOID AGLYCONE DERIVATES

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Abstract

Faithful replication of DNA molecules by DNA polymerases is essential for genome integrity and correct transmission of genetic information in all living organisms. DNA polymerases have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. Herein we report additional synthesis of simplified bicyclic aglycones of iridoids and their biological activity against *Taq* DNA polymerase with the object to find out some of the likely molecular targets implicated in the biological activity showed for this kind of compounds. The compounds 14, 33 and 34 showed inhibitory activity against *Taq* DNA polymerase with IC_{50} values of 13.47, 17.65 and 18.31 µM, respectively. These results would allow proposing to DNA polymerases as the molecular targets implicated in this bioactivity and enhance the iridoid aglycones as leader molecule to develop new drugs for cancer therapy.

Key words: Iridoid aglycones, DNA polymerases, natural products, biological activity.

Article information

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INTRODUCTION

DNA polymerases are enzymes that play a crucial role in DNA metabolism such as replication, transcription, recombination, and chromosome segregation during mitosis. For this motive, it has long been accepted that these enzymes are valuable targets for the development of cancer chemotherapeutic agents. Several inhibitors have been introduced into clinical trials including dideoxynucleotides, phospholipids, fatty acids, flavonoids, iridoids, triterpenoids, camptothecines, anthacyclines, aminoacridines and ellipticines (6,7,10,11). However, more effective agents are still needed.

DNA polymerases from different organisms share common features: all of them have three different activities located on different domains. Bacterial DNA polymerase I enzymes are characterized by multidomain structures, which contain a 5'-3' exonuclease domain, a 3'-5' exonuclease domain and a polymerase domain. According to Astatke *et al.* (3), the last domain has a structure reminiscent of a right hand, with a large cleft formed by the fingers, thumb and palm domains. Comparison of the structures of DNA polymerases from different organisms shows that the hand-shape architecture is a common feature of the polymerase domain among all known polymerases. There is significant evidence that the region covering the junction between the palm and fingers subdomains as being of primary importance in binding an incoming nucleotide. The importance of these domains is also shown by amino acid sequence conservation (2-4).

Iridoid (cyclopentane-[c]-pyranomonoterpenoids) glycosides occur in about 57 families of plants and form a collection with almost 2500 structures with important role in chemotaxonomy. Several biological activities for this kind of compounds have been reported, as well as for iridoid containing plants. Catalpol (1), harpagide (2) and aucubin (3) (Fig. 1) have been isolated from *Scrophularia nodosa* L., a plant used in folk medicine for cancer healing. Managing their five-membered-ring chirality as an adequate synthon, iridoids have also been used as starting materials to produce nucleoside analogues (12-14).

In preliminary studies, the iridoid catalpol (1) has shown significant inhibition of *Taq* DNA polymerase (IC_{50} = 47.80 µM) (13) *In vitro* experiments and theoretical calculations suggest that the mechanism of *Taq* DNA polymerase inhibition may occur in a competitive way with deoxynucleoside triphosphates (dNTPs) at the binding site of the enzyme (9,12). In fact, iridoids show a certain resemblance with a nucleoside framework. The bicyclic aglycone could mimic the purine scaffold present in nucleosides. In contrast to catalpol (1), the natural iridoid harpagide (2) is a weak *Taq* DNA polymerase inhibitor (IC_{50} = 416.90 µM). Considering that the sugar moiety is identical for catalpol (1) and harpagide (2), the aglycone fragment seems to play a role in *Taq* DNA inhibition (13).

Furthermore, we have previously synthesized the bicyclic aglycone derivatives by means of a cyclization reaction catalyzed by L-proline. These compounds represent a simplified scaffold of the aglycone framework of naturally occurring iridoids, and their silylated derivatives showed remarkable biological activity towards human cancer cell lines, including cell cycle arrest and apoptosis induction (5).

Herein we report additional synthesis of simplified bicyclic aglycones of iridoids and their biological activity against *Taq* DNA polymerase with the object to find out some of the likely molecular targets implicated in the biological activity showed for aglycones of iridoids and derivatives in previous studies (5).

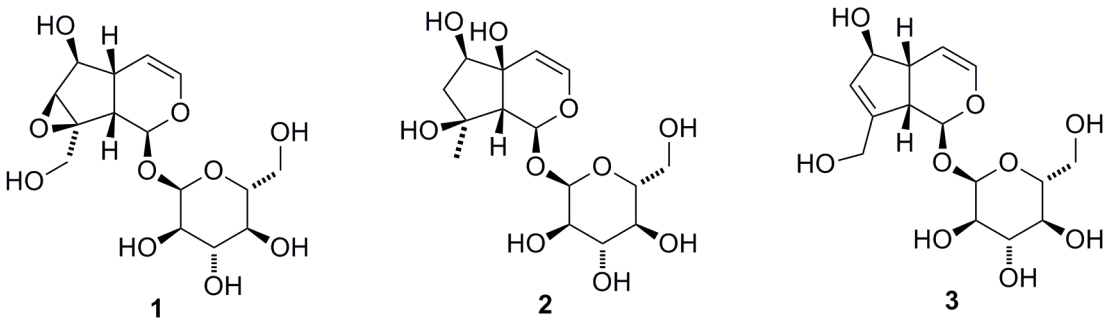

Figure 1. Structures of catalpol (1), harpagide (2) and aucubina (3).

MATERIALS AND METHODS

Chemicals and reagents

Commercial reagents for the preparation of aglycones were obtained from Fluka, Sigma-Aldrich and Merck. Reactions requiring anhydrous conditions were performed under nitrogen or argon. Dichloromethane and diethyl ether were distilled from CaH_2 and Na/benzophenone, respectively, under N_2 prior to use. Other solvents or chemicals were purified by standard techniques (1) Thin-layer chromatography was carried out on Merck and Macherey-Nagel aluminium sheets coated with silica gel 60 F_{254}. Plates were visualized by use of UV light and/or phosphomolybdic acid 20wt. % solution in ethanol with heating. Anhydrous magnesium sulphate was used for drying solutions. Chromatography in column was performed on silica gel Merck grade 9385, 60 Å.

Compounds

All compounds evaluated in this work were obtained under the conditions and using the reagents described in the figure 2, 3 and 4.

Spectroscopic data

NMR spectra were measured at 200 and 400 MHz (^{1}H NMR) and 50 and 100 MHz (^{13}C NMR) with a Bruker AC-200 and 400 instruments, and chemical shifts are reported relative to internal Me_4Si (δ= 0). All intermediates and final products gave satisfactory analytical and spectroscopic data in full according with their assigned structures. Structural characterization by NMR of key compounds: Compound 14: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.43 (d, J = 6.6 Hz, 1H, H_1), 6.38 (dd, J = 6.3, 2.4 Hz, 1H, H_3), 5.05 (dd, J = 6.3, 2.7 Hz, 1H, H_4), 3.97 (m, 1H, H_6), 2.59 (m, 1H, H_5), 2.10 (s, 3H, $OC(O)CH_3$), 2.08-1.88 (m, 3H, H_{7a}, H_8, H_9), 1.24 (m, 1H, H_{7b}), 0.94 (d, J = 6.9 Hz, 3H, H_{10}); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 169.95 ($O\mathbf{C}(O)CH_3$), 140.55 (C_3), 100.83 (C_4), 91.15 (C_1), 76.35 (C_6), 45.93 (C_9), 42.17 (C_7), 41.28 (C_5), 21.03 (C_8), 20.42 ($OC(O)\mathbf{C}H_3$), 18.86 (C_{10}). Compound 36: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.98 (s, 1H, H_3) 4.82 (d, J = 6.6 Hz, 1H, H_1), 2.42 (m, 1H, H_5), 2.05-1.60 (m, 4H, H_9, H_8, H_{6a}, H_{7a}), 1.51 (s, 3H, H_{11}), 1.40-1.10 (m, 2H, H_{6b}, H_{7b}), 1.05 (d, J = 6.7 Hz, 3H, H_{10}); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 133,88 (C_3), 113.52 (C_4), 94.16 (C_1), 50.18 (C_5), 38.57 (C_9), 35.59 (C_8), 33.04 (C_6), 31.05 (C_7), 20.36 (C_{11}), 16.03 (C_{10}).

Bioactivity

Reagents for PCR experiments were obtained from Sigma-Aldrich (dNTP mix, *Taq* recombinant *Thermus aquaticus* expressed in *Escherichia coli* D1806 and water MQ (Millie Q-). All PCRs were carried out in 20 µl reaction volumes, in a Techne TC 3000 thermal cycler. The agarose gel electrophoresis was performed in a horizontal Cuba mini gel Cole-Parmer, with a power supply Thermo LV 105. To view the gels a transilluminator UVP was used.

Polymerization Chain Reaction (PCR)

Sample stock solutions were prepared at a concentration of 10 mM in dimethyl sulfoxide (DMSO) using as final volume 1 ml. The PCR master mix consisted of 40 mM Tris-acetate, pH 8.3, containing 1 mM EDTA, 1.8 mM $MgCl_2$, 1.0 U *Taq* DNA polymerase, 1 µM each oligonucleotide primer, 0.20 mM each deoxynucleotide triphosphates (dNTP) and 0.013 µg/ml of DNA template (*p*RSETA plasmid containing the *pol β* gen). In the experiments the primers were: sense primer 5´GGATCCATGAGCGGGGACCATCTCCA and the antisense primer: 5´TCAAAACTCGTAGTCCTCATAG. 1 µl of compound solution at a concentration of 10 mM was added to the PCR master mix.

The amplification conditions consisted of 35 cycles of denaturation at 94 ºC for 30 sec followed by primers annealing at 53 ºC for 45 sec and primer extension at 72 ºC for 1 min. and 30 sec. Inhibition positive controls were performed using ddNTP set at a final concentration of 500 µM (12). Activity controls were executed without compounds solution in the master mix and solvent controls were performed without compounds but with 1 µl of DMSO solvent. After completion of reaction 2 µl of loading buffer 10x (0.25% of bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll 400 in water) was added.

The amplified DNA sequences were electrophoresed for 60 min. in 1% agarose gel in buffer TBE 1x (Tris-boric-EDTA, pH: 8) at 75-80 V using TBE running buffer 1x. Finally, gels were stained using 0.5 µg of ethidium bromide per ml. Amplified DNA bands were detected visually with UV transilluminator. Each assay was replicated three times.

Analysis of PCR products

The image of stained agarose gels was captured using digital cameras. The digitized band images were processed using the Image processing program (Scion Image, public domain program).

IC_{50} values determination

Serial dilutions (1:2) were performed in DMSO (concentration 500 µM to 7.8125 µM). The PCR conditions (master mix, program cycles, electrophoresis and staining of gels) were the same that in the screening assay. IC_{50} values were determined using Scion Image (public domain)

and GraphPad Prism 4 (demo version).

RESULTS

Chemistry

The synthesis of the compounds reported in this study began from racemic *β*-citronellol (4) and is outlined in Figure 2. Thus, benzoylation of the alcohol group led to ester 5, which was converted into aldehyde 6 by ozonolysis. Applying the proline-catalyzed formyl oxidation protocol (8) with L-proline over aldehyde 6 we obtained the corresponding *S*-oxy-aldehyde 7 with high levels (over 95%) of catalyst-enforced induction. The olefination of the resultant aldehyde 7 using Horner-Wadsworth-Emmons conditions, produced (*E*)-α,β-unsaturated ester 8. The secondary hydroxyl group of compound 8 was protected as *tert*-butyldiphenylsilyl (TBDPS) ether 9. Further reduction with DIBAL-H gave allylic alcohol 10. Dess-Martin oxidation of compound 10 produced the dialdehyde intermediate 11. Exposure of intermediate 11 to L-proline in DMSO (8) provided the lactol 12, which was subsequently, acetylated providing the bicyclic derivative 13. The stereochemistry of the ring fusion was *cis* as confirmed by nOe studies and ^{1}H-^{1}H coupling constants (5). Finally, the cleavage of the TBDPS group of 13 yielded derivative 14.

Additionally, we performed the synthesis of these aglycone starting from *β*-citronellol enantiomeric forms ((+)-*β*-citronellol and (-)-*β*-citronellol) such as it was described in García *et al*, 2010 (5) and fourteen compounds (15 - 28) have been obtained (Figure 3).

Finally, the synthesis of bicyclic aglycone from (*S*)-citronellal (29) and (*R*)-citronellal (30) was initiated and is outlined in Figure 4. In the first reaction, an oxidation with selenium dioxide and *tert*-butyl hydroperoxide was made to produce the dialdehyde intermediates (31-32). Then, the cyclization reactions using secondary amines was generated, which provided the compounds 33-34 with optimal results with regard to both the yield and stability of the products. Finally, the compounds 35-36 by hydrolysis with *p*-toluenesulfonic acid in aqueous tetrahyrofuran were obtained (5,8,16). Further, the compounds 37-38 were obtained from the dialdehyde intermediates using hydroxylamine hydrochloride and *p*-toluenesulfonic acid monohydrate.

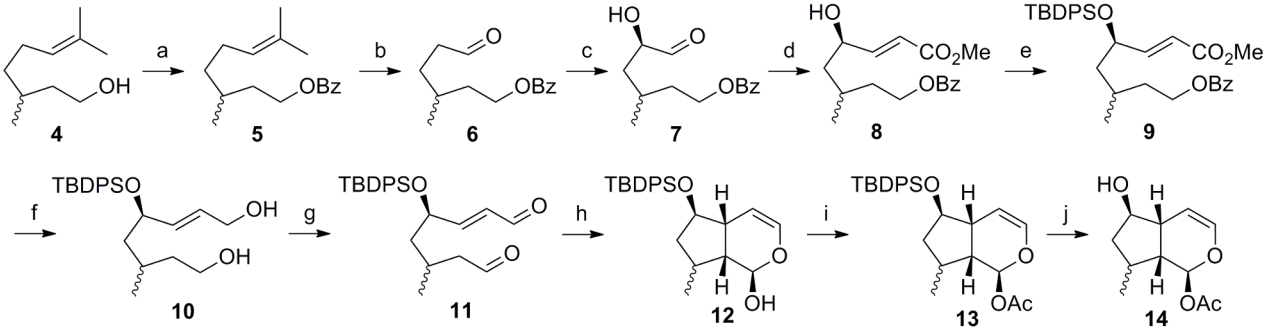

Figure 2. Reagents and conditions. (a) BzCl, Et_3N, CH_2Cl_2, 97%; (b) O_3, MeOH, CH_2Cl_2, –78 ºC, 71%; (c) *L*-proline, PhNO, DMSO, 45 min; (d) (i) $(MeO)_2POCH_2CO_2Me$, DBU, LiCl, CH_3CN, –15 ºC; (ii) NH_4Cl, MeOH, 42% yield from 3; (e) TBDPSCl, imidazole, DMF, 98%; (f) DIBAL-H, Et_2O, –78 ºC, 80%; (g) Dess-Martin, CH_2Cl_2, 1 h, 89%; (h) *L*-proline, DMSO, 40 ºC, 65 h; (i) Ac_2O, DMAP, pyridine, 48% yield from 8; (j) HF·pyridine, THF, 87%.

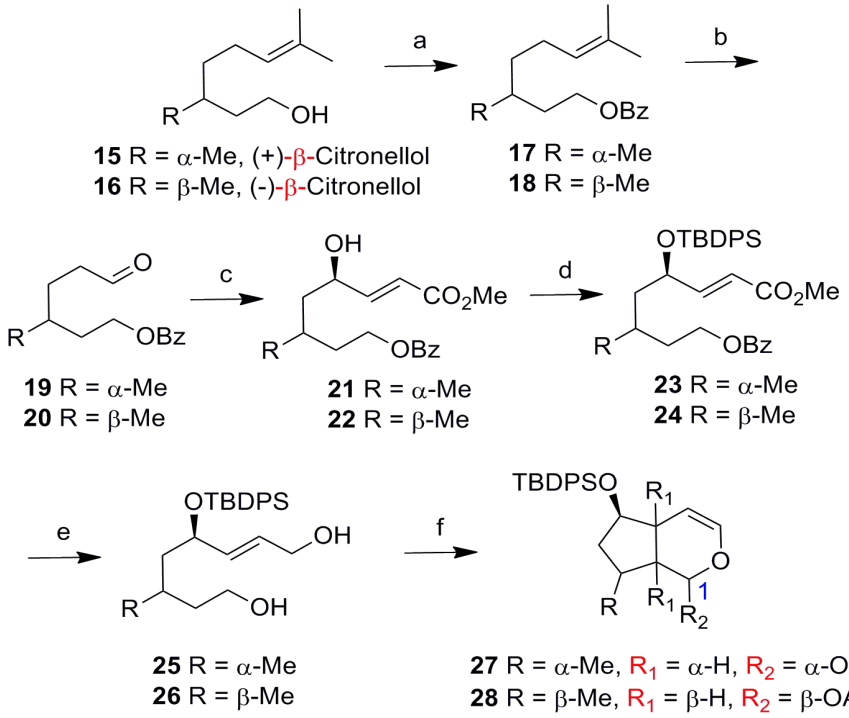

Figure 3. Reagents and conditions. (a) BzCl, Et_3N, CH_2Cl_2, 99%; (b) (i) O_3, Py, CH_2Cl_2/MeOH (9:1), −78 ºC; (ii) PPh_3, −78 ºC to 0 ºC, 91%; (c) (i) L-proline, PhNO, DMSO; (ii) $(MeO)_2P(O)CH_2CO_2Me$, DBU, LiCl, CH_3CN, −15 ºC; (iii) NH_4Cl, MeOH, −15 ºC to rt, 24%; (d) TBDPSCl, imidazole, CH_2Cl_2, rt, 94%; (e) DIBAL-H, Et_2O, −78 ºC, 45%; (f) (i) DMP, CH_2Cl_2, rt; (ii) L-proline, DMSO, 40 ºC, 60 h; (iii) Ac_2O, DMAP, Py, 0 ºC, 15%.

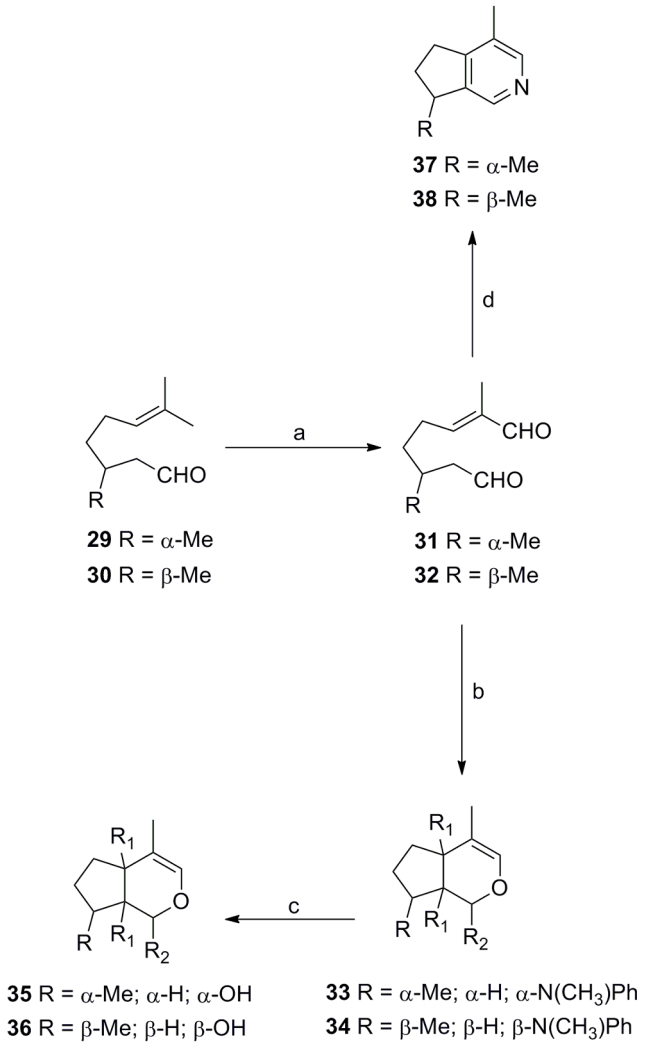

Figure 4. Reagents and conditions. a) SeO_2, *t*-BuOOH, CH_2Cl_2, 0 ºC, 12 h, 40%; (b) MeNHPh, 4Å MS, Et_2O, rt, 24 h, 78%; (c) *p*-TSOH, THF:H_2O (9:1), rt, 24 h, 80%.; (d) NH_2OH, *p*-TSOH, THF, 50 °C, 12 h, 67%.

Bioactivity

A group of 35 compounds at a concentration of 500 µM was assayed and 9 compounds showed activity (25.71% of active compounds) against *Taq* DNA polymerase (Figure 5). The compound 27 was not tested because there was insufficient sample mass. To determinate IC_{50} values, serial dilutions (1:2) were performed. Compound 14 was the most active compound with an IC_{50} of 13.47 µM and compounds 33 and 34 were strong inhibitor with IC_{50} values of 17.65 and 18.31 µM. Compounds 35, 36 and 13 showed good activities with IC_{50} of 31.76, 32.43 and 38.33 µM, respectively. Also, compound 28 generated an inhibition with an IC_{50} value of 42.57 µM. Additionally, the intermediate compounds 21 and 22 showed a weak activity against *Taq* DNA polymerase with IC_{50} values equal to 58.21 and 58.79 µM, respectively. (Tab. 1)

DISCUSSION

During the last century, progress in molecular biology contributed to the increasing understanding of the underlying mechanisms related to cancer initiation, promotion, and progression. As a consequence, monoclonal antibodies and small molecules have been developed. These drugs interfere with specific molecular targets (typically proteins) involved in tumor growth and progression, and therefore have become an important part of the anticancer armamentarium (15). These targets include growth factor receptors, signalling molecules, cell-cycle proteins, apoptosis' modulators, and molecules involved in invasion and angiogenesis, which are essential for development and homeostasis in normal tissues. In cancer cells, they have a gain of function as a consequence of overexpression and/or gene alterations.

One of these molecular targets are DNA polymerases which have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents because of faithful replication of DNA molecules by DNA polymerases is essential for genome integrity and correct transmission of genetic information in all living organisms.

The idea behind molecular targeting is to design treatments that specifically attack the molecular pathways that cause illness, without disrupting the normal functions in our cells. Drugs developed using this approach can be less toxic and more effective than current medicines. Already, for certain types of cancer, targeted drugs have demonstrated superior effectiveness in reducing or eliminating tumors. They have also allowed patients to maintain their quality of life without the side effects of chemotherapy.

Table 1. Inhibitory activity at a concentration of 500 µM and IC_{50} values for the compounds panel.

Compound	IC_{50} (*µ*M)	Compound	IC_{50} (*µ*M)	Compound	IC_{50} (*µ*M)	Compound	IC_{50} (*µ*M)
4	NI	13	38.33	22	58.79	31	NI
5	NI	14	13.47	23	NI	32	NI
6	NI	15	NI	24	NI	33	17.65
7	NI	16	NI	25	NI	34	18.31
8	NI	17	NI	26	NI	35	31.76
9	NI	18	NI	27	NT	36	32.43
10	NI	19	NI	28	42.57	37	NI
11	NI	20	NI	29	NI	38	NI
12	NI	21	58.21	30	NI		

IC_{50} values were determinated by interpolation from plots of enzyme activity *vs*. inhibitor concentration. The IC_{50} values are means from at least three independent experiments and standart deviation never exceeded 20%. NI: No Inhibition. NT: No Tested. Comp.: compound. The results are expressed in *µ*M.

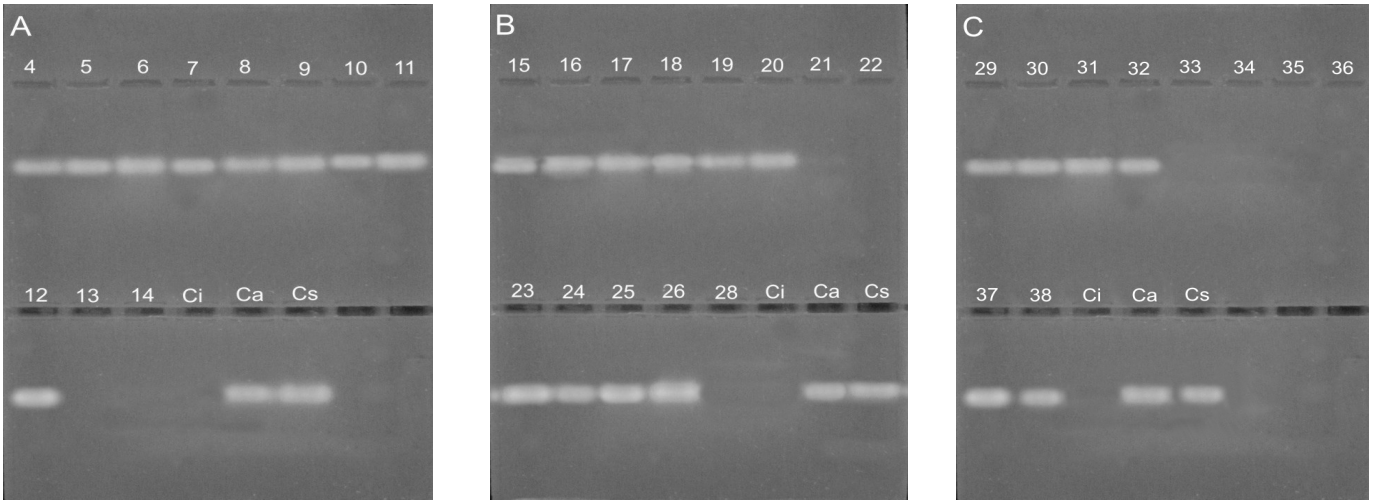

Figure 5. Activity against *Taq* DNA polymerase. A: Compounds 4 - 14. B: Compounds 15 - 28. C: Compounds 29 - 38. Controls (Ci: Inhibition control, Ca: Activity control and Cs: Solvent control)

Examples are dasatinib (Sprycel®) for acute lymphoblastic leukemia in adult with resistance or intolerance to prior therapy and trastuzumab (Herceptin®) for breast cancer in women with overexpression of HER2 protein.

In previous studies, we observed that compound 28 at low doses (1 and 2 μM) produced an increase in the percentage of cells in the G_0/G_1 phase in a cell cycle distribution of epithelial HBL-100 and human lung carcinoma SW1573 cells lines. This was concomitant with a decrease in the S-phase compartment and consistent with DNA polymerase inhibition (5). Now, we observe that compounds with similar structures to iridoid aglycones, including compound 28, have inhibitory activity against *Taq* DNA polymerase; this would indicate that the molecular target implicated in the cytotoxic activity of these compounds is indeed related to inhibiting the activity of DNA polymerases. Also, we can detect that inhibitory activity against this enzyme is better when cyclopentane-[c]-pyrano moiety is present and there are not significant differences in the activity between racemic and enantiomeric forms because the molecules 13 (racemic form) and 28 (enantiomeric form) showed similar IC_{50} values. Similarly, there are not significant differences between the enantiomeric forms as we can observe in the IC_{50} values of the compounds 33 and 34 (17.65 and 18.31 μM, respectively)

These results are consistent with DNA polymerases as the molecular targets implicated in this bioactivity and suggest that iridoid aglycones can serve as lead molecules to develop new drugs for cancer therapy.

In summary, the inhibitory activity of these compounds against *Taq* DNA polymerase would indicate that the molecular target involved in the cytotoxic activity, observed in previous studies, are DNA polymerases.

ACKNOWLEDGMENT

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