*Cellular and Molecular Biology*™ **50** (6), 767-772
DOI 10.1170/T567

ISSN 1165-158X
2004 Cell. Mol. Biol.™

# INHIBITION OF *TAQ* DNA POLYMERASE BY CATALPOL

C.R. PUNGITORE[1], M. JURI AYUB[2], E.J. BORKOWSKI[1], C.E. TONN[1] and G.M. CIUFFO[2✍]

[1] INTEQUI-CONICET, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, 5700 San Luis, Argentina
[2✍] Departamento de Bioquímica y Cs. Biológicas, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, 5700 San Luis, Argentina
*Fax: +54 2652 422 644; E-mail: gciuffo@unsl.edu.ar*

*Received June 15, 2004; Accepted September 28, 2004*

**Abstract** - DNA polymerases have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. This report describes a PCR assay as a method to investigate the action mechanism of the inhibition of *Taq* DNA polymerase by catalpol. This inhibition was not primer or template specific, nor was it due to chelation of $Mg^{2+}$ ions. In assays of hyperchromicity of double-stranded DNA, catalpol did not affect melting profile. The inhibitory effect of catalpol does not appear to depend on DNA concentration. In contrast, increasing dNTP concentration rescue the *Taq* DNA polymerase activity, suggesting that catalpol acts in a competitive way with dNTPs at the binding site of the enzyme. Theoretical calculations reinforce the experimental data and the proposed mode of action of catalpol.

**Key words**: Iridoids, catalpol, *Taq* Polymerase inhibition, molecular properties

## INTRODUCTION

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. In spite that several DNA polymerase inhibitors have been reported, including the nucleotide dideoxy TTP, phospholipids, fatty acids and flavonoids, more potent agents are still needed (25). Therefore information concerning the structural characteristics of inhibitors could provide valuable insight for the design of anti-cancer agents (15) and some probes for understanding the roles of specific enzymes in DNA replication and repair.

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 Astake *et al.* (2), 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 know polymerases. A variety of evidences point to 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 (1,2,3).

Iridoids are cyclopentane-[c]-pyran monoterpenoids occurring as constituents of several plants (4,9). Several biological activities have been described for these compounds, such as antimicrobial, antitumoral, hemodynamic, choleretic, hepatoprotective and anti-inflamatory activities (10,11,18,21).

In a previous paper, we studied the action of the iridoids catalpol (**1**), 8-O-acetylharpagide (**2**), harpagide (**3**) and dihydrocatalpol (**4**) against *Taq* DNA polymerase by using a PCR assay (20). The present paper investigates the action of iridoids as *Taq* DNA polymerase inhibitors, in order to better characterize the action mechanism of these compounds.

## MATERIALS AND METHODS

*Compounds*

Catalpol (**1**) was isolated from *Buddleja cordobensis* Griseb, and compound **2** was supplied by Dr. J. Coll (Dept. Chim. Org., Barcelona, Spain). Compound **3** was prepared from the derivative **2**. Compound **4**

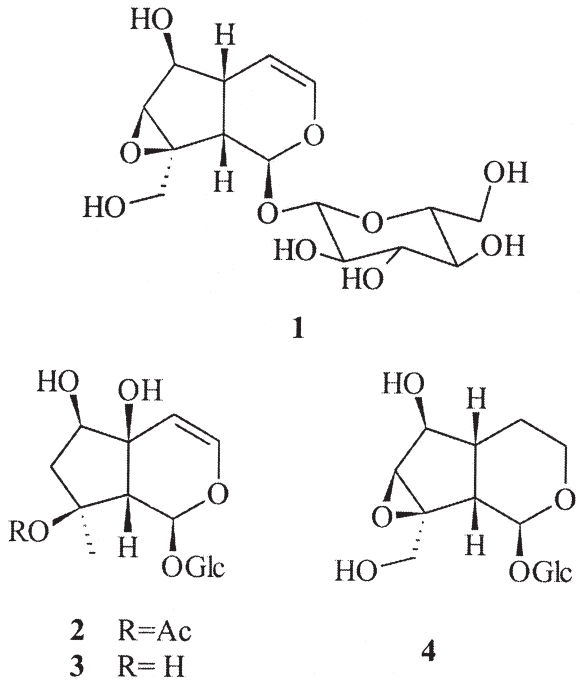


**Fig. 1** Chemical structure of compounds

was derived from catalpol (Fig. 1) (20). The assayed compounds were dissolved in DMSO at a 100x stock solution.

*PCRs assays*

The PCR master mix consist of 40 mM Tris-acetate pH 8.3 containing 1 mM EDTA, 2.5 mM $MgCl_2$, 4.0 U *Taq* DNA polymerase, 20 μM each oligonucleotide primer and 0.25 mM each deoxynucleotide triphosphates (dNTP). All PCRs were carried out in 20 μl reaction volumes, in a Perkin-Elmer Gene Amp 2400.

Inhibition studies were carried out under different conditions, where desoxynucleotides triphosphates, $MgCl_2$ and DNA template concentrations were varied. In the experiments performed with p-GEMT-$AT_2$ (supplied by Dr. Sergio Alvarez from our Molecular Biology Laboratory) as a template, the primers were: sense primer 5′-TTG TTT GGT GTA TGG CTT GT-3′ and the antisense primer: 5′-CTT AGA GAA ATG GAC ACC TT-3′. In a different set of experiments, we also amplified the human β-globin gene, by using suitable primers (5′-CAA CTT CAT CCA CGT TCA CC-3′ and 5′-GAA GAG CCA AGG ACA GGT AC-3′) and human DNA (26). For both set of primers, the amplification conditions consisted of 35 cycles of denaturation at 95ºC for one min. followed by primers annealing at 56ºC and primer extension at 72ºC for 2 min. Inhibition positive controls were performed using ddNTP set.

The amplified DNA sequences were electrophoresed for 45 min. in 1% agarose gel at 60 V in TAE (Tris-acetate-EDTA) running buffer containing 40 mM Tris-acetate, 2 mM $Na_2EDTA.2H_2O$, and 0.5 μg of ethidium bromide per ml. Amplified DNA bands were detected visually with UV transilluminator.

*Analysis of PCR products*

Relative intensities of ethidium bromide stained PCR products were analyzed by using an optical scanner and the Scion-Image program. The image of stained agarose gels was captured using Polaroid camera and then scanned (Hewlett-Packard 3200 C). The digitized band images were processed using the Image processing program (Scion Image, public domain program); $IC_{50}$ values were determined by GraphPad Prism program.

*Thermal transition of DNA*

Thermal transition profiles of double-stranded to single-stranded DNA in the absence or in the presence of catalpol were determined with a Beckman DU640B spectrophotometer equipped with thermostatic system. Plasmid DNA (p-GEMT-$AT_2$, 6 μg/ml) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0). The solution temperature was equilibrated at 75°C for 10 min, and increased by 1° at 2 min intervals for each measurement point and the absorbance was recorded.

*Theoretical calculations*

All theoretical calculations were performed using the PC Spartan Pro® software (19) on a PC with an AMD Athlon(™) processor. The Spartan Pro® routine for conformational search was based on Montecarlo Method and the Merck Molecular Force Field (MMFF94) was used to perform the search. The more stable MMFF conformer was minimized using a Density Functional method: perturbative Becke-Perdew (pBP), with a numerical polarization basis set (DN*). HOMO and LUMO were calculated at 0.032 a.u. as isovalue; the electron density was estimated at 0.002 electrons/a.u.$^3$ as isovalue, and the molecular electrostatic potential was calculated at ± 20 kcal/mol as isovalue. Information in detail on the computational procedures is obtainable from authors by correspondence.

## RESULTS

In a previous paper (20), we reported that the iridoids catalpol (**1**), 8-O-acetylharpagide (**2**) and harpagide (**3**) exhibit inhibitory activity on *Taq* DNA polymerase. Compound **1** was identified as the most active, showing an $IC_{50}$ = 47.8 μM (Fig. 2A). Compound **2** revealed values of $IC_{50}$ = 213.8 μM, having a moderate activity; derivative **3** showed only a weak inhibitory activity ($IC_{50}$ = 416.9 μM) and dihydrocatalpol (**4**) has not inhibitory activity (data not shown). In addition, we also showed that changes in the sugar moiety affected bioactivity. The introduction of *O*-acetyl groups or *O*-oleyl fragments on the D-glucose moiety of compound **1** was accompanied by loss of enzyme inhibitory effect.

In order to better characterize the action mechanism of catalpol (**1**) in the inhibition of the *Taq* DNA polymerase, we performed polymerization chain reaction (PCR) assays under different conditions.

*Catalpol inhibition is independent of DNA template*

Our initial experiments were performed using as template the p-GEMT-$AT_2$ plasmid and a specific primer set. Under these conditions, we could not rule out the possibility that the inhibition could be primer or gene specific. To study this point, we performed amplification of the human β-globin gene from human DNA (26) in the absence or in the presence of catalpol. The amplification of β-globin was completely inhibited by 125 μM of catalpol (**1**). These results suggest that the inhibitor acted independently of the sequence to be amplified and the primer set used (Fig. 2B).

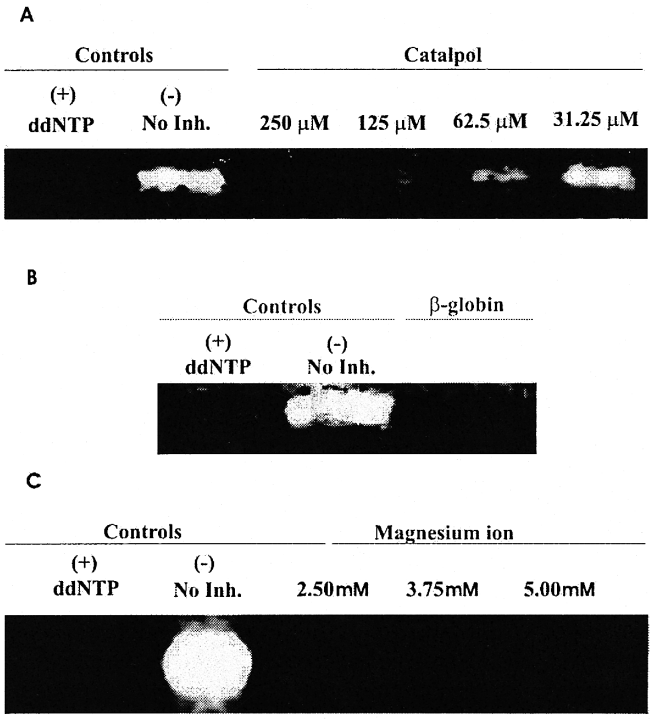


**Fig. 2** **A**) Inhibitory effect of catalpol against *Taq* DNA polymerase. PCR assays were conducted in the presence of different concentrations of catalpol. **B**) Specificity of PCR product inhibition. PCR inhibition assays were performed for human β-globin using human DNA as template in the presence of catalpol (125 μM). **C**) Effect of increasing magnesium concentrations. PCR assays were made with increasing magnesium ion concentrations in the presence of catalpol (125 μM). The figures are representative of three independent experiments.

*Effect of increasing magnesium concentrations*

It is well-known that PCR reaction can also be inhibited by chelating divalent cations such as $Mg^{2+}$. To investigate if the observed inhibition was magnesium dependent, increasing concentrations of $MgCl_2$ (2.5 mM, 3.75 mM and 5.0 mM) were added to the PCR reaction mix containing 125 μM compound **1**. PCR amplifications were done as described under Materials and Methods. Fig. 2C shows the effect of increasing concentrations of $MgCl_2$. As expected, within an acceptable range of $Mg^{2+}$ ion concentrations, from 2.5 to 5 mM, catalpol was able to inhibit *Taq* polymerase, thus suggesting the inhibitory effect of catalpol is not sequestrating $Mg^{2+}$ ions.

*Hyperchromicity of double-stranded DNA*

To investigate whether compound **1** binds to DNA, the melting transition of double-stranded DNA in the presence of 100 μM of the iridoid was determined using a spectrophotometer equipped with a thermostatic system. Bioassay was performed as described in Materials and Methods. At this concentration of catalpol (**1**), no shift in the curve was observed. On the contrary, 100 μM of EtBr, a well-known DNA-intercalating, clearly modified the thermal transition (13,14,15,27) (Fig. 3).

*Effect of increasing DNA template concentrations*

To determine if the inhibitory effect of catalpol was DNA template dependent, increasing concentrations of DNA (0.013 μg/ml, 0.130 μg/ml, 1.300 μg/ml) were added to the PCR master mix, containing catalpol in a concentration, 125 μM, higher than the $IC_{50}$. Since the increase of plasmid DNA concentrations failed to rescue the polymerase activity of reactions containing 125 μM of compound **1** (Fig. 4A), we can discard the possibility that catalpol interacts with DNA in a way to interfere with the enzyme activity.

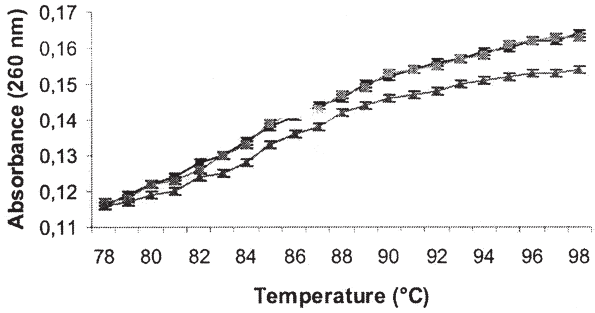


**Fig. 3** Effect of catalpol on the thermal transition of double-stranded DNA. No addition (control: ●), compound 1 (■) and ethidium bromide (▲) (100 μM of each)

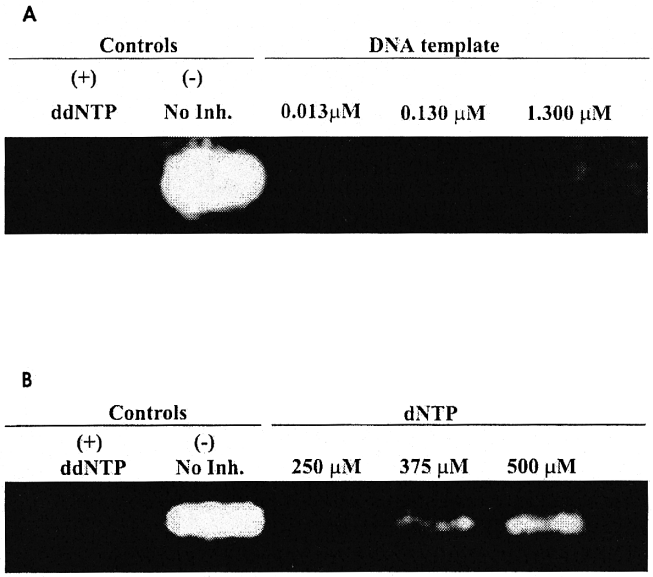


**Fig. 4** **A**) Effect of DNA template concentrations. PCR assays were performed in the presence of catalpol (125 μM) and increasing concentrations DNA template. **B**) Effect of increasing dNTP concentrations. Different concentrations (250 μM, 375 μM and 500 μM) of dNTP were added to the PCR assay in the absence (No inh) or in the presence of 125 μM catalpol. Experiments are representative of two independent assays.

*Effect of increasing dNTP concentrations*

To study the effect of increasing concentrations of dNTP, we performed the amplification in the presence of catalpol and different dNTP concentrations (250 μM, 375 μM and 500 μM). We observed (Fig. 4B) that a concentration of 375 μM dNTP rescue the *Taq* DNA polymerase activity of reactions containing 125 μM of compound **1**, and this effect is dose-dependent, suggesting a competitive mechanism between catalpol and dNTPs.

*Theoretical calculations*

From the more stable MMFF conformer (see Materials and Methods), the isosurfaces were calculated. Fig. 5A shows a significant similarity among the HOMO properties of adenosine, guanosine, and compound **1**. Moreover, it shows that the HOMO of compound **4** exhibits no similarity with that of compound **1,** adenosine and guanosine.

Additionally, the possible hydrogen bonds formed between compound **1** and thymidine, was considered. Fig. 5B shows the calculated hydrogen bonds between adenosine-thymidine compared with the same interaction between catalpol-thymidine and dihydrocatalpol-thymidine, respectively.

## DISCUSSION

Taking into account the *Taq* DNA polymerase properties (1,2,3,12) it is possible to use this model as a screening method to find DNA polymerase inhibitors and also to investigate the action mechanism of inhibition.

The multiple applications of DNA polymerase inhibitors as well as the number of natural products with this activity (5,6,25) such as coumarins and flavonoids (23,24), sulfonosphingolipids and acetylenic fatty acid (8,16) prompted us to investigate the inhibitory role of iridoids.

In the present paper we studied the inhibitory effect of catalpol, using as a model for DNA polymerase, *Taq* polymerase, under different conditions. We observed that catalpol inhibitory activity on *Taq* polymerase is independent of the template and primer set used. In effect, catalpol inhibited both the amplification of a plasmid containing $AT_2$ receptor or the amplification of the β-globin gene on human DNA.

It is well known that dNTPs can work as $Mg^{2+}$ chelating. We test the possibility that catalpol sequestrates $Mg^{2+}$ ions, by adding increasing concentrations of $Mg^{2+}$ during the amplification procedure. Since the increased concentration of $Mg^{2+}$ ions did not revert catalpol inhibition, we can conclude that catalpol act by a different mechanism.

We compared the melting profile of double-stranded DNA (dsDNA) in the presence of the iridoid to determinate whether compound **1** binds to DNA (7,13,14,15,17) and compare with EtBr. While EtBr does affect melting profile of dsDNA, catalpol does not, suggesting that catalpol does not bind dsDNA. From these results, one can hypothesize that the inhibitor does not appear to act as a chelator of magnesium ions or bind to DNA, suggesting that it must inhibit the enzyme activities by interacting with the enzyme directly.

Biochemical and molecular genetic studies have determined dNTP binding site and DNA binding site at the polymerase domain (1). The dNTP binds adjacent to the O-helix interacting principally with three positively charged residues, $Arg^{754}$, $Arg^{682}$ and $Lys^{758}$, plus $His^{734}$ and $Gln^{708}$ (3). The DNA template bind in the groove between the thumb subdomain of the polymerase and the 3'-5' exonuclease domain, rather than in the DNA-binding cleft of the polymerase itself. Moreover, a portion of the duplex region of the DNA interacts with α-helices of the thumb subdomain of the structure (1). In order to investigate the catalpol interaction at the enzyme site, we performed different experiences. To determinate if compound **1** compete with DNA template at the DNA binding site, assays of PCRs with increasing DNA template concentrations were performed. Since increasing DNA template concentrations cannot restore DNA polymerase activity we can assume that catalpol do not compete at the DNA binding site.

On the other hand, increasing dNTP concentrations restore *Taq* polymerase activity in the presence of catalpol, suggesting a competition between them (Fig. 4B). Chemical structure of compound **1** has a certain resemblance with a nucleoside framework. The bicyclic aglycone moiety, possessing both oxygenated functional groups and one desaturation, could mimic a purine-nucleoside electronic model (20). Taking into account this similarity, isosurfaces and the possible hydrogen bonds between catalpol and pyrimidine-nucleoside were calculated (Fig. 5).

Since HOMO is the most external occupied orbital, its shape gives an idea about the electronic distribution, wich is available for recognition. Contrary to dihydrocatalpol (4), adenosine, guanosine and catalpol (1) show alternate negative and positive HOMO lobes over the non-sugar moiety. When theoretical calculation was carried out on (4), the shape and localization of HOMO vary considerably, giving this orbital over the glycosidic part of molecule.

Considering isosurfaces as a possible recognition profile (20,22), together with our experimental results, we can hypothesize that catalpol (**1**) could compete with dNTP at the dNTP binding site of the enzyme.

As we showed earlier, dihydrocatalpol (**4**), the hydrogenated form of catalpol did not inhibit *Taq* DNA polymerase. Additionally, we calculated dihydrocatalpol (**4**)

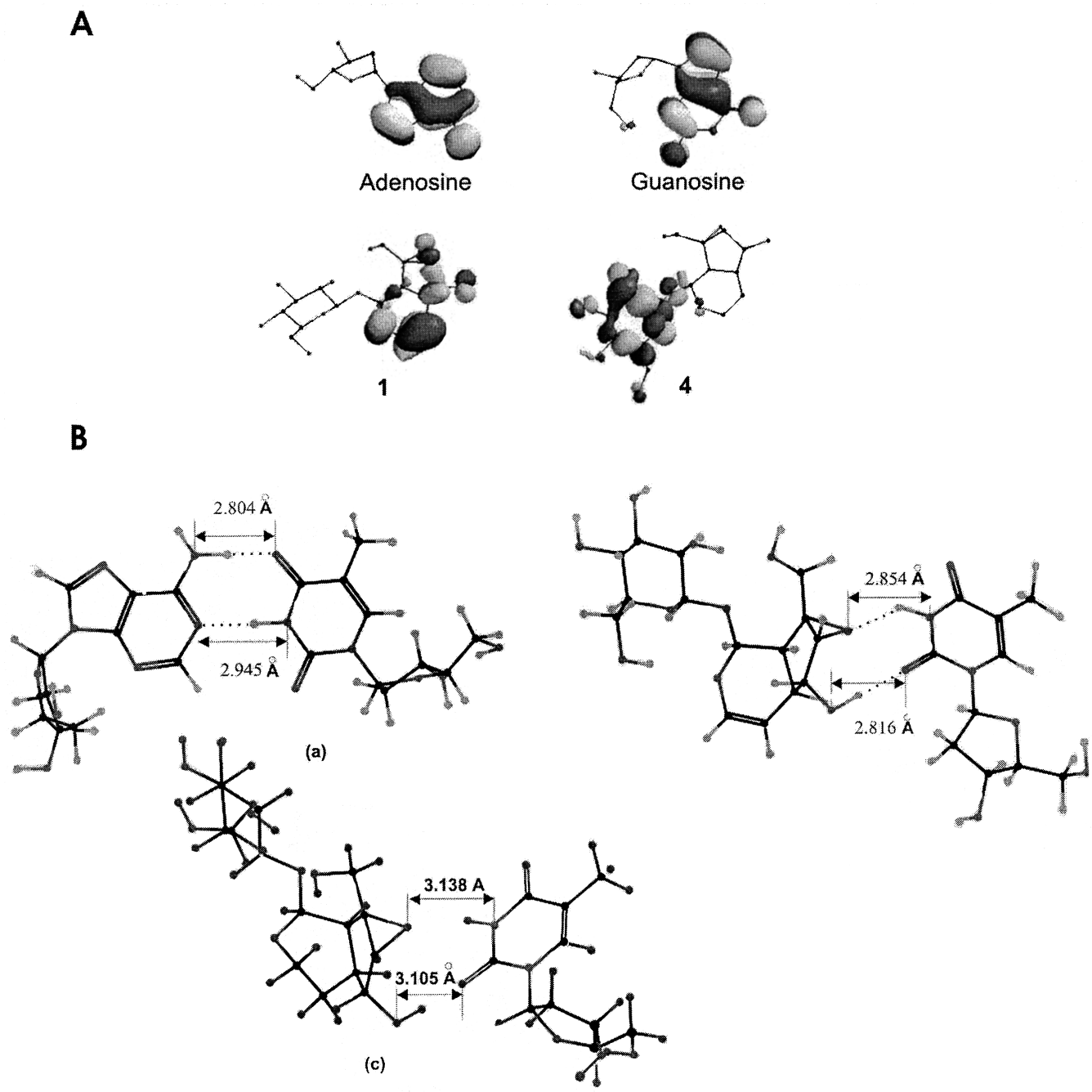


**Fig. 5** **A**) Spatial view of the HOMO orbital of the compounds studied. **B**) Hydrogen bonds interactions between adenosine-thymidine (a), catalpol-thymidine (b) and dihydrocatalpol-thymidine (c).

isosurfaces (Fig. 5A) and the possible hydrogen bonds between compound **4** and pyrimidine-nucleosides (Fig. 5B). The HOMO orbital of compound **4** exhibits no similarity with the HOMO orbital of purine-nucleosides and catalpol, as well as a lower ability to form hydrogen bonds with pyrimidine-nucleosides. Theoretical estimations allow us to propose that catalpol (**1**) could interact directly with the enzyme because it has an adequate electronic profile to compete at the dNTP binding site and the ability to fit within the same hydrogen bond distances as the adenosine-thymidine nucleotide pair.

In summary, catalpol is a *Taq* DNA polymerase inhibitor and the inhibitory effect can be produced by direct interaction at the dNTP binding site of the enzyme. This compound could be an important inhibitor not only to investigate DNA polymerase activities but also, the computer-simulated drug design of this agent, may in theory be useful to develop new anticancer chemotherapy medicines.

**Acknowledgments** – Financial support from CONICET (PIP 5031), UNSL (Project 7301; 22/Q801) is gratefully acknowledged. We wish to thank to Dr. Sergio E. Alvarez, who kindly provide us with the plasmid pGEM-$AT_2$. We thank to the Molecular Biology Laboratory staff and Cecilia Lucero Estrada from Microbiology Laboratory of Universidad Nacional de San Luis for their help. C.R.P. thank CONICET for the fellowship. This work is a part of the doctoral thesis of C.R.P.

## REFERENCES

1. Arnold, E., Ding, J., Hughes, S.H. and Hostomsky, Z., Structures of DNA and RNA polymerases and their interactions with nucleic acid substrates. *Curr. Opin. Struct. Biol.* 1995, **5**: 27-38.
2. Astatke, M., Grindley, N.D.F. and Joyce, C.M., How *E. coli* DNA polymerase I (Klenow fragment) distinguishes between deoxy- dideoxynucleotides. *J. Mol. Biol.* 1998, **278**: 147-165.
3. Beese, L.S., Friedman, J.M. and Steitz, T.A., Crystal structures of the Klenow fragment of DNA polymerase I complexed with deoxynucleoside triphospate and pyrophosphate. *Biochemistry* 1993, **32**: 14095-14101.
4. Boros, C.A. and Stermitz, F.R., Iridoids. An updated review, part I. *J. Nat. Prod.* 1990, **53**: 1055-1147.
5. Deng, J.Z., Starck, S., Hecht, S.M., Ijames, C.F. and Hemling, M.E., Harbinatic acid, a novel and potent DNA polymerase β inhibitor from *Hardwickia binata*. *J. Nat. Prod.* 1999, **62**: 1000-1002.
6. Deng, J.Z., Starck, S.R. and Hecht, S.M., DNA polymerase β inhibitors from *Baeckea gunniana. J. Nat. Prod.* 1999, **62**: 1624-1626.
7. Dervan, P.B., Molecular recognition of DNA by small molecules. *Bioorg. Med. Chem.* 2001, **9**: 2215-2235.
8. Ishiyama, H., Ishibashi, M., Ogawa, A., Yoshida, S. and Kobayashi, J., Taurospongin A, a novel acetylenic acid derivative inhibiting DNA polymerase β and HIV reverse transcriptase from sponge *Hippospongia* sp. *J. Org. Chem.* 1997, **62**: 3831-3836.
9. Jensen, S.R., In: *Ecological Chemistry and Biochemistry of Plant Terpenoids*, Harborne, J.B. and Tomas-Barberan, F.A. (eds.), Clarendon Press, Oxford, 1991, pp. 133-154.
10. Kapadia, G.J., Sharma, S.C., Tokuda, H., Nishino, H. and Ueda, S., Inhibitory effect of iridoids on Epstein-Barr activation by a short-term *in vitro* assay for anti-tumor promoters. *Cancer Lett.* 1996, **102**: 223-226.
11. Konoshima, T., Takasaki, M., Tokuda, H. and Nishino, H., Cancer chemopreventive activity of an iridoid glycosilated, 8-acetylharpagide, from *Ajuga decumbens*. *Cancer Lett.* 2000, **157**: 87-92.
12. Li, Y. and Waksman, G., Crystal structures of a ddATP-, ddTTP-, ddCTP- and ddGTP-trapped ternary complex of Klentaq 1: insights into nucleotide incorporation and selectivity. *Prot. Sci.* 2001, **10**: 1225-1233.
13. Mizushina, Y., Iida, A., Ohta, K., Sugawara, F. and Sakaguchi, K., Novel triterpenoids inhibit both DNA polymerase and DNA topoisomerase. *Biochem. J.* 2000, **350**: 757-763.
14. Mizushina, Y., Ikuta, A., Endoh, K., Oshige, M., Kasai, N., Kamiya, K., Satake, T., Takazawa, H., Morita, H., Tomiyasu, H., Yoshida, H., Sugawara, F. and Sakaguchi, K., Inhibition of DNA polymerases and DNA topoisomerase II by triterpenes produced by plant callus. *Biochem. Biophys. Res. Commun.* 2003, **305**: 365-373.
15. Mizushina, Y., Murakami, Ch., Ohta, K., Takikawa, H., Mori, K., Yoshida, H., Sugawara, F. and Sakaguchi, K., Selective inhibition of the activities of both eukaryotic DNA polymerases and DNA topoisomerases by elenic acid. *Biochem. Pharm.* 2002, **63**: 399-407.
16. Mizushina, Y., Watanabe, I., Ohta, K., Takemura, M., Sahara, H., Takahashi, N., Gasa, S., Sugawara, F., Matsukage, A., Yoshida, S. and Sakaguchi, K., Studies on inhibitors of mammalian DNA polymerase α and β. *Biochem. Pharm.* 1998, **55**: 537-541.
17. Neidle, S., DNA minor-groove recognition by small molecules. *Nat. Prod. Rep.* 2001, **18**: 291-309.
18. Ortiz de Urbina, A.V., Martín, M.L., Fernández, B., San Román, L. and Cubillo, L., *In vitro* antispasmodic activity of peracetylated penstemonoside, aucubin and catalpol. *Planta Med.* 1994, **60**: 512-515.
19. PC Spartan ProVer.1.Wavefunction, Inc., Irvine, CA, 1999. Reg. # Ppro1-002353.
20. Pungitore, C.R., Juri Ayub, M., García, M., Borkowski, E.J., Sosa, M.E., Ciuffo, G., Giordano, O.S. and Tonn, C.E., Iridoids as allelochemicals and DNA polymerases inhibitors. *J. Nat. Prod.* 2004, **67**: 357-361.
21. Recio, M. del C., Giner, R.M., Máñez, S. and Rios, J.L., Structural considerations on the iridoids as anti-inflamatory agents. *Planta Med.* 1994**, 60**: 232-234.
22. Stezer, W.N., Holland, M.T., Bozeman, C.A., Glenn, G.F., Setzer, M.C., Moriarity, D.M., Reeb, S., Vogler, B., Bates, R.B. and Haber, W.A., Isolation and frontier molecular orbital investigation of bioactive quinone-methide triterpenoids from the bark of *Salacia petenensis. Planta Med.* 2001, **67**: 65-69.
23. Sun, D.A., Starck, S., Locke, E.P. and Hecht, S.M., DNA polymerase β inhibitors from *Sandoricum koetjape. J. Nat. Prod.* 1999, **62**: 1110-1113.
24. Sun, N.J., Woo, S.H., Cassady, J.M. and Snapka, R.M., DNA polymerase and topoisomerase II inhibitors from *Psoralea coryfolia*. *J. Nat. Prod.* 1998, **61**: 362-366.
25. Tanaka, N., Kitamura, A., Mizushina, Y., Sugawara, F. and Sakaguchi, K., Fomitellic acids, triterpenoid inhibitors of eukaryotic DNA polymerases from a basidiomycete, *Fomitellia fraxinea. J. Nat. Prod.* 1998, **61**: 193-197.
26. Wiedbrauk, D.L., Werner, J.C. and Drevon, A.M., Inhibition of PCR by aqueous vitreous fluids. *J. Clin. Microbiol.*1995, **33**: 2643-2646.
27. Williams, M.C., Rouzina, I. and Bloomfield, V.A., Thermodynamics of DNA interactions form simple molecule stretching experiments. *Acc. Chem. Res.* 2002, **35**: 159-166.