Molecular Pharmacology Fast Forward. Published on February 9, 2005 as doi:10.1124/mol.104.001776

Modulation of cellular response to cisplatin by a novel inhibitor of DNA polymerase β

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Running title: Inhibition of DNA polymerase β

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Number of text pages: 25

Number of figures: 4

Number of tables: 1

Number of References: 47

Words in the abstract: 206

Words in the Introduction: 844

Words in the Discussion: 875

List of abbreviations: Pol β (DNA polymerase β), trans-communic acid (CA), masticadienonic acid (MA), mahureone A (MH), base excision repair (BER), translesion synthesis (TLS)

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Abstract

DNA polymerase β (Pol β) is an error-prone enzyme whose up-regulation has been shown to be a genetic instability enhancer as well as a contributor to cisplatin resistance in tumor cells. In this work we describe the isolation of new Pol β inhibitors after high throughput screening of 8,448 semi purified natural extracts. *In vitro* the selected molecules affect specifically Pol β-mediated DNA synthesis as compared to replicative extracts from cell nuclei. One of them, masticadienonic acid (MA), is particularly attractive since it perturbs neither the activity of the purified replicative Pol δ nor that of nuclear HeLa cell extracts. With an IC_{50} value of 8 μM, MA is the most potent of the Pol β inhibitors known to-date. Docking simulation revealed that this molecule could substitute for single strand DNA in the binding site of Pol β by binding Lys35, Lys68 and Lys 60 which are the main residues involved in the interaction Pol β/single-strand DNA. Selected inhibitors also affect the Pol β-mediated translesion synthesis across cisplatin adducts, MA being still the most efficient. As a consequence masticadienonic acid sensitised the cisplatin resistant 2008 C13*5.25 human tumour cells. Our data suggest that molecules such as masticadienonic acid could be suitable in conjunction with cisplatin to enhance anticancer treatments.

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Introduction

DNA polymerase β (Pol β) is the smallest human DNA polymerase with a molecular mass of 39 kDa. It contains two domains, first an N-terminal fragment (8 kDa) which possesses the binding affinity for single strand and double strand DNA, 5'-phosphate recognition in gapped DNA and dRP lyase activity, and second a C-terminal domain (31 kDa) which contains the catalytic activity. In somatic cells Pol β is an essential enzyme of the base excision repair (BER) and single strand break (SSB) repair pathways (Hubscher et al., 2002). We also demonstrated (Bergoglio et al., 2001; Canitrot et al., 1998) that Pol β overexpression allows cells to tolerate bifunctional DNA damage generated for example by cisplatin, which is used in the treatment of many cancers (Pil and Lippard, 1997). Indeed Pol β facilitates the error-prone translesion replication of these adducts which otherwise would block the replicative machinery and kill the cells (Canitrot et al., 1998; Hoffmann et al., 1995). A large number of mechanisms can contribute to cisplatin resistance such as decreased drug uptake, enhanced repair of the adducts or increased levels of detoxifying agents like glutathione (Andrews and Howell, 1990). The resistant phenotype of 2008 C13*5.25 cells used in this study, generated from 2008 human ovarian carcinomas by *in vitro* selection with cisplatin (Andrews et al., 1985) has been attributed to impaired uptake, decreased DNA platination (Jekunen et al., 1994), as well as translesion synthesis across cisplatin adducts. Such replicative bypass has been measured either indirectly by quantifying radiolabeled nascent DNA (Mamenta et al., 1994) or more directly by showing that extracts from these cells displayed an enhanced Pol β-mediated capacity to replicate a DNA oligonucleotide carrying a cisplatin adduct (Bergoglio et al., 2001). Moreover, down-regulation of *pol β* by siRNA clearly sensitised cisplatin-resistant Hela and SKOV-3 cancer cells to cisplatin (M. Albertella, KuDOS, UK, communicated in J. Monod conference, Roscoff, 2004).

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In addition to its contribution to the mutagenic translesion process, up-regulated Pol β induces an overall untargeted genetic instability by competing with replicative DNA polymerases in DNA transactions where normally it is not involved (Canitrot et al., 2000; Servant et al., 2002a). It thus contributes to the emergence of variant clones with a proliferating phenotype (Bergoglio et al., 2003; Bergoglio et al., 2002; Canitrot et al., 1998; Canitrot et al., 1999; Servant et al., 2002a). In accordance with these data, inhibition of Pol β-mediated DNA synthesis by using dideoxycytidine, which is efficiently incorporated into DNA by this enzyme (Copeland et al., 1992), increased the survival of mice previously inoculated with Pol β-overexpressing melanomas (Louat et al., 2001).

Pol β shares infidelity and TLS capacity with the newly-discovered error-prone DNA polymerases such as Pol κ, η, ζ, ι, θ, μ and λ (Radman, 1999), which seem also devoted *in vivo* to help cells to tolerate DNA damage. Among those "adaptive" DNA polymerases, Pol λ, whose primary sequence is very close to that of Pol β, also possesses an intrinsic 5'-deoxyribose-5'-phosphate lyase function (Garcia-Diaz et al., 2001) and a distributive DNA synthesis activity (Garcia-Diaz et al., 2002). Moreover Pol λ can substitute *in vitro* Pol β in BER, suggesting that it also participates to this pathway (Garcia-Diaz et al., 2001). In this work we have searched for natural compounds that can selectively inhibit Pol β as i) tools to investigate its specific role *in vivo* by distinguishing it from other DNA polymerases such as replicative or beta-like DNA polymerase and ii) new potential drugs likely to reverse the Pol β-associated tumor resistance to cisplatin. To our knowledge, only two Pol β inhibitors have been considered to-date as specific i.e. prunasin (IC_{50} of 93μM), isolated from a red Perila and a mugwort (Mizushina et al., 1999) and solanapyrone A (IC_{50} of 30μM), a plant phytotoxin (Mizushina et al., 2002). After screening 8,448 semi purified extracts from plants and marine organisms, we purified from *Juniperus communis, Pistacia lentiscus*, and *Mahurea palustris* the three inhibitors *trans*-communic acid (CA), masticadienonic acid (MA) and the novel

mahureone A (MH), respectively. MA was the most effective inhibitor (IC_{50} of 8 μM) identified to-date. Moreover it only poorly altered the action of either the replicative Pol δ or that of HeLa cell nuclear extracts. In addition, *pol β*–deficient mouse embryonic fibroblasts (MEF) were not sensitive to the action of MA. In contrast MA alters the response to cisplatin of isogenic wild type MEF cells, conferring to these cells a *pol* $\beta^{-/-}$ phenotype. Finally, MA inhibited the Pol β-mediated translesion DNA synthesis across a cisplatin lesion. We showed by docking that MA could compete with the single strand DNA (ssDNA) for binding into the ssDNA binding site of Pol β. MA potentially binds three lysine residues (Lys35, Lys60 and Lys 68) involved in the ssDNA binding activity of the amino-terminal 8-kDa domain (Prasad et al., 1998). Cellular experiments showed that treatment by MA in the presence of cisplatin sensitised the Pol β-overexpressing-cisplatin-resistant 2008 C13*5.25 human tumour cells whereas it does not alter the response to cisplatin of *pol β* minus MEF cells. In summary we have isolated a molecule likely to target the Pol β-directed transactions and thus reduce tumoral cisplatin resistance.

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Material and Methods

Plant extracts and compounds. Extracts from either the stems of *J. communis* and *P. lentiscus* or the leaves of *M. palustris* were prepared by overnight maceration in ethyl acetate (1/10 w/v), filtration and evaporation. These extracts (50 mg) were first coarsely fractionated on 1.5 mg (6ml) SPE SiO_2 cartridges Upti-clean (Interchim) using hexane, and a gradient of methanol in chloroform as eluents. Aliquots were dissolved in 100% DMSO and diluted 1/1200 in the enzyme assay. Then 50 g of the plant material were submitted to a bioguided fractionation by combining chromatographies (normal and reverse C18 phases) to lead respectively to 50 mg of CA, 60 mg of MA and 400 mg of MH as pure compounds. These products were dissolved in appropriated buffer to achieve 10^{-2} M to 10^{-7} M solutions.

Cells and cell extracts. The *pol β* null and the corresponding wild-type mouse embryonic fibroblasts (MEF) were purchased from ATCC and described previously (Sobol et al., 1996). They were cultivated in DMEM. Human 2008 and 2008 C13*5.25 cells were grown as described in RPMI1640 medium (Bergoglio et al., 2001). HeLa cells (ATCC) were grown as spinner cultures in the same medium. Replicative extracts were prepared from either Hela or MEF cells as described (Roberts et al., 1993) after harvesting cells in the upper part of their log phase growth.

Enzymes. Rat Pol β for the HTS was purified as described (Skandalis and Loeb, 2001). The 8-kDa domain of human Pol β (residues 1-88) was cloned into pIVEX2.4d (Roche) to obtain the p1968 plasmid which was sequenced and introduced into BL21(DE3) bacteria before purification as described (Skandalis and Loeb, 2001). Purification of Pol κ was carried out by GTP Technology (Toulouse, France). Human Pol β for replication assays was purchased from Trevigen (USA). Pol α was purchased from ChimerX. The recombinant four subunits of Pol δ were isolated from infected insect baculovirus Sf9 cells as described (Podust et al., 2002). Full length recombinant single subunit Pol λ have been purified according to

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Ramadan et al. (Ramadan et al., 2003). Purification of the Human Immunodeficiency Virus reverse transcriptase (HIV RT) was as published (Preston et al., 1988).

Micro-assay for HTS. For HTS the DNA polymerase activity was determined as the amount of fluorescein-12-dCTP incorporated in a 60-mer-biotinylated-oligonucleotide template hybridized to a 5' 17-mer synthetic primer. This substrate was immobilised in a streptavidin coated combiplates C8 (Thermolabsystem). The standard reaction mixture (100 µl) contained 25 mM Hepes (pH 8.5), 5 mM $MgCl_2$, 125 mM NaCl, 25 pmol biotinylated hybridised oligonucleotide and 5 µg rat Pol β in the presence of extracts or compounds. The reaction was started with the simultaneous addition of 10 µM dNTP and 1µM fluorescein-12-dCTP. Incubation was for 150 minutes at 37°C and the products were washed three times with 200 µl of 25 mM Hepes (pH 8.5), 5 mM $MgCl_2$, 125 mM NaCl, 0.05% (v/v) Tween. The fluorescence was measured in a Fluostar fluorimeter (BMG). The HTS experiments were run on a Beckman Sagian system. Plate handling was performed with the ORCA (Optimized Robot for Chemical Analysis) robotic arm. Each positive fraction was manually controlled with the same protocol.

DNA replication assay

Cell extracts preparation and replication conditions for these extracts were previously described (Servant et al., 2002a). All purified DNA polymerases were tested by using specific conditions for their optimal activity. One unit of DNA polymerase corresponds to 1 pmol of dNTP incorporated into acid-insoluble materials at 37°C in 60 min, by using as a substrate an activated calf thymus DNA pre-incubated with DNAse I. Calf thymus DNA pre-incubated with DNase I (activated DNA from Sigma) was used as a template in a final volume of 20 µl at 37°C for various incubation times in the presence of inhibitors. Human Pol β (0.5 units) was added to a reaction buffer containing 25 mM Hepes KOH pH 8.5, 10mM $MgCl_2$, 25 mM NaCl, 1mM DTT, 100 µM of each dATP, dGTP, dCTP, 0.2 µCi dTTP and 1 µg template.

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Wild-type human Pol λ (0.5 unit) was used in 50 mM Tris pH 8.5, 50 mM NaCl, 1 mM DTT, 20 μg/ml BSA, 0.75 mM $MnCl_2$, 5 μM of each dATP, dGTP, dCTP, 0.2 μCi [^{3}H]dTTP and 1 μg template. The assay conditions with human Pol δ (0.05 units), Pol κ (1.5 unit), Pol α (1 units) or HIV RT (1 unit) were 50 mM Tris pH 6.5, 1 mM DTT, 0.25 mg/ml BSA, 6 mM $MgCl_2$, 4.6 μg/ml PCNA (if Pol δ), 100μM of each dATP, dGTP, dCTP, 0.2 μCi [^{3}H]dTTP and 1 μg template. The amounts of DNA polymerases were chosen in order to obtain comparable nucleotide incorporation without inhibitor. After incubation, reaction was stopped with 10 mM EDTA and the radioactive DNA products were collected on GF/C glass fibre filters (Whatman). Filters were washed twice with 0.5% TCA solution and then used for determination of radioactivity. IC_{50} was calculated by using GraphPad Prism software (San Diego, CA).

Docking of masticadienonic acid on human DNA polymerase β

Molecular modelling was performed by using SYBYL 6.9 (from TRIPOS Inc., St Louis, MO) by running on a Silicon Graphics Octane2 workstation. Docking calculations were performed utilising FlexX, version1.11. The FlexX scoring function was used during the complex construction phase. DrugScore as implemented in FlexX reranked the obtained solutions. Thirty docking solutions were generated. These solutions were checked to find the solution with the best score that matched the original pharmacophore hypothesis. Figures were generated using Grasp (Nicholls et al., 1991) or Pymol (http://www.pymol.org).

Translesion synthesis assay

The unmodified or cisplatin-modified 60-mer template were prepared as described (Hoffmann et al., 1995) and hybridised to a 5'-^{32}P-labeled 17-mer primer. Translesion reactions were carried out as described (Bergoglio et al., 2001). At the end of the reaction, the samples were denatured for 10 min at 70°C and loaded onto a 15 % polyacrylamide / 7 M urea / 30 % formamide gel.

Proliferation and clonogeny assays

For growth rate analysis, the control MEF and MEF *pol* $\beta^{-/-}$ cells were seeded at 300 and 600 cells per well in 6-well dishes, respectively. Cells were allowed to attach overnight at 34°C in a MEM medium supplemented with glutamine/10% foetal calf serum/penicillin/streptomycin and treated with cisplatin (Sigma) for 1h at 37°C and /or masticadienonic acid at various concentrations during 6 days. Cells were then trypsinised and counted. Cellular proliferation was expressed as the ratio of the cell number in the untreated wells versus that in the treated wells.

Cytotoxicity for the 2008 and 2008/C13.25 cells was determined by a clonogenic assay as described (Bergoglio et al., 2001). For resistance reversion assays, cells were treated with inhibitors at DL_{20} all experiment long, cisplatin (Sigma) being administrated for 1h at 37°C 24h after plating.

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Results

High throughput screening (HTS) of partially purified plant extracts.

An automated assay was designed to identify Pol β inhibitors by selecting compounds that decreased the incorporation of fluorescent nucleotides into a DNA template. The ddCTP chain terminator, which inhibits DNA synthesis mediated by Pol β (Bouayadi et al., 1997) was used as a reference (Fig. 1A). The "z' factor" determination proposed by Zhang et al (Zhang et al., 1999) is an indicator of the performance of the assay system. In the present case we measured a z' factor average value of 0. 55. During the run, the interplate coefficient of variability (CV) of the DMSO control signal value averaged 7.5 % (data not shown). This simple assay enabled the screening of 8,448 partially purified natural extract fractions, from which 71 active extracts were identified. Subsequent ^{32}P-labelled-primer extension procedure was carried out with Pol β and replicative CHO cell extracts to select specific inhibitory extracts. Three of them were selected (Fig 1A): *Juniperus communis* (EX1), *Pistacia lentiscus* (EX2), and *Mahurea palustris* (EX3) from which we isolated and purified the corresponding active molecules, *trans*-communic acid, masticadienonic acid and mahureone A, respectively.

Structural characterization of the three inhibitors

The compound isolated from *Juniperus communis* (Cupressaceae) was found to be a ubiquitous diterpene of the labdane series: *trans* communic acid (CA). Its structure (Fig. 1B) was established by comparison of its spectral data (1 and 2D NMR and MS) with that from an authentic sample (Arya et al., 1961). A second molecule was isolated in a straightforward manner from *Pistacia lentiscus* (Anarcardiaceae), a small tree growing in the Mediterranean basin and the exudate of which, known as mastic, is used as a chewing gum. The active compound was found to be masticadienonic acid (MA), 3-oxotirucalla-7, 24-dien-26-oic acid, for which the structure (Fig. 1C) was known (Barton and Soane, 1956). Worthy of note is the

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fact that the isomer isomasticadienonic acid, also present in the extract, which only differs by the position of the double bond in ring B (Δ7 vs. Δ8) is totally inactive in the test (data not shown). The structure is also very close to another Pol β inhibitor isolated by Hecht et al. which is characterised by a rare E double bond in the chain (Deng et al., 2000). The Z configuration of the Δ24 double bond in the mastic compound was ascertained by the NMR chemical shift of H-24 (6.10-ppm vs. 6.89 in the E compound). The third product was isolated from a plant from Guyana *Mahurea palustris*, a species belonging to the Guttiferae family and which has so far not been the object of chemical investigations. Mass spectrometry indicated that this molecule, for which we propose the trivial name of mahureone A (MH), had a $C_{35}H_{46}O_6$ composition (exact mass analysis) and the molecular ion was characterised by its decomposition with losses of 69 (isoprene chain) and 128 mass units. UV was not very informative and simply indicated the presence of a phenyl ring (λmax 283 nm) while IR displayed vibrations for a complex carbonyl system. Literature search showed a compound, laxifloranone (Fig. 2B), also isolated from a Guttiferae, *Marila laxiflora*, with similar data (Bokesch et al., 1999) but direct comparison of the compounds on HPLC proved that they were different. The ^{1}H and ^{13}C NMR spectra and full analysis of 2D NMR experiments led to the proposed structure (Fig. 1D), salient feature of which being the central six member ring which contained a rare α triketone (enolised form) and no less than eight contiguous quaternary carbon atoms. The validity of the structure was further supported by the isolation of four other compounds from the same series (data not shown). The differences between mahureone A and laxifloranone may be explained by a ring opening in basic medium followed by ring closure of an intermediate enol in a series of events compatible with the rule of the biosynthesis. They most probably belong to the same optical series.

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Effect of CA, MA and MH on DNA polymerase β, other DNA polymerases and replicative nuclear extracts.

To assess the efficiency of the three inhibitors on Pol β, we carried out an *in vitro* replication assay by using an activated gapped genomic DNA. DNA synthesis signals obtained with Pol β were compared to that of nuclear extracts from HeLa cells, which contain all the replicative DNA polymerases able to support bi-directional and semi-conservative genomic DNA replication, as well as to the purified replicative Pol δ and the error-prone Pol κ and Pol λ. As shown in Fig. 2 (A-C), all three molecules inhibited Pol β with IC_{50} values of 92 μM for CA, 8 μM for MA and 118 μM for MH. Nuclear extracts from HeLa cells were much more resistant to inhibition by those molecules, with IC_{50} values of 2 mM, 3 mM and 2 mM respectively. Pol δ activity was also resistant to MA, with IC_{50} values of 60 mM, whereas CA and MH inhibited Pol δ. Furthermore MH altered the activity of Pol κ and Pol λ, these enzymes being less affected than Pol β by the two other inhibitors CA and MA (Fig. 2 A-C). We also tested the inhibitory action of MA on the other replicative enzyme Pol α, involved in the synthesis of RNA/DNA primers, and found that this enzyme is less affected (IC_{50} of 93 μM) than Pol β (Fig. 2 B). Taken together, these data indicate that masticadienonic acid (MA) is an attractive Pol β inhibitor.

Effect of MA on pol β null cells

Although *pol β*-deficient null mice are not viable (Sugo et al., 2000), suggesting that correctly performed BER is highly important for maintaining development, the corresponding embryonic cells survive in culture (Sobol et al., 1996). However, pβ is likely required in responding to endogenous oxidative genotoxic stress (Horton et al., 2002), and we predicted that inhibition of this enzyme in cells would affect their survival as compared to wild type

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cells grown in the same conditions. Therefore, we used a *pol β* knockout mice fibroblast cell line and its isogenic control wild type cell and conducted growth analysis for both cell lines in the absence or presence of the inhibitor during 6 days. We found that cells lacking Pol β are less sensitive to MA as compared to the control cells, suggesting an alteration of essential Pol β- mediated DNA transactions (BER or SSB repair). This is illustrated in cellular proliferation experiments as a function of dose of MA shown in Fig 2D. As an additional control we also measured the MA activity on replicative MEF and MEF *pol* $\beta^{-/-}$ cell extracts and found that both extracts are resistant to MA (Table 1), probably because Pol β at a basal level does not belong to the replication machinery. From this experiment, we suggest that MA could alter the Pol β-mediated process of endogenous oxidative DNA damage.

MA best fits the ssDNA binding site of the 8-kDa domain of DNA polymerase β

The crystal and solution structure of the human Pol β have been determined (Maciejewski et al., 2000; Sawaya et al., 1997). We used the Pol β pdb file 1BPY without DNA to dock MA using FlexX. This software is designed to identify the positive charges likely to bind carboxylate. *In silico* analysis indicated that MA is likely to interact with the ssDNA binding site of the 8-kDa N-terminal domain of Pol β (Fig. 3A-C). Figure 3A shows the ssDNA-binding site of Pol β in the presence of the ssDNA. Figure 3B shows MA alone in the ssDNA binding site of Pol β. Figure 3C represents the superposition of ssDNA and MA, showing that the inhibitor interacts with Lys60, Lys68 and Lys35. Lys60 makes a hydrogen bond with MA and Lys68 and Lys35 make ionic contact with the carbonyl of MA. The distances between those residues and MA are 2.86, 2.85, 2.81 Å, respectively. Interestingly, Lys68 and Lys35 are known to be the main residues recognising directly ssDNA (Prasad et al., 1998). We checked that the ssDNA-binding site of Pol β does not differ in the absence of DNA by superposing the two structures (1BPY and 1DK3 without DNA, data not shown).

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Moreover we found that MA does not bind the dNTP site of the active site in the palm domain and band shift assays indicated that MA interacts with the 8-kDa domain (data not shown), further suggesting a competitive inhibiting action of MA because of its association with the ssDNA-binding site.

Effect of the three inhibitors on the translesion synthesis (TLS) across a cisplatin adduct

In vitro, Pol β efficiently replicates past a single Pt-(dGpG) lesion (Fig 4B ; compare lanes 1 and 2) during a 17-mer primer extension reaction on a platinated 60-mer template (Fig. 4A). We investigated the impact of the inhibitors during this specific Pol β-mediated bypass synthesis. We determined inhibitory concentrations able to affect the translesion synthesis (TLS) without inhibitory effect on undamaged DNA. As indicated in Fig 4B (lanes 3-8), MA is the most effective TLS inhibitor since only 30 μM MA were sufficient for altering the bypass (Fig. 4B, lanes 3-4) whereas the inhibition was achieved with 100 μM CA and 200 μM MH (Fig. 4B, lanes 5-8). It has to be noted that the presence of the adduct changes the synthesis from distributive to processive in nature. We discussed this aspect in previous papers (Hoffmann et al., 1996; Hoffmann et al., 1995), explaining that when the damage is present, the enzyme dissociates frequently from the adducted bases, resulting in more availability of Pol β to re-initiate primer extension and to extend most of the [3'-OH] termini to the site of the lesion. Further, the synthesis upstream the lesion is not altered in the presence of inhibitors. However concentrations of the inhibitors used for this TLS experiment could not be compared to those used in the calf thymus activated DNA assay (Fig 2) since the nature of DNA substrates are different and the ratio [Pol β / 3'hydroxy termini of the DNA substrate] is much lower in the latter case.

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Cell resistance to cisplatin in the presence of inhibitors

We previously demonstrated (Bergoglio et al., 2001) that a Pol β-dependent TLS across platinated DNA crosslinks occurred in the 2008C13*5.25 human tumour cells, known to display a tolerant phenotype towards cisplatin. We proposed that this TLS process could contribute to the cisplatin resistance phenotype exhibited by these cells as compared to the parental 2008 cells (Bergoglio et al., 2001). The potential of the three inhibitors to prevent Pol β from bypassing a cisplatin adduct *in vitro* prompted us to examine whether they could reduce the resistance to cisplatin of the 2008C13*5.25 cells. Clonogenic experiments were performed by incubating the cells with both cisplatin and a sublethal (80% cell survival) concentration of inhibitors (Fig 4C). In the presence of MA, the strongest TLS inhibitor *in vitro*, we observed a significant sensitization of 2008 C13*5.25 cells to cisplatin while the response of parental 2008 cells to cisplatin was not altered. In contrast, MA and MH, which were less efficient for inhibiting TLS *in vitro* (see Fig. 4B), had poor effect.

Finally we wondered whether the deletion of *pol β* had any impact on the ability of MA to sensitize the cells to cisplatin. Fig. 4D indicates that the response of *pol β* minus MEF cells to cisplatin was not modified by the presence of MA, whereas such response is altered in wild-type MEF cells, cisplatin survival of *pol* $\beta^{+/+}$ cells treated with MA being similar to that of *pol β* minus cells. These data are in accordance with another study recently published, showing that a synthetic Pol β inhibitor did not affect the sensitivity of *pol β* knockout MEF cells to MMS whereas it alters the cell survival of wild-type cells (Hu et al., 2004). Our results further reinforce the *in vitro* findings showing the specific anti-Pol β inhibitory action of MA.

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Discussion

We described here the identification of three new natural molecules that inhibited Pol β , an error-prone enzyme frequently overexpressed in tumor cells (Canitrot et al., 2000; Scanlon et al., 1989; Servant et al., 2002b; Srivastava et al., 1999). Among those molecules, we identified a product that we named mahureone A since it was purified from the leaves of *Mahurea palustris*. The two other compounds identified were *trans*-communic acid, which is a ubiquitous diterpene of the labdane series, isolated from *Juniperus communis*, and masticadienonic acid, from *Pistacia lentiscus*. To our knowledge, the latter is the most efficient Pol β inhibitor identified to-date, with an IC_{50} value of 8 μM. Interestingly it altered in a much lesser extent the activity of either nuclear cell extracts or purified replicative Pol α and Pol δ, or error-prone DNA polymerases κ and λ. MA could thus be of particular interest as a tool able to distinguish the biochemical features of the two closely related enzymes Pol β and Pol λ. Docking simulation shows that it could be located at the ssDNA-binding site of the 8-kDa N-terminus domain. This domain also called dRPlyase domain is only present in the X-family DNA polymerases as well as in Pol ι from the Y-family, therefore possibly explaining the specificity of inhibition compared to the replicative Pol δ. The ssDNA-binding site of Pol λ differs from that of Pol β, presenting a lower positive charge, which might explain the different IC_{50} of MA between Pol β and Pol λ. Before this work, Prunasin was considered as the best Pol β inhibitor with an IC_{50} of 98 μM (Mizushina et al., 1999). Our study reports a natural compound whose inhibitory action is more potent and preferentially targets Pol β compared to a wide range of other DNA polymerases. This was confirmed by using a *pol β*-deficient null embryonic cell line, which was less sensitive to the action of MA than its isogenic wild type cell line. Therefore, masticadienonic acid can be considered as a potential useful molecular tool for investigating the role of Pol β among all the X-family DNA

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polymerases in normal somatic cells, for example in helping to distinguish its specific role in base excision repair pathway from Pol λ.

The anticancer agent cisplatin is widely used in the treatment of ovarian, testicular, head and neck carcinomas. Its therapeutic effects result from covalent binding to DNA thus inhibiting replication and/or transcription (Pil and Lippard, 1997). Nevertheless, the cisplatin lesions are also mutagenic and could thus play a role in the generation of second tumours in cancer patients treated with this agent. Another major obstacle for the successful treatment of cancer by cisplatin is drug resistance. Most of the studies on platinum resistance have focused on decreased drug uptake, enhanced nucleotide excision repair, or loss of DNA mismatch repair (Andrews and Howell, 1990). It has also been proposed that an alternative tumor resistance may be due to an increased capacity of the cell to tolerate platinum-DNA lesions. We (Canitrot et al., 1998; Hoffmann et al., 1995) and others (Vaisman and Chaney, 2000; Vaisman et al., 1999) have shown that Pol β has the potential to efficiently catalyse error-prone translesion synthesis *in vitro* across the major intrastrand cross-link at the N-7 positions of adjacent guanine bases. In addition to alternative mechanisms such as drug uptake and DNA platination (Jekunen et al., 1994) this translesion mechanism has been proposed to contribute to the cisplatin resistance of the human ovarian carcinoma 2008/C13∗5.25 cells (Bergoglio et al., 2001; Mamenta et al., 1994), extracts from these cells displaying enhanced replicative bypass and Pol β-mediated translesion synthesis of platinum lesions. We found here that the three selected inhibitors affect the *in vitro* ability of Pol β to bypass a cisplatin adduct, MA being the most potent. As a consequence, we showed that MA decreased the ability of 2008/C13∗5.25 to adapt to cisplatin pressure. In contrast MA did not affect the response to cisplatin of MEF *pol* $\beta^{-/-}$ cells and confers to wild-type cells a pol $\beta^{-/-}$ phenotype of resistance to cisplatin. Surprisingly, we also observed in the control experiments that MA protected from cisplatin toxicity wild-type MEF cells, known to be sensitive to this agent

(Raaphorst et al., 2002). So far we cannot explain this discrepancy between cells that overexpress Pol β and cells that only express the enzyme at a basal level. However these data suggest that i) the intracellular level of Pol β protein in the response of cells to genotoxic stresses and ii) the nature of the cells (tumorigenic and human in the case of 2008C13, embryonic and murine for MEF cells), are certainly of importance in the modulation of the adjuvant effect of masticadienonic acid. Another explanation (Raaphorst et al., 2002) could be that depletion of Pol β and by extension Base Excision Repair favors recombination repair and cell survival.

From a pharmaceutical point of view, masticadienonic acid could thus be considered as a potentially useful adjuvant likely to reinforce the cisplatin action against tumours for which a Pol β overexpression « signature » would have been first diagnosed. It also could be considered as an antimutagenic drug likely to prevent side effects of cisplatin administration *i.e.* emergence of secondary tumours in a context of new individual therapeutic strategies.

Acknowledgements

We acknowledge very much S.B.Howell (San Diego) for the gift of 2008 and 2008C13 cells. We also thank V. Masson (CCP, Toulouse) and K. Ramadan (IVBMB, Zurich) for their technical assistance. C. Moretti (IRD, Cayenne) collected Mahurea.

Molecular Pharmacology Fast Forward. Published on February 9, 2005 as DOI: 10.1124/mol.104.001776
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Footnotes : C Cazaux and JS Hoffmann are both corresponding authors. This work was supported by la Ligue Nationale Contre le Cancer (Labellisation d'équipe), le Ministère de la Recherche et de l'Education Nationale (ACI Nouvelles cibles thérapeutiques) and le Plan Cancer (Cancéropôle Grand Sud-Ouest). F. Boudsocq was supported by the Association de Recherche contre le Cancer and the Fondation pour la Recherche Médicale. U. Hübsher, E. Friedrich-Heinecken and I. Shevelev are supported by the Swiss National Foundation (grant 31-61361.00) and by the Kanton of Zürich.

Legends of figures

Figure 1 : (A) HTS to screen for Pol β inhibitors. *Juniperus communis* (EX1), *Pistacia lentiscus* (EX2), and *Mahurea palustris* (EX3) fraction activity measurement. All fractions were dissolved in 100% DMSO and diluted 1200 times into the assay. Each value represents the mean ± s.d. of three separate experiments. Control experiment was carried out with ddCTP (10μM). **(B-D) Chemical structures of the isolated compounds** : trans-communic acid (CA) **(B) ;** masticadienonic acid (MA) **(C)** ; and mahureone A (MH) **(D).**

Figure 2 : Effect of CA **(A)**, MA **(B)** and MH **(C)** *in vitro* on Pol β, Pol δ, Pol κ, Pol λ, and nuclear extracts. 100% represents the maximal activity in the control without inhibitor. IC_{50} was calculated by using a GraphPad Prism software. Each value represents the mean ± s.d. of three separate experiments in triplicate. **(D):** Viability of pol $\beta^{-/-}$ and isogenic wild type MEF cells in the presence of MA. Cells were exposed to MA at various concentrations for one week and counted. Each value represents the mean ± s.d. of three separate experiments performed in duplicate.

Figure 3 : (A-C) Structure of Pol β and docking simulation. (A) Electrostatic surface potential of the crystal structure of the human Pol β in the presence of ssDNA (1BPY). Blue and red represent positive and negative potentials, respectively. The 5'-phosphate of the ssDNA is situated between Lys68 and Lys35. **(B)** Docking simulation of MA interaction interface with the ssDNA-binding site of Pol β. **(C)** Superposition of ssDNA (carbon in orange) and MA (carbon in green) with the main residues recognising ssDNA i.e. Lys60, Lys68 and Lys35 (carbon in white). Grasp generated panels B and C and panel D was generated by Pymol.

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Figure 4 : (A) Sequence of the single-stranded DNA damaged with a unique cisplatin adduct used in the primer extension reactions **(B)** Translesion synthesis of a single Pt-d(GpG) adduct by Pol β in the presence of trans-communic acid (CA), masticadienonic acid (MA) and mahureone A (MH). The concentrations used were 10^{-4} M (CA), 3.10^{-5} M (MA) and $2.\ 10^{-4}$ M (MH) inhibitors. Arrows indicate positions of the 17-mer (primer), 39-mer (site of the lesion) and 60-mer (full-size product). **(C) (D)** Viability of 2008/2008 C13*5.25 (C) or MEF/pol β knockout MEF cells (D) in the presence of cisplatin and CA, MA or MH. Cells were treated for 1 hour by cisplatin then continuously exposed to inhibitors at concentration corresponding to DL_{20}. After 1 week, 2008/2008C13*5.25 colonies of more than 50 cells were fixed, stained then scored whereas MEF/*pol β* knockout MEF cells were counted. Each value represents the mean ± s.d. of three separate experiments.

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Table 1: Inhibitory effect of masticadienonic acid on pol $\beta^{-/-}$ MEF cell extracts

Cell extracts	IC_{50} (μM)
MEF	15,000
MEF pol$\beta^{-/-}$	4,000

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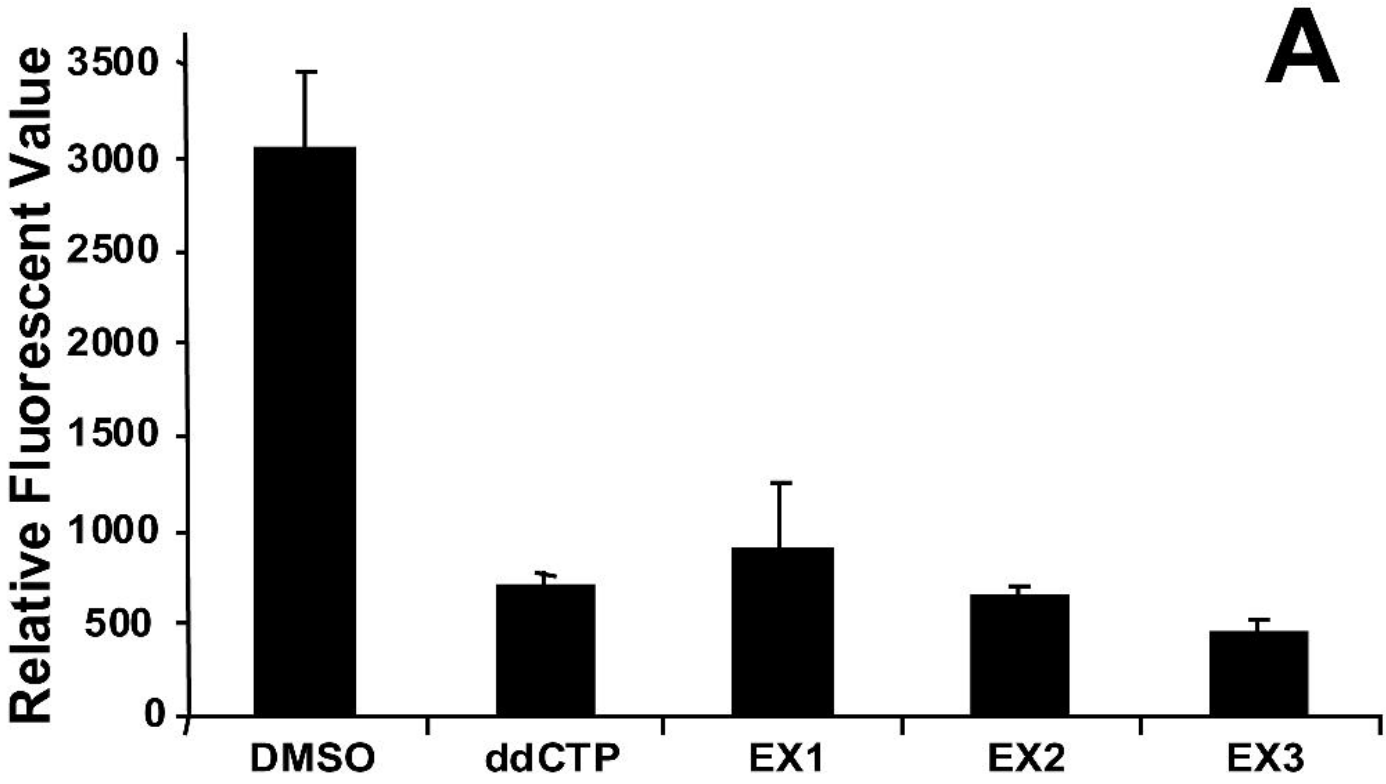
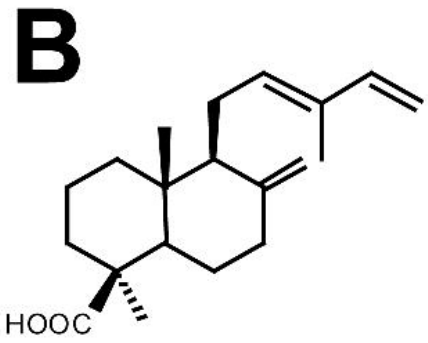
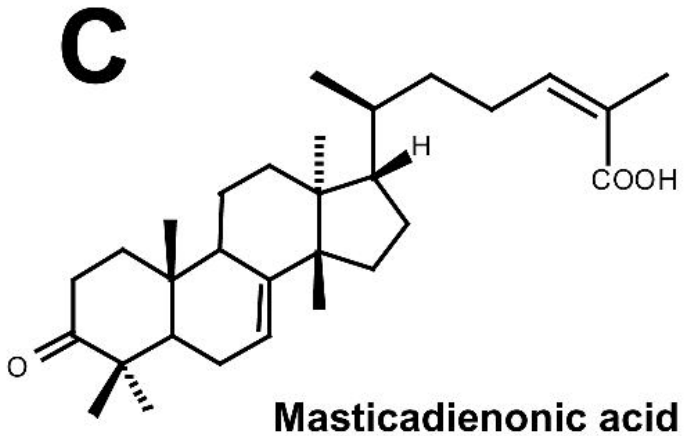
Figure 1

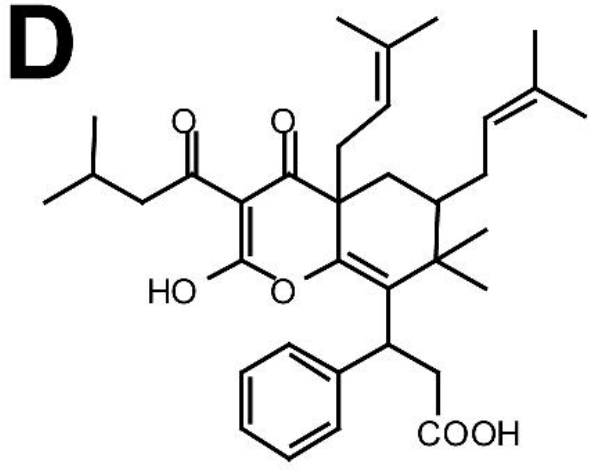




trans **Communic acid**



Masticadienonic acid



Mahureone A

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Figure 2

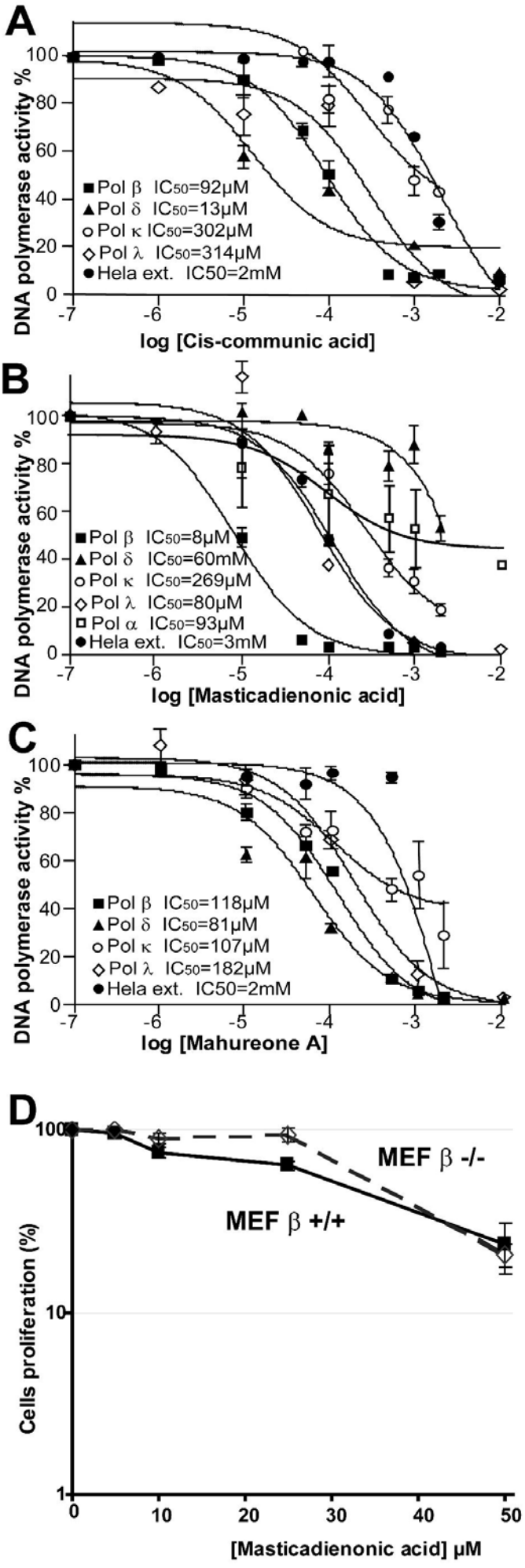

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Figure 3