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Modulation of Cellular Response to Cisplatin by a Novel Inhibitor of DNA Polymerase β

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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 8448 semipurified natural extracts. In vitro, the selected molecules affect specifically Pol β-mediated DNA synthesis compared with replicative extracts from cell nuclei. One of them, masticadienonic acid (MA), is particularly attractive because 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 found so far. Docking simulation revealed that this molecule could substitute for single-strand DNA in the binding site of Pol β by binding Lys35, Lys68, and Lys60, which are the main residues involved in the interaction Pol β/single-strand DNA. Selected inhibitors also affect the Pol β-mediated translesion synthesis (TLS) across cisplatin adducts; MA was still the most efficient. Therefore, masticadienonic acid sensitized the cisplatin-resistant 2008C13*5.25 human tumor cells. Our data suggest that molecules such as masticadienonic acid could be suitable in conjunction with cisplatin to enhance anticancer treatments.

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) that 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) that contains the catalytic activity. In somatic cells, Pol β is an essential enzyme of the base excision repair (BER) and single-strand break repair pathways (Hubscher et al., 2002). We also demonstrated (Canitrot et al., 1998; Bergoglio et al., 2001) 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 that otherwise would block the replicative machinery and kill the cells (Hoffmann et al., 1995; Canitrot et al., 1998). 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 2008C13*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

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ABBREVIATIONS: Pol β, DNA polymerase β; CA, *trans*-communic acid; MA, masticadienonic acid; MH, mahureone A; BER, base excision repair; TLS, translesion synthesis; MEF, mouse embryonic fibroblast; ssDNA, single-strand DNA; DMSO, dimethyl sulfoxide; HTS, high-throughput screening; DTT, dithiothreitol.

uptake, decreased DNA platination (Jekunen et al., 1994), and 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 small inducible RNA clearly sensitized cisplatin-resistant HeLa and SKOV-3 cancer cells to cisplatin (M. Albertella, KuDOS Pharmaceuticals, Cambridge, England, UK, unpublished data; communicated in Jacques Monod Conference, Roscoff, France, 2004).

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 (Canitrot et al., 1998, 1999; Bergoglio et al., 2002, 2003; 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 inoculated previously with Pol β-overexpressing melanomas (Louat et al., 2001).

Pol β shares infidelity and translesion synthesis 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 in this pathway (Garcia-Diaz et al., 2001). In this work, we sought natural compounds that can selectively inhibit Pol β as 1) tools to investigate its specific role in vivo by distinguishing it from other DNA polymerases such as replicative or β-like DNA polymerase; and 2) new potential drugs that are 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 93 μM), isolated from a red Perila and a mugwort (Mizushina et al., 1999), and solanapyrone A (IC_{50} of 30 μM), a plant phytotoxin (Mizushina et al., 2002)]. After screening 8448 semipurified 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 Lys68) 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 sensitized the Pol β-overexpressing cisplatin-resistant 2008C13*5.25 human tumor cells, whereas it does not alter the response to cisplatin of *pol* β minus MEF cells. In summary, we have isolated a molecule that is likely to target the Pol β-directed transactions and thus reduce tumoral cisplatin resistance.

Materials 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 (6 ml) solid-phase epitaxial SiO_2 cartridges in an Upti-clean column (Interchim, Montluçon, France) 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 was submitted to a bioguided fractionation by combining chromatographies (normal and reverse C18 phases) to lead to 50 mg of CA, 60 mg of MA, and 400 mg of MH, respectively, as pure compounds. These products were dissolved in appropriated buffer to achieve 10^{-2} to 10^{-7} M solutions.

Cells and Cell Extracts. The *pol* β null and the corresponding wild-type MEFs were purchased from the American Type Culture Collection (Manassas, VA) as described previously (Sobol et al., 1996). They were cultivated in DMEM. Human 2008 and 2008C13*5.25 cells were grown in RPMI 1640 medium as described previously (Bergoglio et al., 2001). HeLa cells (American Type Culture Collection) were grown as spinner cultures in the same medium. Replicative extracts were prepared from either HeLa or MEF cells as described previously (Roberts et al., 1993) after harvesting cells in the upper part of their log-phase growth.

Enzymes. Rat Pol β for the high-throughput screening (HTS) was purified as described previously (Skandalis and Loeb, 2001). The 8-kDa domain of human Pol β (residues 1–88) was cloned into pIVEX2.4d (Roche Diagnostics, Indianapolis, IN) to obtain the p1968 plasmid, which was sequenced and introduced into BL21(DE3) bacteria before purification as described previously (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 (Gaithersburg, MD). Pol α was purchased from CHIMERx (Milwaukee, WI). The recombinant four subunits of Pol δ were isolated from infected insect baculovirus Sf9 cells as described previously (Podust et al., 2002). Full-length recombinant single-subunit Pol λ was purified according to procedures described by Ramadan et al. (2003). Purification of the human immunodeficiency virus reverse transcriptase was done as published previously (Preston et al., 1988).

Microassay for HTS. For HTS, the DNA polymerase activity was determined as the amount of fluorescein-12-dCTP incorporated into a 60-mer biotinylated oligonucleotide template hybridized to a 5′ 17-mer synthetic primer. This substrate was immobilized in a streptavidin-coated combiplate C8 (ThermoLabsystem, Franklin, MA). The standard reaction mixture (100 μl) contained 25 mM HEPES, pH 8.5, 5 mM $MgCl_2$, 125 mM NaCl, 25 pmol biotinylated hybridized oligonucleotide, and 5 μg of rat Pol β in the presence of extracts or compounds. The reaction was started with the simultaneous addition of 10 μM dNTP and 1 μM fluorescein-12-dCTP. Incubation was for 150 min 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, and 0.05% (v/v) Tween 20. The fluorescence was measured in a Fluostar fluorimeter (BMG Labtechnologies Inc., Durham, NC). The HTS experiments were run on a Beckman Sagian system (Beck-

man Coulter, Fullerton, CA). Plate-handling was performed with the Optimized Robot for Chemical Analysis robotic arm (Beckman Coulter). Each positive fraction was manually controlled with the same protocol.

DNA Replication Assay. Cell-extract preparation and replication conditions for these extracts were described previously (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 preincubated with DNase I. Calf thymus DNA preincubated with DNase I (activated DNA; Sigma-Aldrich, St. Louis, MO) 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, 10 mM $MgCl_2$, 25 mM NaCl, 1 mM DTT, 100 μM dATP, 100 μM dGTP, 100 μM dCTP, 0.2 μCi dTTP, and 1 μg of template. 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 bovine serum albumin, 0.75 mM $MnCl_2$, 5 μM dATP, 5 μM dGTP, 5 μM dCTP, 0.2 μCi [^{3}H]dTTP, and 1 μg of template. The assay conditions with human Pol δ (0.05 units), Pol κ (1.5 unit), Pol α (1 units), or human immunodeficiency virus reverse transcriptase (1 unit) were 50 mM Tris, pH 6.5, 1 mM DTT, 0.25 mg/ml bovine serum albumin, 6 mM $MgCl_2$, 4.6 μg/ml proliferating cell nuclear antigen (if Pol δ), 100 μM dATP, 100 μM dGTP, 100 μM dCTP, 0.2 μCi [^{3}H]dTTP, and 1 μg of template. The amounts of DNA polymerases were chosen 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 fiber filters (Whatman, Clifton, NJ). Filters were washed twice with 0.5% trichloroacetic acid solution and then used for determination of radioactivity. IC_{50} value was calculated by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Docking of Masticadienonic Acid on Human DNA Polymerase β. Molecular modeling was performed by using SYBYL 6.9 (Tripos, St Louis, MO) by running on a Silicon Graphics Octane 2 workstation. Docking calculations were performed using FlexX, version 1.11 (Tripos). 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 previously (Hoffmann et al., 1995) and hybridized to a 5′-^{32}P-labeled 17-mer primer. Translesion reactions were carried out as described previously (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/well in six-well dishes, respectively. Cells were allowed to attach overnight at 34°C in a minimal essential medium supplemented with glutamine/10% fetal calf serum/penicillin/streptomycin and treated with cisplatin (Sigma-Aldrich) for 1 h at 37°C and/or masticadienonic acid at various concentrations during 6 days. Cells were then trypsinized 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 2008C13*5.25 cells was determined by a clonogenic assay as described previously (Bergoglio et al., 2001). For resistance-reversion assays, cells were treated with inhibitors at DL_{20} all experiment long, with cisplatin (Sigma-Aldrich) being administrated for 1 h at 37°C 24 h after plating.

Results

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. (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 CV of the DMSO control signal value averaged 7.5% (data not shown). This simple assay enabled the screening of 8448 partially purified natural extract fractions, from which 71 active extracts were identified. Subsequent ^{32}P-labeled primer extension procedure was carried out with Pol β and replicative Chinese hamster ovary cell extracts to select specific inhibitory extracts. Three of them were selected (Fig. 1A): *J. communis* (EX1), *P. lentiscus* (EX2), and *M. 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 *J. communis* (Cupressaceae) was found to be a ubiquitous diterpene of the labdane series, CA. Its structure (Fig. 1B) was established by com-

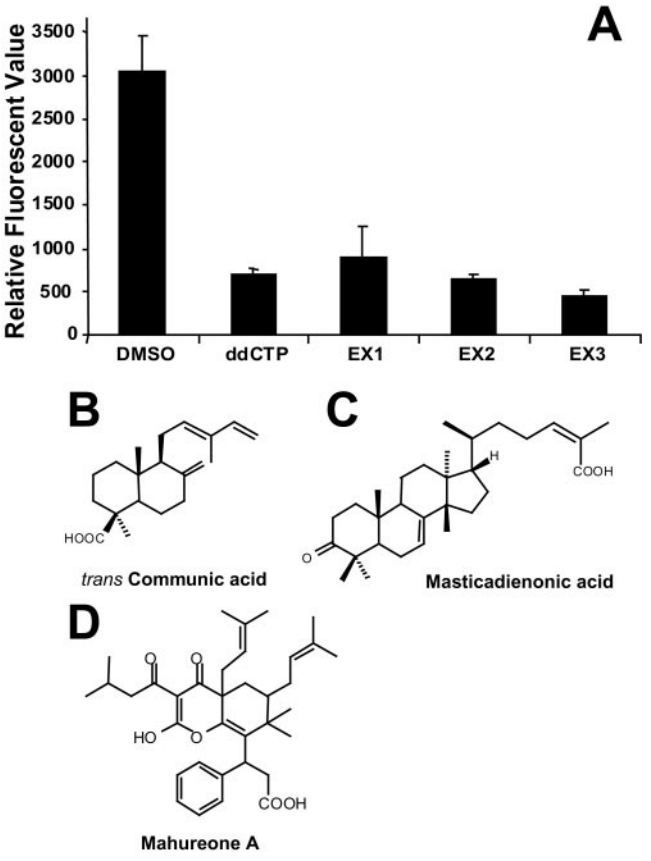

Fig. 1. A, HTS to screen for Pol β inhibitors. *J. communis* (EX1), *P. lentiscus* (EX2), and *M. palustris* (EX3) fraction activity measurement. All fractions were dissolved in 100% DMSO and diluted 1200 times into the assay. Each value represents the mean ± S.D. of three separate experiments. Control experiment was carried out with ddCTP (10 μM). B to D, chemical structures of the isolated compounds CA (B), MA (C), and MH (D).

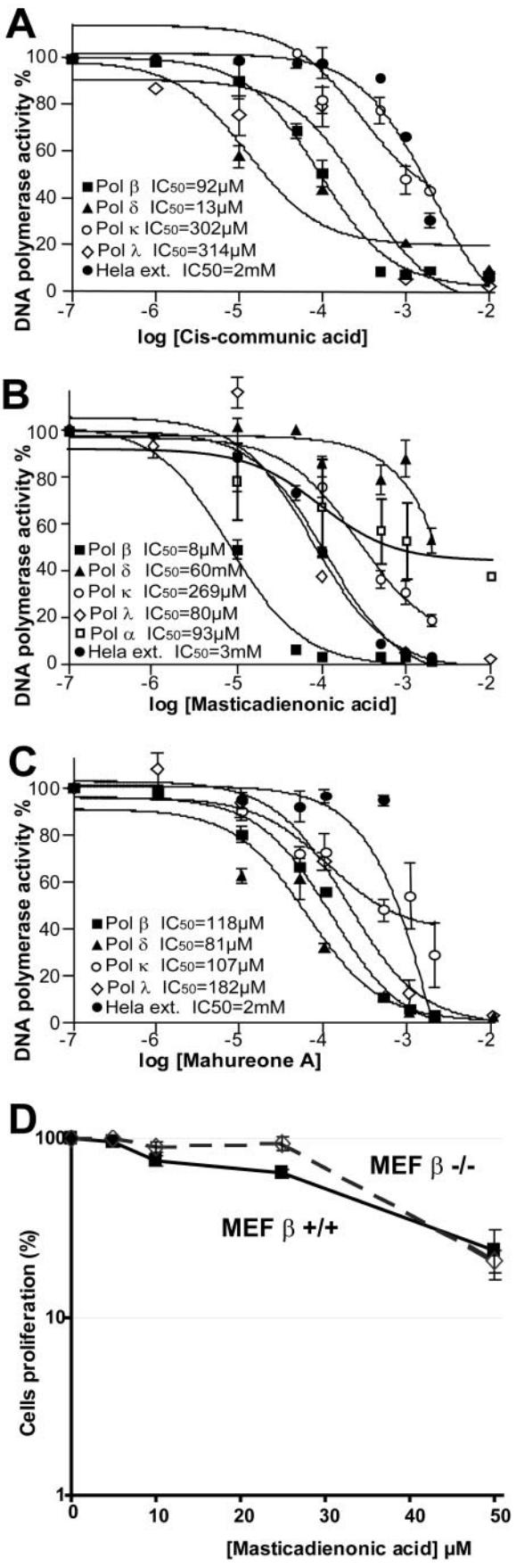

Fig. 2. Effect of CA (A), MA (B), and MH (C) in vitro on Pol β, Pol δ, Pol κ, Pol λ, and nuclear extracts. For each, 100% represents the maximal activity in the control without inhibitor. IC_{50} value was calculated by using GraphPad Prism software. Each value represents the mean ± S.D. of three separate experiments performed 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 1 week and counted. Each value represents the mean ± S.D. of three separate experiments performed in duplicate.

parison of its spectral data (1 and 2D NMR and mass spectrometry) with that from an authentic sample (Arya et al., 1961). A second molecule was isolated in a straightforward manner from *P. 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 MA (3-oxotirucalla-7,24-dien-26-oic acid), for which the structure (Fig. 1C) was known (Barton and Soane, 1956). It is noteworthy that the isomer isomasticadienonic acid, also present in the extract, which only differs by the position of the double bond in ring B (Δ7 versus 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 characterized 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 versus 6.89 ppm in the E compound). The third product was isolated from a plant from Guyana, *M. 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 characterized 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 (λ_{max} = 283 nm), whereas infrared displayed vibrations for a complex carbonyl system. Literature search showed that 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 high-performance liquid chromatography 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 were the central six-member ring that 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.

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 with that of nuclear extracts from HeLa cells, which contain all the replicative DNA polymerases able to support bidirectional and semiconservative genomic DNA replication, and with the purified replica-

tive Pol δ and the error-prone Pol κ and Pol λ. As shown in Fig. 2, A to C, all three molecules inhibited Pol β with IC_{50} values for CA, MA, and MH of 92, 8, and 118 μM, respectively. Nuclear extracts from HeLa cells were much more resistant to inhibition by those molecules, with IC_{50} values of 2, 3, and 2 mM, respectively. Pol δ activity was also resistant to MA, with an IC_{50} value of 60 mM, whereas CA and MH inhibited Pol δ. Furthermore, MH altered the activity of Pol κ and Pol λ, with 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} = 93 μM) than Pol β (Fig. 2B). Taken together, these data indicate that 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, *pol* β is probably 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 compared with wild-type 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 for 6 days. We found that cells lacking Pol β are less sensitive to MA compared with the control cells, suggesting an alteration of essential Pol β-mediated DNA transactions (BER or single-strand break 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 (IC_{50} values of 15,000 and 4000 μM, respectively), 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 previously (Sawaya et al., 1997; Maciejewski et al., 2000). We used the Pol β Protein Data Bank file 1BPY without DNA to dock MA using FlexX. This software is designed to identify the positive charges that are 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. 3, A–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, and 2.81 Å, respectively. It is interesting that Lys68 and Lys35 are known to be the main residues recognizing 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). 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 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 TLS without inhibitory effect on undamaged DNA. As indicated in Fig. 4B (lanes 3–8), MA is the most effective TLS inhibitor because only 30 μM MA was sufficient for altering the bypass (Fig. 4B, lanes 3 and 4), whereas the inhibition was achieved with 100 μM CA and 200 μM MH (Fig. 4B, lanes 5–8). It is noteworthy that the presence of the adduct changes the synthesis from distributive to processive in nature. We discussed this aspect in previous articles (Hoffmann et al., 1995, 1996), explaining that when the damage is present, the enzyme dissociates frequently from the adducted bases, resulting in more availability of Pol β to reinitiate primer extension and to extend most of the [3′-OH] termini to

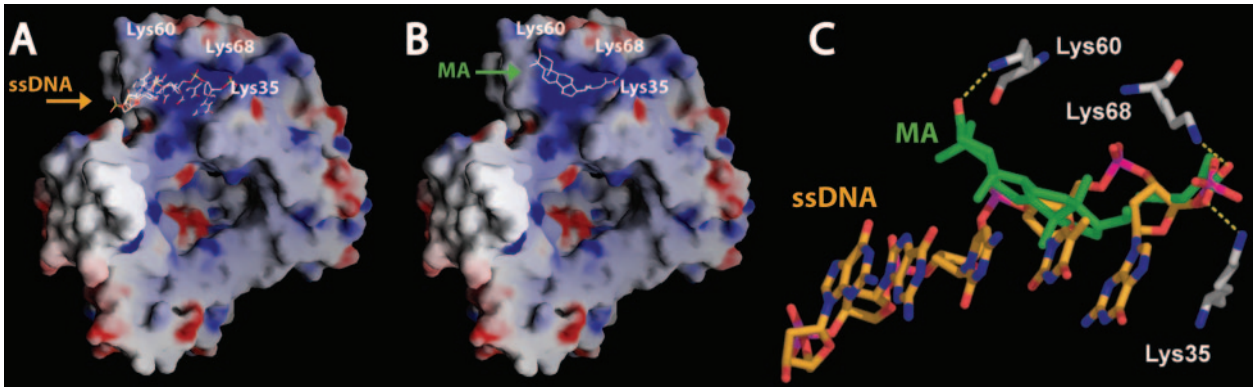

Fig. 3. Structure of Pol β and docking simulation. A, electrostatic surface potential of the crystal structure of the human Pol β in the presence of ssDNA (1BPY). Blue and red represent positive and negative potentials, respectively. The 5′-phosphate of the ssDNA is situated between Lys68 and Lys35. 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 recognizing ssDNA (i.e., Lys60, Lys68, and Lys35) (carbon in white). Grasp was used to generate A and B, and C was generated by Pymol.

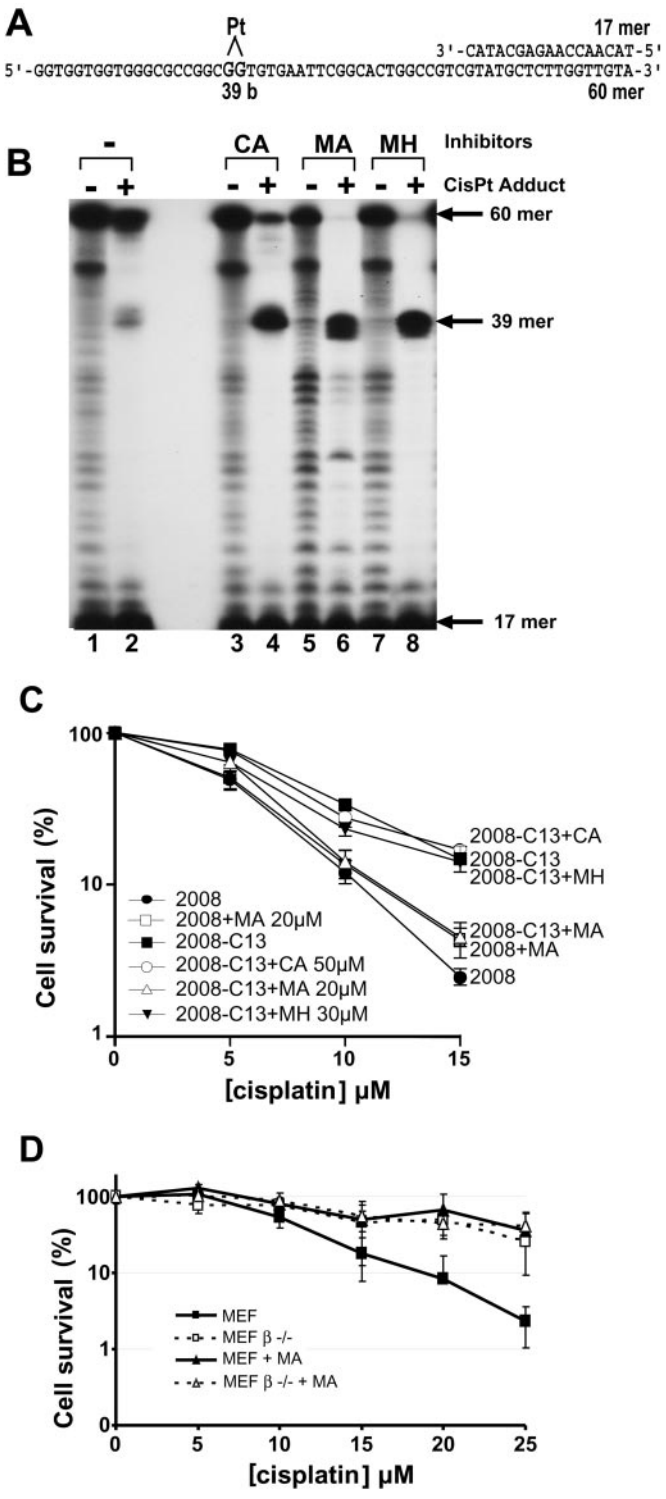

Fig. 4. A, sequence of the single-stranded DNA damaged with a unique cisplatin adduct used in the primer extension reactions. B, translesion synthesis of a single Pt-d(GpG) adduct by Pol β in the presence of CA, MA, and MH. The concentrations used were 10^{-4} M (CA), 3.10^{-5} M (MA), and 2×10^{-4}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

the site of the lesion. Furthermore, the synthesis upstream of the lesion is not altered in the presence of inhibitors. However concentrations of the inhibitors used for this TLS experiment could not be compared with those used in the calf thymus-activated DNA assay (Fig. 2), because the nature of DNA substrates is different, and the ratio [Pol β/3′hydroxy termini of the DNA substrate] is much lower in the latter case.

Cell Resistance to Cisplatin in the Presence of Inhibitors. We demonstrated previously (Bergoglio et al., 2001) that a Pol β-dependent TLS across platinated DNA crosslinks occurred in the 2008C13*5.25 human tumor cells, known to display a tolerant phenotype toward cisplatin. We proposed that this TLS process could contribute to the cisplatin resistance phenotype exhibited by these cells compared with 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 2008C13*5.25 cells to cisplatin, whereas 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 (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. Figure 4D indicates that the response of *pol* β minus MEF cells to cisplatin was not modified by the presence of MA, whereas such a response is altered in wild-type MEF cells, with cisplatin survival of *pol* $\beta^{+/+}$ cells treated with MA being similar to that of *pol* β minus MEF 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 methyl methane sulfonate, 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.

Discussion

We describe here the identification of three new natural molecules that inhibited Pol β, an error-prone enzyme frequently overexpressed in tumor cells (Scanlon et al., 1989; Srivastava et al., 1999; Canitrot et al., 2000; Servant et al., 2002b). Among those molecules, we identified a product that we named mahureone A because it was purified from the leaves of *M. palustris*. The two other compounds identified were *trans*-communic acid, which is a ubiquitous diterpene of the labdane series isolated from *J. communis*, and masticadienonic acid, from *P. lentiscus*. To our knowledge, the latter is the most efficient Pol β inhibitor identified to date, with an IC_{50} value of 8 μM. It is interesting that it altered to 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-terminal domain. This domain, also called dRPlyase domain, is only present in the X family DNA polymerases and Pol ι from the Y family, therefore possibly explaining the specificity of inhibition compared with 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} values of MA between Pol β and Pol λ. Before this work, prunasin was considered to be 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 with 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 to be a potential useful molecular tool for investigating the role of Pol β among all of the X family DNA polymerases in normal somatic cells, such as 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 tumors in patients with cancer 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 caused by an increased capacity of the cell to tolerate platinum/DNA lesions. We (Hoffmann et al., 1995; Canitrot et al., 1998) and others (Vaisman et al., 1999; Vaisman and Chaney, 2000) have shown that Pol β has the potential to efficiently catalyze 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 2008C13*5.25 cells (Mamenta et al., 1994; Bergoglio et al., 2001), with 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, with MA being the most potent. Therefore, we showed that MA decreased the ability of 2008C13*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. We were surprised to observe in the control experiments that MA protected wild-type MEF cells from cisplatin toxicity, which are 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 those that only express the enzyme at a basal level. However, these data suggest that the intracellular level of Pol β protein in the response of cells to genotoxic stresses and 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 favor recombination repair and cell survival.

From a pharmaceutical point of view, masticadienonic acid could thus be considered a potentially useful adjuvant that is likely to reinforce the cisplatin action against tumors 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 tumors) in the context of new individual therapeutic strategies.

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