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INHIBITION OF DNA TOPOISOMERASE I AND GROWTH INHIBITION OF HUMAN CANCER CELL LINES BY AN OLEANANE FROM *JUNELLIA ASPERA* (VERBENACEAE)

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Abstract – DNA topoisomerases and DNA polymerases are enzymes that play a crucial role in DNA metabolism events such as replication, transcription, recombination, and chromosome segregation during mitosis. Thus, DNA topoisomerases and DNA polymerases inhibitors could be expected to have antitumor effects. Naturally occurring triterpenoids isolated from *Junellia aspera* (Gillies & Hook; Moldenke) (Verbenaceae) were assayed for human DNA topoisomerase I and *Taq* DNA polymerase inhibitory activities. Maslinic acid (**2**) and its diacetyl derivative (**7**) showed human DNA topoisomerase I inhibitory activity with IC_{50} values in the range of 76-80 μM and growth inhibition against various human solid tumour cell lines with GI_{50} values in the range of 5-18 μM. The triterpene frames could be used for screening new inhibitors of the enzyme, and computer-simulated drug design using the frame and pocket structure of enzyme may in theory be a possible approach to develop new inhibitors.

Keywords: DNA topoisomerase inhibitors, solid tumours, anticancer drugs, triterpenes, *Junellia aspera*.

INTRODUCTION

It is well known that natural products play a highly significant role in drug discovery and development processes. This is particularly evident for cancer, where over 60% of the drugs currently used in chemotherapy are from natural origin (12). The finding and development of new lead compounds is a consequence of the rapid escalation in the discovery of molecular targets; that may be applied to the discovery of novel tools for diagnosis, prevention and treatment of human diseases.

DNA polymerases and DNA topoisomerases are essential for genome integrity and correct transmission of genetic information in all living organisms. DNA topoisomerases, that catalyse the interconversion of various topological states of DNA, were originally discovered as enzymes that change the super helical structure of closed circular DNA. Based on their functional mechanisms, DNA topoisomerases have been classified into two types. Type I DNA topoisomerases break and rejoin only one of the two strands during catalysis, while type II DNA topoisomerases break and rejoin both strands for each DNA strand-passing reaction.

Topoisomerase I seems to be associated with actively transcribed genes, whereas, topoisomerase II is required for DNA replication and for successful traverse of mitosis. Through these two fundamentally different mechanisms, DNA topoisomerases modify the topological states, which facilitate various DNA transactions such as DNA replication, RNA transcription, recombination, chromosome condensation/decondensation, and chromosome segregation (4,10,17,19)

It has long been accepted that topoisomerases are valuable targets for the development of cancer chemotherapeutic agents. Several topoisomerase inhibitors have been introduced

***** Equal Contribution

into clinical trials as potent anticancer drugs, including camptothecin derivatives inhibiting topoisomerase I, anthacyclines, epipodophyllotoxins, aminoacridines and ellipticines targeting topoisomerase II (11,18,20). However, more potent agents are still needed. Therefore, information concerning the structural characteristics of inhibitors could provide valuable insight for the design of anticancer agents and a tool for better understanding the roles of specific enzymes in DNA replication and repair.

In the search of novel bioactive constituents from plants, we have focused on novel topoisomerase I inhibitors as potential lead structures for anticancer drug development (14,15). In this paper we report on the ability of maslinic acid isolated from *Junellia aspera* (Gilles & Hook) Mold. (Verbenaceae) and its diacetyl derivate to inhibit topoisomerase I. Additionally, the "*in vitro*" antiproliferative activity of these compounds was evaluated against several human solid tumour cell lines: A2780 (ovarian cancer), SW1573 (non-small cell lung cancer, NSCLC), WiDr (colon cancer), T-47D (breast cancer) and HBL-100 (breast cancer). The genus *Junellia* includes 45 species endemic to Los Andes Mountains and Patagonian regions of South America. *J. aspera* is a shrub that was collected 1520 m above sea level (13).

MATERIALS AND METHODS

Chemicals and reagents

Molecular Biology Laboratory from UNSL supplied p-GEMT-AT2; *Taq* DNA polymerase was prepared in this laboratory (6). Human DNA topoisomerase I (5 U/μL) was obtained from Amersham Biosciences UK Limited. Supercoiled pBr 322 plasmid DNA and camptothecin were supplied by Dr. Maximiliano Juri Ayub (Instituto Leloir/CONICET). All starting materials were commercially available research-grade chemicals and used without further purification. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) was from Gibco (Grand Island, NY), trichloroacetic acid (TCA), glutamine and gentamicin were from Merck (Darmstadt, Germany), and dimethyl sulfoxide (DMSO) and sulforhodamine B (SRB) were from Sigma (St Louis, MO).

Compounds

Aerial parts of *J. aspera* were collected during December 2001, in Villavicencio (lat.: 32:31:16S; lon.: 68:59:43W), Las Heras, Mendoza, Argentina. *J. aspera* dry aerial parts were chopped and macerated twice for seven day periods each time with MeOH at r. t.. The solvent was evaporated under reduced pressure, the residue taken up in $CHCl_3$ and partitioned against H_2O. The organic layer was dried (Na_2SO_4), concentrated, and the brown amorphous residue was purified by silica gel column chromatography. After several purifications oleanolic acid (**1**), maslinic acid (**2**) and daucosterol (**3**), were obtained (Fig. 1) (13). The compounds oleanolic acid acetate (**4**), methyl oleanate (**5**), 3ß-acetoxyolean-12α-bromine-(28→13)-olide (**6**) and maslinic acid diacetate (**7**) were obtained by chemical transformations (Fig. 1) (13).

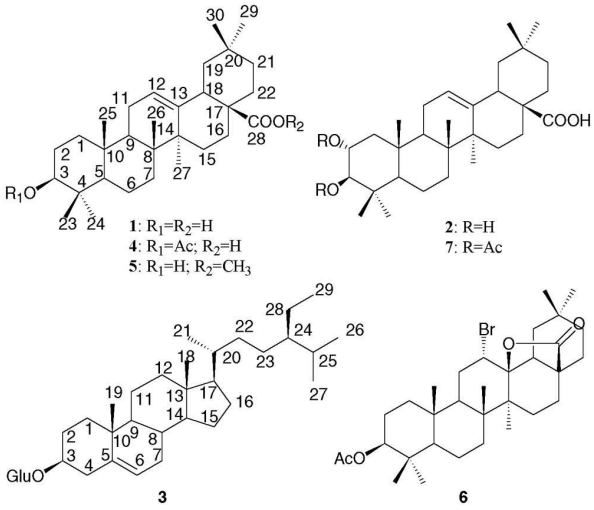

Figure 1. Chemical structure of triterpenoids 1–7.

Spectroscopic data

Identities of **1**, **2** y **3** were confirmed by comparison of both spectroscopic and physical data with previously published values (1,3,5). Oleanolic acid acetate (**4**): ^{1}H RMN (200 MHz, $CDCl_3$) δ 5.26 (*brs*, 1 H, H_{12}), δ 4.50 (*t*, *J*= 10.0; 6.0 Hz, 1 H, H_3), δ 2.81 (*dd J*= 13.5; 4.5 Hz, 1H, H_{18}) and δ 2.05 (*s*, 3 H, Ac); ^{13}C NMR (50 MHz, $CDCl_3$) δ 183.5 (C_{28}), 171.1 (C=O, Ac), 143.6 (C_{13}), 122.5 (C_{12}), 80.9 (C_3), 55.4 (C_5), 47.6 (C_9), 46.6 (C_{17}), 45.7 (C_{19}), 41.6 (C_{14}), 41.0 (C_{18}), 39.4 (C_8), 38.1 (C_1), 37.7 (C_4), 37.0 (C_{10}), 33.6 (C_{21}), 33.1 (C_{29}), 32.6 (C_7), 32.5 (C_{22}), 30.7 (C_{20}), 28.1 (C_{23}), 27.7 (C_{15}), 25.9 (C_{27}), 23.6 (C_{30} and C_2), 23.4 (C_{11}), 22.9 (C_{16}), 21.3 (CH_3, Ac), 18.2 (C_6), 17.2 (C_{24}), 16.7 (C_{26}) and 15.2 (C_{25}). Methyl oleanate (**5**): ^{1}H RMN (200 MHz, $CDCl_3$) δ 5.26 (*t*, *J*= 3.5 Hz, 1 H, H_{12}), δ 3.62 (*s*, 3 H, O-CH_3), δ 3.23 (*dd*, *J*= 10.0; 6.0 Hz, 1 H, H_3) and δ 2.85 (*dd J*= 13.5; 4.5 Hz, 1H, H_{18}); ^{13}C NMR (50 MHz, $CDCl_3$) δ 177.9 (C_{28}), 143.8 (C_{13}), 122.3 (C_{12}), 80.0 (C_3), 55.2 (C_5), 47.6 (C_9), 46.6 (C_{17}), 45.8 (C_{19}), 41.6 (C_{14}), 41.3 (C_{18}), 39.3 (C_8), 38.7 (C_4), 38.5 (C_1), 37.0 (C_{10}), 33.8 (C_{21}), 33.1 (C_{29}), 32.6 (C_7), 32.3 (C_{22} and O-CH_3), 30.6 (C_{20}), 28.1 (C_{23}), 27.7 (C_{15}), 27.1 (C_2), 26.0 (C_{27}), 23.6 (C_{30}), 23.4 (C_{16}), 23.1 (C_{11}), 18.3 (C_6), 16.8 (C_{26}), 15.6 (C_{24}) and 15.3 (C_{25}). 3ß-Acetoxyolean-12α-bromine-(28→13)-olide (**6**): ^{1}H-NMR (200 MHz, $CDCl_3$) δ 4.51 (*dd*, *J*= 9.1, 5.2 Hz, 1 H, H_3), δ 4.30 (*brs*, 1 H, H_{12}) and δ 2.08 (*s*, 3 H, Ac). ^{13}C-NMR (50 MHz, $CDCl_3$) δ 178.9 (C_{28}), 171.0 (C, Ac), 91.6 (C_{13}), 80.5 (C_3), 56.1 (C_{12}), 55.2 (C_5), 52.2 (C_{18}), 45.4 (C_9 and C_{17}), 43.3 (C_{14}), 42.3 (C_8), 39.8 (C_{19}), 37.9 (C_4), 37.7 (C_1), 36.4 (C_{10}), 33.8 (C_{22}), 33.2 (C_{20}), 33.0 (C_{29}), 31.8 (C_7), 30.5 (C_{21}), 30.3 (C_{15}), 29.1 (C_{23}), 27.8 (C_{11}), 27.4 (C_{27}), 23.5 (C_2), 23.4 (C_{30}), 21.3 (C_{16}), 21.1 (CH_3, Ac), 19.0 (C_{24}), 17.5 (C_6), 17.0 (C_{26}) and 16.4 (C_{25}). Maslinic acid diacetate (**7**): ^{1}H RMN (200 MHz, $CDCl_3$) δ 5.20 (*brs*, 1 H, H_{12}), δ 4.90 (*m*, 1 H, H_2), δ 4.20 (*d*, *J*= 9.5 Hz, 1 H, H_3), δ 2.08 (*s*, 3 H, Ac) and δ 2.01 (*s*, 3 H, Ac); ^{13}C NMR (50 MHz, $CDCl_3$) δ 180.7 (C_{28}), 171.3 (C=O,

Ac), 170.9 (C=O, Ac), 143.7 (C_{13}), 121.9 (C_{12}), 83.3 (C_3), 68.4 (C_2), 55.9 (C_5), 48.2 (C_9), 47.7 (C_{17}), 46.6 (C_{19} and C_1), 42.0 (C_{14}), 41.9 (C_{18}), 39.8 (C_8 and C_4), 38.5 (C_{10}), 34.3 (C_{21}), 33.7 (C_7), 33.3 (C_{29} and C_{22}), 30.9 (C_{20}), 29.3 (C_{23}), 28.3 (C_{15}), 26.2 (C_{27}), 23.8 (C_{30} and C_{11}), 23.6 (C_{16}), 21.8 (CH_3, Ac), 21.1 (CH_3, Ac), 18.8 (C_6), 17.5 (C_{24} and C_{26}) and 16.8 (C_{25}).

Human DNA topoisomerase I assay

Topoisomerase I activity was determined by relaxation of supercoiled pBr 322 plasmid DNA. The reaction mixture in 20 µl contained 35 mM Tris-HCl, pH 8, 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin, 0.64 µg/20 µl supercoiled pBr 322 DNA, 1.5 U human DNA topoisomerase I and 1 µl of test compound diluted with DMSO. The reaction mixture was incubated for 30 min at 37 ºC and stopped by addition of 5 µl of a mixture of 0.1% bromophenol blue, and 25% glycerol. Inhibition positive controls were performed using camptothecin. Reaction products were submitted to 45 min electrophoresis on a 1% agarose gel in TAE (Tris-acetate-EDTA) running buffer at 60 V. Gels were stained with ethidium bromide (0.5 µg/ml) for 60 min. IC_{50} values were determinate by interpolation from plots of enzyme activity *vs.* inhibitor concentration. The IC_{50} values are means from at least three independent experiments and standard deviation never exceeded 20% (9,17,18,19).

PCR assays

The assayed compounds were dissolved in DMSO. The PCR master mixture consisted of 40 mM Tris-acetate pH 8.3 containing 1 mM EDTA, 25 mM $MgCl_2$, 4 U of *Taq* DNA polymerase, 20 mM each oligonucleotide primer, and 2.5 mM each deoxynucleotide triphosphate (dNTP). PCR assays were performed in 20 µl reaction volume. The experiments were performed with p-GEMT-AT_2 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'. The conditions consisted of 35 cycles of denaturation at 95 ºC for 1 min followed by primer annealing at 56 ºC and primer extension at 72 ºC for 2 min. Inhibition positive controls were performed using dideoxynucleotides (ddNTP) set. The amplified DNA sequences were electrophoresed for 45 min in 1% agarosa gel at 60V in TAE (Tris-acetate-EDTA) running buffer containing 40 mM Tris-acetate, 2 mM $Na_2EDTA.2H_2O$, and 0.3 µg of ethidium bromide per ml (14,15).

Analysis of topoisomerase and PCR products. Relative intensities of ethidium bromide stained 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.

Cells, culture and plating

The human solid tumor cell lines A2780 (ovarian cancer), SW1573 (non-small cell lung cancer, NSCLC), WiDr (colon cancer), T-47D (breast cancer) and HBL-100 (breast cancer) were used in this study. Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum and 2 mM *L*-glutamine in a 37 ºC, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic containing medium (100 U penicillin G and 0.1 mg of streptomycin per ml). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to give the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 µl per well at densities of 7,000 (A2780), 6,000 (SW1573 and HBL-100), 12,000 (WiDr) and 15,000 (T-47D) cells per well, based on their doubling times.

Chemosensitivity testing

Chemosensitivity tests were performed using the sulforhodamine B (SRB) assay of the NCI with slight modifications (7). Pure compounds were initially dissolved in DMSO at 400 times the desired final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicates at different dilutions in the range 1–100 µM. The drug treatment was started on day 1 after plating. Drug incubation times were 48 h, after which time cells were precipitated with 25 µl ice-cold 50% (w/v) trichloroacetic acid and fixed for 60 min at 4 ºC. Then the SRB assay was performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing medium. The percentage growth (PG) was calculated with respect to untreated control cells (C) at each of the drug concentration levels based on the difference in OD at the start (T_0) and end of drug exposure (T), according to NCI formulas. Briefly, if T is greater than or equal to T_0 the calculation is $100 \times [(T-T_0)/(C-T_0)]$. If T is less than T_0 denoting cell killing the calculation is $100 \times [(T-T_0)/(T_0)]$. With these calculations a PG value of 0 corresponds to the amount of cells present at the start of drug exposure, while negative PG values denote net cell kill.

RESULTS

The action of the triterpenoids (**1-7**) as human DNA topoisomerase I and *Taq* DNA polymerase inhibitors was studied. The conversion of supercoiled plasmid DNA to relax DNA by human topoisomerase I was examined (Fig. 2).

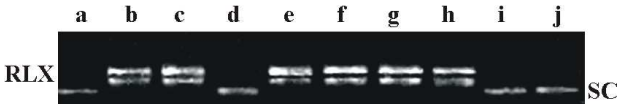

Figure 2. Effect of compounds 1-7 on the DNA relaxation activity by human DNA topoisomerase I. Lane a, supercoiled DNA alone (no drug, no enzyme); lane b, negative control (no drug); lanes c, d, e, f, g, h and i, 200 µM of 1, 2, 3, 4, 5, 6 and 7, respectively; lane j, positive control (camptothecin, 2 µM) SC: supercoiled DNA and RLX: relaxed DNA topoisomers.

Compounds **2** and **7** showed complete inhibitory activity against human DNA topoisomerase I at a concentration of 200 µM. The remaining compounds do not have inhibitory

activity at that concentration. The inhibitory effects of compounds **2** and **7** are dose-dependent with IC_{50} values of 76.6 and 79.3 μM, respectively (Table 1).

Table 1. IC_{50} Values of triterpenoids on topoisomerase I catalytic activity

Compounds	IC_{50}, μM
1	>200.00
2	76.60
3	>200.00
4	>200.00
5	>200.00
6	>200.00
7	79.30

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.

None of these compounds was able to inhibit DNA polymerisation by *Taq* DNA polymerase using a PCR assay (13,14) at a concentration of 200 μM (Fig. 3).

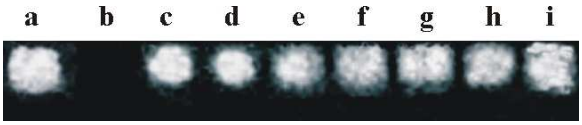

Figure 3. Effect of compounds 1-7 against *Taq* DNA polymerase. Lane a, negative control (no drug); lane b, positive control (ddNTP); lanes c, d, e, f, g, h and i, 200 μM of 1, 2, 3, 4, 5, 6 and 7, respectively.

Additionally, we evaluated maslinic acid (**2**) and maslinic acid diacetate (**7**) for growth inhibition against the human solid tumor cell lines A2780, SW1573, WiDr, T-47D and HBL-100 after 48 h of drug exposure. The antiproliferative activities for each compound expressed as GI_{50} are reported in Table 2. The overall results showed that compounds **2** and **7** inhibit proliferation of all cell lines with GI_{50} values in the range 5–18 μM.

Table 2. Growth inhibition (GI_{50}) against human solid tumour cells.[a]

Compounds	Cell line				
	A2780	SW1573	WiDr	T-47D	HBL-100
2	15±0.4	16±3.5	18±1.6	18±0.9	16±3.1
7	5.6±1.2	12±6.3	11±0.1	11±1.6	11±6.4

[a]Values are given in μM ± standard deviation and are means of two to three experiments.

DISCUSSION

Human DNA topoisomerase I catalyses the intraconversion of topological states of DNA, breaking and rejoining only one of the two strands by a transesterification reaction. For the cleavage reaction, the active site tyrosine residue attacks the scissile DNA phosphodiester bond to produce a covalent intermediate and expulses the leaving 5'-hydroxy polynucleotide strand. After the relaxation of superhelical tension in DNA by the enzyme (through a controlled rotation mechanism), the relinking reaction proceeds by the attack of the covalent intermediate by the 5'-OH strand partner to form a phosphodiester bond and expel the tyrosine. The successive cleavage/religation reactions preserve the stereochemistry of the phosphodiester bond. Arg ^{488}, Arg ^{590}, Lys ^{532} and His ^{652} are the key amino acids implicated in the enzyme-DNA bonding and cleaving process (2). Flavones are known to be weak inhibitors of both DNA polymerase and topoisomerase II (16). We would, however, like to emphasize here that the flavone at first intercalates into the DNA molecule as a template-primer, and subsequently inhibits both activities indirectly through the induction of a conformational change in the DNA. Besides, it has been demonstrated that penta-cyclic triterpenes such as compounds **2** and **7** effected no thermal transition of melting temperature, thus, none of the triterpenes bound to the dsDNA, suggesting that they must inhibit the enzyme activities by interacting with the enzymes directly (8). On the other hand, DNA topoisomerases and DNA polymerases have structural homology at the DNA-binding sites (9). Taking into account this similarity we could postulate that compounds **2** and **7** are not inhibiting the DNA topoisomerase I activities by interacting with the DNA binding site because if they were acting this way, they should inhibit both enzymes.

Our results suggest that the 2α-oxygen-bearing groups of compounds **2** and **7** might be important for the inhibitory activity because compounds **1** and **4**, which have the same framework, do not show an inhibitory effect. This result demonstrates that the absence of 2α-oxygen-bearing groups produces a dramatic decrease of DNA topoisomerase I inhibitory activity. However, in order to determine the mechanism of action of compound **2** more and specific experiments will be necessary.

It is evident from Tables 1 and 2 that the interaction between human topoisomerase I and the assayed compounds correlate well with the biological activities against the human cancer cell lines, these results suggest that DNA topoisomerase I is possibly the cellular target for compounds **2** and **7**. The biggest value in GI_{50} observed for compound **7**, is probably because of the higher lipophilicity of compound **7** in comparison to the compound **2**.

In summary, these results suggest that maslinic acid (**2**) and maslinic acid diacetate (**7**) behave as DNA topoisomerase I inhibitors and growth inhibitors against human cancer cell lines. This kind of compound could be a tool, not only to investigate DNA topoisomerases I activities but also, might be useful in the development of new anticancer agents by computer-simulated drug design based on the chemical structure of these agents.

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