Influence of the Stereoisomeric Position of the Reactive Acetate Groups of the Benzo[b]Acronycine derivative S23906-1 on its DNA Alkylation, Helix-Opening, Cytotoxic, and Antitumor Activities

Sabine Depauw, Thomas Gaslonde, Stéphane Léonce, Laurence Kraus-Berthier, William Laine, Gaëlle Lenglet, Angèle Chiaroni, Bruno Pfeiffer, Christian Bailly, Sylvie Michel, François Tillequin, Alain Pierré, and Marie-Hélène David-Cordonnier

Institut National de la Santé et de la Recherche Médicale U-837-Jean-Pierre Aubert Research Centre, Team 4 Molecular and Cellular Targeting for Cancer Treatment, Institut pour la Recherche sur le Cancer de Lille, Lille, France and IFR114-Institut de Chimie des Substances Naturelles, CNRS Unité Propres de Recherche 2301, Gif-sur-Yvette, France (A.C.); Division Recherche Cancérologie, Institut de Recherches SERVIER, Croissy-sur-Seine, France (S.L., B.P., A.P.); and Institut de Recherche Pierre Fabre, Toulouse, France.

Received June 19, 2009; accepted September 14, 2009

ABSTRACT

S23906-1 is a benzo[b]acronycine derivative acting as a DNA-alkylating agent through covalent bonding to the exocyclic amino group of guanines and subsequent local opening of the DNA helix. This compound was selected for phase I clinical trials based on its efficient antitumor activity in experimental models and its unique mode of action. S23906-1 is the racemate of cis-1,2-diacetoxy-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one. Here, we evaluated the cytotoxic and antitumor activities of the two pure cis-enantiomers and investigated the mechanism of action of both cis- and trans-racemates and their enantiomers in terms of DNA alkylation potency and locally drug-induced DNA helix opening process. Reaction with glutathione, as a detoxification process, was also studied. The trans-compounds, both as racemate or separated enantiomers, were found less potent than the corresponding cis-derivatives. Among the cis-enantiomers, the most efficient one regarding DNA alkylation bears the acetate on the reactive C1 position in the R configuration, both on purified DNA and genomic DNA extracted from cell cultures. By contrast, the most cytotoxic and tumor-active enantiomer bears the C1-acetate in the S configuration. Distinct cellular DNA-alkylation levels or covalent bonding to glutathione could not explain the differences. However, we showed that the S and R orientations of the acetate on C1 asymmetric carbon lead to different local opening of the DNA, as visualized using nuclease S1 mapping. These different interactions could lead to modulated DNA-repair, protein/DNA interaction, and apoptosis processes.

The stereoisomeric position of functional groups in bioactive molecules has been known for more than 100 years to exert an impact on the reactivity of biologically relevant compounds, including drugs. The presence of an asymmetric center in a drug requires specific studies to assess the potency of each isomer. Several cases can be encountered. 1) Both enantiomers display similar activities (qualitative and quantitative), fully justifying the clinical use of a racemate, exemplified by flecainide and fluoxetine (antiarrhythmic and antidepressant drugs, respectively) (Kroemer et al., 1989; Magyar et al., 2003). 2) The enantiomers have similar pharmacological effects but different concentration-response efficiencies, such as S-(−)-warfarin 3- to 5-fold more potent as an
anticoagulant agent than the \( R \)-(-)-form (Takahashi and Echizen, 2001). 3) One form is active, whereas the other is inactive, because the antiepilepsy drug (S)-levetiracetam is merely inactive in its \( R \) form (Gower et al., 1992). 4) Finally, one form is active, whereas the other is toxic, such as ethambutol, the (+)-isomer of which exhibits potent antitubercular properties, whereas the corresponding (-)-isomer is responsible for optical toxicity in humans (Blessington, 1997) or thalidomide (Melchert and List, 2007). In the field of anticancer agents, chiral specificity is also well documented. The 20(S)-isomer of the topoisomerase I inhibitor camptothecin displays significant activity, whereas its 20(R) counterpart is 10- to 100-fold less active (Wani et al., 1987). Likewise, the \( R \)-isomer of the bis-alkylating agent isophosphamide displays an antitumor activity, whereas the S-isomer is responsible for the neurotoxicity of racemic isophosphamide (Williams and Wainer, 1999). Stereoselectivity is associated with DNA monoalkylating agents such as duocarmycin analogs (Cimino et al., 2006) or benzo[\( h \)]pyrene (Buening et al., 1978). Sequence selectivity also depends on the configuration of alkylating agents: the (2S,3S) cis-isomer of the azinomycin epoxide reacts with guanines at TG sites, whereas its enantiomer (2F,3R) fails (David-Cordonnier et al., 2006).

Altogether, those results and many others evidence the need for determining the precise mechanism of action of all possible isomers of a potential therapeutic agent. This is the purpose of this study. The benzo[b]acryvinone derivative S23906-1 was selected for its potent cytotoxicity in vitro and antitumor activity in vivo (Guilbaud et al., 2002). It bears two acetoxy groups on asymmetric carbons at positions 1 and 2 in the cis configuration of the drug at C1 and C2 on DNA bonding and selective bonding of S23906-1 to the exocyclic NH2 group of guanines as does the marine natural product (and recently registered anticancer drug) eteineasidin 743 (ET743; trabectedin [Yondelis]) (Pommier et al., 1996). S23906-1 bonding to DNA induces a local destabilization of the DNA helix (David-Cordonnier et al., 2005). However, the impact of the absolute configuration of the drug at C1 and C2 on DNA bonding and destabilization, but also on cytotoxic and antitumor activities, was not addressed; this is the purpose of the present study.

### Materials and Methods

**Chemical Methods.** Mass spectra were recorded with a Waters ZQ 2000 spectrometer using electrospray ionization. UV spectra (\( \lambda_{\text{max}} \) in nanometers) were recorded in spectroscopic grade MeOH on a UV-Vis spectrophotometer (PU 8730; Philips, Kassel, Germany). IR spectra (\( \nu_{\text{max}} \) in centimeters\(^{-1}\)) were obtained from potassium bromide pellets on an infrared spectrophotometer (257; PerkinElmer Life and Analytical Sciences, Waltham, MA). \(^1\)H-NMR (d parts per million), \( \nu_{\text{max}} \) (Hertz) spectra were run at 400 MHz and \(^{13}\)C-NMR spectra at 100 MHz, using an AVANCE-400 spectrometer (Bruker, Newark, DE). The structures of the novel compounds were insured and the signals unambiguously assigned by two-dimensional NMR techniques: \(^1\)H-\(^1\)H correlation spectroscopy, \(^1\)H-\(^1\)H Overhauser enhancement spectroscopy, \(^1\)C-\(^1\)H heteromeric multiple quantum coherence spectroscopy, and \(^1\)C-\(^1\)H heteromeric multiple-peak-bond correlation spectroscopy. These experiments were performed using standard Bruker microprograms. Chromatographic columns were conducted using silica gel 60 Merck (20–45 \( \mu \)m) with an overpressure of 0.5 mbar. CD spectra were recorded with a chiro-optical spectrometer (J-810; Jasco, Tokyo, Japan) between 300 and 400 nm, at room temperature. The samples were prepared in methanol at 10\(^{-4}\) g/ml.

**Chemicals.** The syntheses of S23906-1 and S23968-7 have been reported previously (Costes et al., 2000). S23906-1 is a racemate with two acetoxy groups on asymmetric carbons at positions 1 and 2 in the relative cis configuration. S23968-7 is the racemic cis-diol monoacetylated at position 2 (Scheme 1). The corresponding trans-racemates S29983-1 and S29850-1, respectively, were also synthesized (Thi Mai et al., 2003). The racemic diesters S23906-1 and S29983-1 were resolved by HPLC on a ChiralCell O.C. column (Chiral Technologies, Strasbourg, France) to give the two corresponding enantiomers. Bromination of S27589-1, the (+)-enantiomer of S23906-1 gave the corresponding 5-bromo and 5,15-dibromo derivatives. This latter gave suitable crystals permitting X-ray diffraction analysis and establishment of the absolute configuration as (1S,2S). The absolute configuration of trans-derivatives was determined further on from CD analysis, in comparison with the cis-isomers (Fig. 1).

**Bromination of (+)-cis-1,2-Diacetoxy-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one.** N-bromosuccinimide (82 mg; 0.46 mmol) and 2,2′-azobisis(2-methylpropionitrile) (1 mg) were added to a solution of (+)-cis-1,2-diacetoxy-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one (101.6 mg; 0.54 mmol) in carbon tetrachloride (14 ml) (Scheme 2). The resulting mixture was stirred 24 h in darkness. After filtration, the solvent was evaporated under reduced pressure. The solid residue was extracted with CH\(_2\)Cl\(_2\) (5 \( \times \) 50 ml). The organic layers were dried over anhydrous MgSO\(_4\), filtered, and evaporated under reduced pressure. Column chromatography over silica gel (elucent CH\(_2\)Cl\(_2\) then CH\(_2\)Cl\(_2\)/MeOH mixtures of increasing polarity) gave (+)-cis-1,2-diacetoxy-5,13-dibromo-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one (101.6 mg; 51%) and (+)-cis-1,2-diacetoxy-5-bromo-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one (75.4 mg; 43%) successively. The dibrominated product was crystallized in benzene to give yellow plates, which were analyzed by X-ray diffraction.
(+)-cis-1,2-Diacetoxy-5,13-dibromo-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one. Yellow plates, hexagonal-shaped. \( [\alpha]^{20}_{D} (\text{CHCl}_3) = +57^\circ \). UV: \( \lambda \) nm in MeOH (log e) 208 (4.52); 214 (4.32); 289 (4.57); 414 (3.52). IR: 3060; 2978; 2935; 2850; 1747; 1654; 1592; 1592; 1456; 1401; 1234; 1153; 1083; 730. MS (positive-ion electrospray): 568 and 570 \([M + H]^+\); 590 and 592 \([M + Na]^+\); 606 and 608 \([M + K]^+\). RMN \(^1\)H (CDCl\(_3\)): 1.52 (s, 3H, C3MeB); 1.56 (s, 3H, C3MeA); 2.12 (s, 3H, COMe1); 2.12 (s, 3H, COMe2); 3.60 (s, 3H, NMe); 4.06 (s, 3H, OMe); 5.46 (d, 1H, J = 5 Hz, H10); 7.70 (t, 1H, \( J = 8 \) Hz, H11); 8.00 (d, 1H, \( J = 8 \) Hz, H12); 8.36 (d, 1H, \( J = 8 \) Hz, H9); 8.75 (s, 1H, H8). RMN \(^{13}\)C (CDCl\(_3\)): 20.8 (COMe2); 21.7 (COMe1); 23.3 (C3MeB); 24.8 (C3MeA); 48.7 (NMe); 61.8 (OMe); 63.5 (C1); 70.2 (C2); 78.1 (C3); 105.6 (C5); 107.5 (C14b); 113.6 (C13); 117.3 (C6a); 126.3 (C10); 126.8 (C9); 128.0 (C8); 129.2 (C7a); 130.2 (C11 and C12); 130.6 (C12a); 135.1 (C8a); 144.5 (C3a); 152.7 (C14a); 156.8 (C4a); 159.5 (C6); 170.4 (OCO2); 171.2 (OCO1); 178.9 (C7). Anal. Calcd. for C\(_{28}\)H\(_{25}\)NO\(_7\)Br\(_2\): C 51.95; H 3.89; N 2.16%. Found: C 51.97; H 3.95; N 2.12%. (Scheme 2, compound a.)

(-)-cis-1,2-Diacetoxy-5,13-dibromo-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one. Yellow amorphous solid. \( [\alpha]^{20}_{D} (\text{CHCl}_3) = -92^\circ \). UV: \( \lambda \) nm in MeOH (log e) 207 (4.50); 238 (4.34); 263 (4.32); 295 (4.79); 440 (3.62). IR: 3056; 2978; 2928; 2850; 1751; 1650; 1619; 1596; 1557; 1401; 1374; 1234; 1219; 1149; 1021. MS (positive-ion electrospray): 568 and 570 \([M + H]^+\); 590 and 592 \([M + Na]^+\); 606 and 608 \([M + K]^+\). RMN \(^1\)H (CDCl\(_3\)): 1.55 (s, 3H, C3MeB); 1.61 (s, 3H, C3MeA); 1.96 (s, 3H, COMe1); 2.04 (s, 3H, COMe2); 3.72 (s, 3H, NMe); 4.07 (s, 3H, OMe); 5.49 (d, 1H, \( J = 5 \) Hz, H2); 6.63 (d, 1H, \( J = 5 \) Hz, H1); 7.43 (t, 1H, \( J = 8 \) Hz, H10); 7.55 (s, 1H, H13); 7.57 (t, 1H, \( J = 8 \) Hz, H11); 7.68 (d, 1H, \( J = 8 \) Hz, H12); 8.00 (d, 1H, \( J = 8 \) Hz, H9); 8.88 (s, 1H, H8). RMN \(^{13}\)C (CDCl\(_3\)): 20.8 (COMe2); 21.1 (COMe1); 23.9 (C3MeA); 24.5 (C3MeB); 43.2 (NMe); 61.7 (OMe); 65.7 (C1); 69.4 (C2); 77.8 (C3); 102.8 and 103.2 (C5 and C14b); 112.0 (C13); 115.3 (C6a); 125.0 (C10 and C7a); 126.9 (C12); 128.5 (C8); 128.8 (C11); 128.9 (C12a); 129.8 (C9); 136.2 (C8a); 142.3 (C13a); 148.7 (C14a); 156.6 (C4a); 159.8 (C6); 170.6 (OCO2); 170.9 (OCO1); 177.4 (C7). Anal. Calcd. for C\(_{28}\)H\(_{25}\)NO\(_7\)Br\(_2\): C 59.16; H 4.61; N 2.46%. Found: C 59.18; H 4.66; N 2.43%. (Scheme 2, compound b.)

**Crystallography.** A yellow little plate, hexagon-shaped, of dimensions 0.25 \( \times \) 0.25 \( \times \) 0.1 mm, crystallized from benzene, was chosen for the data collection. The molecular formula was confirmed as C\(_{26}\)H\(_{25}\)Br\(_2\)NO\(_2\) giving mol. wt. = 647.31. The compound crystallizes in the space group P 2\(_{1}\)2\(_{1}\)2\(_{1}\) of the orthorhombic system, with
four identical molecules in the unit-cell of parameters: \( a = 9.493(4), b = 13.760(6), c = 20.889(8) \) Å, \( V = 2729 \) Å\(^3\); \( \alpha = 90^\circ\), \( \beta = 94.975(5)\), \( \gamma = 90^\circ\), \( \rho = 1.576 \) g/cm\(^3\), \( F(000) = 1304, \lambda (\text{Mo K}\alpha) = 0.71073 \) Å, \( \mu = 3.018 \) mm\(^{-1}\) were deduced.

Data were measured with a Nonius \( \kappa \)-charge-coupled device area-detector diffractometer, using a graphite monochromated Mo K\( \alpha \) radiation, in \( \phi \) and \( \omega \) scans, up to \( \theta = 27.50^\circ\). A full sphere of 43,155 data points was measured leading to 6242 independent orthorhombic reflections (\( R_{\text{int}} = 0.052\)), of which 4926 were considered to be observed, having \( I \geq 2\sigma(I)\). The structure was solved by the Patterson method with program SHELXS86 (http://shelx.uni-ac.gwdg.de/SHELXS/) and refined by full-matrix least squares, based upon all the unique \( F^2 \) with program SHELXL97 (http://shelx.uni-ac.gwdg.de/SHELXL/). All hydrogen atoms could be located on Fourier difference maps, but they were fitted at theoretical positions (\( dC-H = 0.93-0.98 \) Å) and treated as riding, assigned of an isotropic displacement parameter equivalent to 1.2 times that of the bonded atom (1.5 for the H-methyls). The absolute configuration was calculated by the Bijvoet method from the differences observed between the \( F_o \) on the one hand and the \( F_{\text{calc}} \) on the other hand, on the Friedel pairs (\( hkl \) and \(-h-k-l\)), due to the anomalous diffusion of the Br atoms, and the result was confirmed by the zero value of Flack factor (0.010).

Thus, refinement of all atomic coordinates and temperature factors of nonhydrogen atoms (349 parameters) converged to \( R(1 F_o) = 0.0371 \) calculated with the 4926 observed reflections and \( wR2(F^2) = 0.0777 \) considering all the 6242 unique data, with a goodness-of-fit \( S \) factor of 1.057 [these values became \( R1(F_o) = 0.0656 \) and \( wR2(F^2) = 0.1470 \) in the inverse configuration]. The residual density was found between –0.71 and 0.75 eÅ\(^{-3}\), near the Br2 atom, the second residue being of 0.21 eÅ\(^{-3}\). The Cambridge Crystallographic Data Centre file 661564 contains the Supplementary Crystallographic data for this article. These data can be obtained free of charge at http://www.ccdc.cam.ac.uk/deposit (or from the Cambridge Crystallographic Data Centre, Cambridge, UK).

**Standard Proliferation Assay.** S29306-1-sensitive (KB3-1) or -resistant (KB-500) human epidermoid carcinoma cell lines (Leonce et al., 2006) and the human large-cell lung carcinoma NCI-H460 cell line were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 \( \mu \)g/ml streptomycin, and 10 mM Hepes buffer, pH 7.4. Cytotoxicity was measured by the microculture tetrazolium assay (Sigma-Aldrich, St. Quentin Fallavier, France) as described previously (Leonce et al., 1992). Cells were exposed to graded concentrations of drug (nine serial dilutions in triplicate) for four doubling times. The IC\(_{50}\) for optical density at 540 nm was calculated by a linear regression performed on the linear zone of the dose-response curve. All the measurements were performed in triplicate.

**Cell Cycle Analysis.** L1210 cells (5 \( \times \) 10^5 cells/ml) were incubated for 21 h with various concentrations of drugs. Cells were then fixed by 70% ethanol (v/v), washed, and incubated in PBS containing 100 \( \mu \)g/ml RNase and 50 \( \mu \)g/ml propidium iodide for 30 min at 20°C. For each sample, 10^4 cells were analyzed on a XLMCL flow cytometer (Beckman Coulter, Villepinte, France).

**Antitumor Activity and Survival Assays.** The antitumor activity of the cis-racemate and isomers was evaluated on murine colon C38 adenocarcinoma implanted in B6D2F1 (C57Bl/6 \& DBA2) mice or human NCI-H460 (HTB177) non–small-cell lung carcinoma (American Type Culture Collection, Manassas, VA) in nude mice. A C38 tumor fragment of approximately 50 mg was subcutaneously implanted into the dorsal flanks of seven mice. For each sample, 10^4 cells were analyzed on a XLmcl flow cytometer (Beckman Coulter, Villepinte, France).

**Scheme 2.** Bromination reaction and structure of the dibromide (a) and monobromide (b) substituted compounds.
administered intravenously, 10 days apart; the first day of treatment was 7 days after the tumor cell injection. Animal mortality was checked daily, and the MST (median survival time, in days) of each group was calculated. Results were expressed in terms of both the percentage T/C value and number of long-term survivors. Median percentage of T/C = (MST of treated group/MST of control group) × 100, and long-term survivors were defined as surviving animals sacrificed at the end of the experiment in which no tumors could be detected by macroscopic examination. The studies have been carried out in accordance with the Declaration of Helsinki.

DNA Fragments. The radiolabeled 117- and 198-bp DNA fragments were obtained from pBS or pMSI plasmid digestions (David-Cordonnier et al., 2002) using EcoRI and PvuII or HindIII and XbaI restriction enzymes (Bio Labs, Saint-Quentin Yvelines, France), respectively, in their corresponding digestion buffers and labeling at the EcoRI or HindIII sites with [α-32P]ATP (3000 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and avian myeloblastosis virus reverse transcriptase (Ozyme, Saint-Quentin Yvelines, France). The double stranded XH-24 oligonucleotide was prepared by labeling one strand using polynucleotide kinase (Invitrogen) in the presence of [γ-32P]ATP (3000 Ci/mmol; GE Healthcare) before annealing to the complementary oligonucleotide as described previously (David-Cordonnier et al., 2005). The hairpin oligonucleotide HP-AGA was obtained by heating at 80°C the oligonucleotide followed by a slow decrease of temperature up to 4°C. HP-AGA was then labeled on the 5'-end using T4 polynucleotide kinase, as described previously. Both oligonucleotides were ordered from Eurogentec (Seraing, Belgium). The radio-abeled DNA fragments were removed from the excess of radioactive nucleotide by electrophoresis on a nondenaturing 10% (w/v) polyacrylamide gel and subsequent excision of the desired portion of the gel containing the radiolabeled products. This gel portion was then crushed, and the radiolabeled DNA was eluted overnight against 400 μl of elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) before being filtered and ethanol-precipitated.

Gel Shift Studies. A typical DNA alkylation reaction consisted of incubating 8 μl of radiolabeled DNA with 2 μl of 10× buffer (10 mM sodium cacodylate, pH 7.0) and 10 μl of the drug at the desired concentration for the indicated time in the dark at 37°C before adding 5 μl of loading buffer containing tracking dyes. The DNA samples were resolved by electrophoresis under nondenaturing conditions in 10% polyacrylamide gels for approximately 5 h at 300 V at room temperature in Tris-borate-EDTA buffer (89 mM Tris base, 89 mM boric acid, and 2.5 mM Na2EDTA, pH 8.3). Gels were then transferred to a Whatman 3MM paper, dried under vacuum at 80°C, and analyzed using a PhosphorImager. The radiolabeled 117-bp DNA fragments were incubated alone or with 50 μl of native loading buffer containing tracking dyes and subsequently excised of the desired portion of the gel containing the radiolabeled products. This gel portion was then crushed, and the radiolabeled DNA was eluted overnight against 400 μl of elution buffer (500 mM ammonium acetate, 10 mM magnesium acetate) before being filtered and ethanol-precipitated.

HPLC and Mass Spectra Analysis. For HPLC-fluorometric analysis, high-performance liquid chromatographic analyses were carried out using a Jasco HPLC system equipped with a quaternary pump with on-line autosampler, Jasco UV-975 UV/vis detector (Jasco) and RF-551 spectrofluorometric detector (Shimadzu, Tokyo, Japan). The cis-racemate S23906-1 (50 μM) or purified cis-enantiomer S27589-1 and S27590-1 were incubated alone or with 50 μM GSH (Sigma) for 24 h at 37°C in ammonium acetate 1 mM, pH 7.15. 50 μl of samples were injected in a moduulo-cart QS Uptispher 5-PT column (150 × 2 mm; Interchim, Montluçon, France) at a flow rate of 0.2 ml/min of mobile phase (18% acetonitrile, 23% methanol, 59% H2O, and 0.1% formic acid, pH 2.9). Fluorometric spectra were recorded at 510 nm for an excitation wavelength of 354 nm. For HPLC-EI-MS, the HPLC analysis were performed using a PerkinElmer series 200 LC pump equipped with an on-line PerkinElmer series 200 LC pump equipped with an on-line

Detection of DNA Adducts in Cells. KB3-1 epidermoid carcinoma cells were grown in DMEM-GluMax medium (Invitrogen) supplemented with 10% fetal calf serum, 100 IU/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) in a humidified atmosphere at 37°C under 5% CO2. KB-3-1 cells (108) were grown for 24 h in 100-mm diameter dishes with 5 ml of culture medium, before the treatment of the cells using increasing concentrations of the compounds of interest. The radiolabeled DNA fragments were subjected to electrophoresis and analyzed with the use of the PhosphorImager. The radiolabeled DNA fragments were subjected to electrophoresis and analyzed with the use of the PhosphorImager. For studying the competition for DNA alkylation using fragments was then added, and the samples were further incubated for a further 5 min of mild agitation. Finally, 200 μl of 0.1 M EDTA, pH 7.5, were added, and the mixture was incubated 4 h at 37°C. After addition of 80 μl of 5 M NaCl, the genomic DNA was extracted using 3 ml of phenol/chloroform/isooamylic alcohol (25:24:1) mixture. Finally, the genomic DNA was precipitated with cold ethanol (−20°C) and centrifugation at 11,000 rpm for 30 min. The UV absorption of the final solution at 260 nm was used to estimate the quantity of collected DNA. The fluorescence of the benzo-b-acrycline core molecule covalently linked to DNA was measured using a SPEX Fluorolog spectrofluorometer (Ivon Jobin, Longjumeau, France) with an excitation wavelength at 300 nm and an emission range from 450 to 560 nm. The values at the emission peak of 510 nm were used to draw the graph.

Nuclease S1 Digestion. The radiolabeled 117-bp DNA fragments were incubated overnight with various diacetate derivatives at various concentrations at 37°C in 20 μl of sodium cacodylate (1 mM, pH 7.0). The digestion was performed by the addition of 4 units of S1 nuclease (Roche, Neuilly sur Seine, France) in the appropriate nuclease S1 specific buffer for further 15 min incubation. The DNA samples were then mixed with 5 μl of native loading buffer containing tracking dyes subjected to electrophoresis on a 10% native polyacrylamide gel at 300 V at room temperature in Tris-borate-EDTA buffer. The gel was then dried and analyzed on the 445SI PhosphorImager (GE Healthcare).

Results

Structural Characterization of the Molecules. The racemic diesters S23906-1 and S29983-1 were resolved into the corresponding enantiomers using chiral HPLC. To determine the absolute configuration using X-ray diffraction, the S27589-1 (+)-enantiomer of S23906-1 was subjected to bromination with N-bromosuccinimide. This reaction led to the corresponding 5-bromo and 5,13-dibromo derivatives, which could be easily separated on a preparative scale using column chromatography over silica gel. (+)cis-1,2-Diacetoxy-5,13-dibromo-6-methoxy-3,3,14-trimethyl-1,2,3,14-tetrahydro-7H-benzo[b]pyrano[3,2-h]acridin-7-one crystallized from benzene as yellow hexagonal-shaped plates that were submitted to X-ray crystallographic analysis. An ORTEP view of
the molecule is shown in Fig. 1A, with the absolute configuration and the atomic numbering. The analysis of the mean planes and torsion angles values indicates that the five rings are roughly coplanar if are omitted the C3 atom and the nitrogen N14 situated at, respectively 0.820(3) and −0.611(3) Å from the mean plan of the other nineteen atoms. More precisely, the molecule can be considered as two planar entities, the napthalene part (of which the C7 and N14 atoms are deviated by 0.164(3) and −0.171(3) Å, respectively) and the remaining other ring atoms—excluding C3-linked in C7 and N14, and bent by 163° along the N14-C7 direction. The pyrano ring exhibits an envelope conformation with atom C3 deviated by 0.610(3) Å from the mean plane of the other five atoms. The two H atoms fixed at the chiral carbon atoms C1 and C2 appear in cis position, below the mean plane of this ring in bisecting and axial positions, respectively. By contrast, the two acetate substituents are situated above that mean plane, respectively, in an axial position for the one linked to C1, with oxygen O21 at 1.365(2) Å, and in an equatorial position for the second linked to C2, with oxygen O22 at 0.432(2) Å. Therefore, the absolute configuration of atoms C1 and C2 can be deduced unambiguously as S.

The pyramidal conformation of nitrogen atom N14 appears clearly with the methyl group C20 located at −1.897(4) Å from the mean plane of the molecule. In the crystal packing, there are no π–π stacking interactions between the phenyl rings of the different molecules. Only contacts of types CH–π can be observed between the hydrogen atoms of the methyl group C15 (x,y,z) directed toward the phenyl ring [including atoms C9, C10 to C16] of molecule (1 − x, 0.5 − y, 0.5 + z) with distances: CH3-Cg centroid = 3.469 Å, H-Cg = 2.82 Å, angle C-H-Cg = 131°, strengthening the crystalline cohesion.

The configurations of the enantiomers of S29983-1 were deduced from the CD curves (Fig. 1B) and compared with those obtained from S27589-1 and S27590-1 of known 1R,2R and 1S,2S configurations, respectively. Compound S70256-1, which exhibits a positive maximum at λ = 356 nm, possesses the same 1S configuration as S27589-1. Likewise, S70255-1 and S27590-1 exhibit a negative maximum reliable to the 1R configuration.

Cytotoxicity and Antitumor Activities. We investigated the pharmacological properties of the different isomers (Scheme 1). For this purpose, both cytotoxic (Table 1) and in vivo antitumor activities (Fig. 2) were studied. Both stereoisomeric compounds were cytotoxic for the sensitive cell lines KB3-1, HCT116, MDA-MB-468, and NCI-H460 with IC50 values in the same range, with the 1S-isomers being slightly more potent (approximately 2- to 3-fold) than the 1R-counterparts using both cis and trans series. Cell cycle analysis on the L1210 cell line evidenced similar induced cell cycle effects using both compounds, a G2/M phase arrest at 1 μM evolving in S phase accumulation at higher concentrations (10 μM) (data not shown). Antitumor activities were measured on a C38 colon adenocarcinoma model. As evidenced in Fig. 2A, S27589-1 is more active than the opposite cis-pure enantiomers S27590-1 on C38 model expressed as tumor growth (Fig. 2A). As expected, the cis-racemate S23906-1 presents an intermediate profile. S23906-1, administered at 4 mg/kg twice to mice bearing NCI-H460 tumor, induced a prolongation of survival with a T/C of 241%, this increase was significantly different from the control (p = 0.002) (Fig. 2B). The mouse, which survived on day 180, showed a tumor mass in the pleural space at the autopsy. The administration of 6 mg/kg of the enantiomers was toxic; early deaths were recorded with five and six mice who died after the first treatment with S27589-1 and S27590-1, respectively. The dose of 4 mg/kg was thus considered the maximal tolerable dose for the two enantiomers, even if S27590-1 was more toxic than S27589-1, inducing one death on day 17 at 4 mg/kg. S27589-1 and S27590-1, administered at 2 or 4 mg/kg, induced an increase in life span with maximal T/C of 229 and 185%, respectively. In the group treated by 2 mg/kg S27590-1, two mice survived at the end of the experiment (day 180). The autopsies revealed that these two mice were not tumor-free. The increase in life span observed for the 4 mg/kg S27589-1 treated group was statistically different from the control with p < 0.01.

The efficacy of S27589-1 (4 mg/kg, T/C = 229%) was superior to that of S27590-1 (4 mg/kg, T/C = 173%), the differences in T/C being statistically different between the two groups (p < 0.01). When administered at their maximal tolerated dose (4 mg/kg), S23906-1 and S27589-1 induced a T/C of 241 and 229%, respectively, showing that S23906-1 was slightly more active than S27589-1. To understand why and how one of the two pure cis-enantiomers is more potent than the other, we investigated the mechanism of action of both cis and trans pure or racemate compounds.

**Influence of the cis or trans Isomeric Position of Racemate Derivatives on DNA Alkylation Efficiency.** The comparison of the DNA alkylation potencies of the various cis- or trans-racemate derivatives of the diacetate or mono-acetate forms of benzo[b]acronycine (Scheme 1) was investigated using an assay described previously (David-Cordonnier et al., 2002, 2003). In these initial studies, we focused on the cis-racemate forms of the mono- or diacetate derivatives, S23906-1 and S28687-1, respectively, and clearly evidenced the DNA bonding rather than noncovalent binding of the interaction using SDS, NaCl, or DNA competition experiments. Here, their respective trans-racemate homologs S29983-1 and S29850-1 were synthesized. A comparison of their covalent DNA bonding properties is presented in Fig. 3. The trans-diacetylated racemate S29983-1 alkylates DNA with similar efficiency than the cis-diacetylated racemate S23906-1. By contrast, the trans-monoacetylated racemate S29850-1 totally fails to react with DNA. This result is in total agreement with previously published data demonstrating that a transesterification process is required to displace the acetate group from position 2 to the reactive position 1 of the 1S-racemate compound S28687-1 (David-Cordonnier et al., 2004b). In the case of S29850-1, this transesterification process could not occur because of the high-energy cost, and this compound is consequently inactive. In the case of S29983-1, the departing acetate is present at the reactive position 1 and could potently react with guanines to form covalent DNA adducts.

**DNA Alkylation Potency of the Pure cis- or trans-Enantiomers.** Both pure enantiomers of the diacetylated forms of benzo[b]acronycine were assessed for their covalent bonding to DNA using electrophoretic mobility shift assays (EMSA). Figure 4 shows a comparison between the two pure cis- (S27589-1 and S27590-1, A) or trans- (S70255-1 and S70256-1, B) enantiomers for their ability to form a covalent complex with a radiolabeled 117-bp DNA fragment. All four
isomers present comparable DNA-alkylating efficiencies. A quantitative analysis of the migration delay upon DNA alkylation (Fig. 4B) evidenced that DNA alkylation efficiency, proportional to the retardation of the gel migration induced by the various compounds, is in the order S27590-1 > S27589-1 > S70255-1 > S70256-1, corresponding to the acetate positions (1R,2R) > (1S,2S) > (1R,2S) > (1S,2R). Therefore, the cis-isomers S27589-1 and S27590-1 are more potent than the trans-isomers S70255-1 and S70256-1. Likewise, compounds bearing the acetate group in the 1R orientation (S27590-1 and S70255-1) are slightly more potent than their counterparts bearing the acetate in the 1S orientation (S27589-1 and S70256-1) using the 117-bp DNA probe (Fig. 4) or any other tested radiolabeled DNA fragments (Supplemental Fig. S1 and data not shown). The kinetics of reaction assessed using 50 μM concentrations of either compound for a reaction time of up to 2 h (Fig. 5) reveal that the alkylation reaction is faster with the two cis-enantiomers (Fig. 5, A and C) than with the two trans-enantiomers (Fig. 5, B and C). A faster reaction was observed using the two cis-isomers compared with the trans-isomers. It is also more rapid with compounds bearing the acetate group at the 1R than with the 1S compounds. In terms of kinetics, the compounds rank in the order (1R,2R) > (1S,2S) > (1R,2S) > (1S,2R), which is consistent with the dose-dependent effects (Fig. 3). The better reactivity of 1R versus 1S versions of the compound is in opposite correlation with the antitumor activity of the compounds for which the 1S version is more potent than the 1R (Fig. 2).

Covalent Binding to GSH. A direct correlation between DNA alkylation and cytotoxic/antitumor activities has previously been established for several chemical series of benzo[a]acronyine derivatives (David-Cordonnier et al., 2002, 2004a; Thi Mai et al., 2003; Nguyen et al., 2006). This is apparently not the case here. Therefore, we searched for an alternative explanation and, to this end, we investigated the reactivity against GSH, which has been considered a potential detoxification process for S23906-1 and related molecules (David-Cordonnier et al., 2003).

All pure cis-enantiomers were tested for their bonding to GSH. Both S27589-1 and S27590-1 were previously tested for their abilities to bond to glutathione using CD and mass spectrometry (David-Cordonnier et al., 2003). HPLC was used to separate the different reaction products and isomers. In a first instance, the racemate S23906-1 compound was incubated in the presence of GSH before the separation on a QS Uptisphere 5-FT HPLC column and localization of the peaks corresponding to S23906-1 and metabolites or to the adducts bound to glutathione using the fluorescence properties of the benzo[a]acronyine core (Fig. 6A). GSH alone was eluted from the column very early (3–4 min) and was detected by UV absorption at 280 nm (data not shown). Figure 6A evidences the formation of different adducts with more or less retarded migration in the column. The di- or monoacetylated and diol forms (open arrows) were assigned from a comparison of the retention profile of the mono-acetate S28687-1 and diol S23907-1 compounds (data not shown). Two series of double peaks were identified, coming out of the column at 6.2 to 8.5 and 17 to 19 min (double full arrows). Once the mobile phase was optimized, it was used to separate the adduct from the free compounds using an HPLC apparatus equipped with an ion-spray nebulizer coupled simple-
quadrupole mass spectrometer. The diacetate ([M+H] = 490), monoacetate ([M+H] = 448), and diol ([M+H] = 406) derivatives were localized within the retention time attempted from fluorescence studies (Fig. 6A, blue arrows). Two structurally different adducts could be obtained from alkylation of GSH by S23906-1: an adduct at [M+H] = 737 bearing an acetate group at position 2 of S23906-1 or an adduct at [M+H] = 695 bearing an OH group at position 2 obtained from the hydrolysis of S23906-1 as described previously (David-Cordonnier et al., 2002, 2003). From the HPLC separation, four peaks corresponding to the adduct at [M+H] = 737 were retained on the column and are labeled a1, a2, b1, and b2. Likewise, two close double peaks corresponding to the adduct at [M+H] = 695 were found and labeled c1, c2, d1, and d2 (Fig. 6B). HPLC separation of the adducts formed using the pure cis-enantiomers S27589-1 and S27590-1 discriminates between each generated adducts: peaks a1 (8.5 min), b1 (19.3 min), c1 (6.9 min), and d1 (8.0 min) could be attributed to the adduct generated from the (1S,2S) isomer S27589-1 (Fig. 6C), whereas peaks a2 (7.4 min), b2 (19.9 min), c2 (6.4 min), and d2 (8.2 min) are attributed to the adduct generated from (1R,2R) counterpart S27590-1 (Fig. 6D). From this study, it can be seen that both cis-enantiomers react with GSH in a similar manner. It is noteworthy that the formation of two kind of adducts of each type (adducts a or b for [M+H] = 737 and c or d for [M+H] = 595) is in agreement with the formation of an intermediate reactive carbocation generated after the release of the reactive acetate group in position 1 (Scheme 1). This carbocation has no asymmetric carbon and the resulting adduct could therefore be linked in the 1S or 1R orientation. Peaks a1 and b1 correspond to either (1S,2S) or (1R,2S) enantiomers, whereas peaks a2 and b2 correspond to either (1S,2R) or (1R,2R) enantiomers bearing an acetate in position 2 (C2-acetate). A similar correlation could be established for peaks c1 and d1 and peaks c2 and d2 corresponding to the (1S,2S) or (1R,2S) and either (1S,2R) or (1R,2R) enantiomers in the series bearing a C2-OH group. These data evidence the racemization of the adduct formed after the release of the reactive C1-acetate to form the reactive carbocation intermediate, as previously hypothesized (David-Cordonnier et al., 2002, 2004b).

Because both cis-enantiomers react with GSH in a similar manner and give similar orientation of GSH toward the core acronyshine (the sole difference being the orientation of the acetate or -OH group), we looked at inhibition of the DNA alkylation efficiency with increasing concentrations of each of the cis-enantiomers in the presence of a fixed concentration of GSH. Each cis-enantiomer previously incubated with GSH present an important decrease in their DNA alkylation efficiency, with equal orders of magnitude (Supplemental Fig. S2). Therefore, both compounds have similar reactivity profiles on either DNA or GSH. As a consequence, GSH bonding

![Figure 2](molpharm.aspetjournals.org)
could not be used as a criterion to select which of the enantiomer could be the best one for clinical use.

**Alkylation of Genomic DNA.** To evaluate the influence of the stereoisomeric position of the two acetate groups on DNA alklylation within cells, we exploited the fluorescence property of the benzo-acronycine core molecule to quantify the DNA adduct formation on extracted genomic DNA, as described previously (David-Cordonnier et al., 2002). The alkylation efficiency of genomic DNA of KB3-1 cells treated with increasing concentrations of the various derivatives was quantified using fluorescence excitation at 300 nm and emission at 510 nm. As shown in Fig. 7, both compounds can enter the nucleus and alkylate genomic DNA. The alkylation efficiency of genomic DNA of KB3-1 cells treated with increasing concentrations of the various derivatives was quantified using fluorescence excitation at 300 nm and emission at 510 nm. As shown in Fig. 7, both compounds can enter the nucleus and alkylate genomic DNA. The cis-isomers (black symbols) are more potent than the trans-isomers (open symbols). These cellular results are fully consistent with gel shift data (Figs. 3 and 5).

**Drug-Induced DNA Destabilization.** The ability of the various enantiomers to destabilize the DNA helix was evaluated using EMSA (Fig. 8). Electrophoretic migration of reaction samples containing a radiolabeled 24-bp DNA oligonucleotide (designated XH-24) incubated with increasing amounts of diastereoisomers resulted in the creation of alkylated single-stranded DNA that migrates faster than the alkylated double-stranded DNA. Both cis-diacetates are more potent in destabilizing this small double-strand DNA than any of the three trans-diacetates (both as racemate or pure enantiomers). Indeed, a 50% denaturation is observed using 5 to 7.5 μM cis-derivatives versus 15 to 20 μM trans-isomers. Moreover, at 50 μM concentrations of any of the cis-enantiomers, all double-stranded alkylated DNA is converted into a single-stranded alkylated fragment, whereas at this concentration, the double stranded alkylated DNA (ds-f) remains observed in gels. This suggests that the cis-enantiomers are more potent in destabilizing the DNA helix than their relative trans-isomers. Such DNA destabilization ability was confirmed for either diastereoisomer using melting temperature studies. A decrease in the double strand DNA melting temperature was observed in the presence of any of the pure cis- or trans-enantiomers or racemate forms (data not shown).

To further differentiate the mechanism of alkylation by the various diastereoisomers, each molecule was incubated for 1 h with a short hairpin oligonucleotide (HP-AGA), and subsequent alkylated products were separated on native polyacrylamide gel (Fig. 9). The HP-AGA oligonucleotide was designed as a hairpin DNA with a single guanine in the stem portion, providing a unique alkylation site within an AT-rich sequence. However, EMSA reveals two retarded bands in the presence of the racemate cis- (S23906-1) or trans-isomers (S29983-1). It is noteworthy that each pure enantiomer could not be used as a criterion to select which of the enantiomer could be the best one for clinical use.

Fig. 3. Gel shift retardation of DNA by trans-racemat of the mono-OH-monooacetylated or diacetylated forms of benzo[β]acronycine. Increasing concentrations from 2 to 50 μM of the racemate trans-monooacetate (S29850-1) or trans-diacetate (S29983-1) were incubated with a 117-bp radiolabeled DNA fragment for 16 h from comparison with the racemate cis-monooacetate (S28687-1), cis-diacetate (S23906-1), or the cis-diol (S23907-1). Free (f) and alkylated (bound, b) DNAs were resolved on a 10% polyacrylamide gel.

Fig. 4. Gel shift retardation of DNA by the various pure enantiomers of the diacetate derivative of benzo[β]acronycine. A, increasing concentrations (from 1 to 50 μM) of the various pure cis- and trans-enantiomers were incubated with a 117-bp radiolabeled DNA fragment for 16 h. Free (f) and alkylated (bound, b) DNAs were resolved on a 10% polyacrylamide gel. B, quantification of the percentage of migration using alkylated DNA from comparison with the maximum migration distance using free DNA (lanes DNA). The percentage of migration ∆L = 1 - L_{Alk}/L_{DNA} where L_{Alk} and L_{DNA} correspond to the medium distance of the band from the wells of the gel for alkylated DNA samples or control nonalkylated DNA, respectively.
form only one band, migrating at a different level depending on the position of the acetate group relative to the core of the molecule. But each retard band comigrates with one or the other shifted position observed for the respective racemate compounds. Because only one adduct could be formed per oligonucleotide at the unique guanine site, this result suggests that different structural positioning of the adduct induces a different level of gel shift. Adduct formed with a link in the 1S configuration (S27589-1) leads to a greater delay of migration than that formed through a bond at the 1R orientation (S27590-1) of the core benzo-acronycine. One possible explanation is that the presence of the adduct in the different configurations induces a distinct orientation relative to the DNA helix and thus differentially destabilizes the double-stranded stem of the hairpin to give single-stranded DNA. The higher level of retardation using the 1S configuration correlates with the higher level of DNA opening using compounds bearing the reactive group in the 1S configuration relatively to the 1R configuration. This increase of the delay of migration for a more potent DNA destabilizing compound is reinforced with the more important DNA retardation observed using the dicarbamate S29385-1 derivative previously evidenced as a stronger DNA destabilizing compound that S23906-1 (David-Cordonnier et al., 2005). Nuclease S1 digestion of the alkylated DNA fragment was performed to get an insight into the strength of each diastereoisomer to locally open the DNA helix. As presented in Fig. 9, several nuclease S1 digestion sites could be identified using S23906-1. Similar cleavage sites are observed using the trans-isomer S29983-1, suggesting that they display the same mode of bonding. It is noteworthy that the two pure cis-compounds or the two pure trans-compounds induce different cleavage sites, each of them being obtained using both racemate molecules. Therefore, cleavages sites obtained using S23906-1 represent an accumulation of that induced using S27589-1 and S27590-1.

**Discussion**

In the actual pharmacopoeia, the presence of an isomeric reactive group within a molecule requires further studies to assess the real medical potency of each of the isomers. Different stereoisomeric positions may give rise to different biological properties, pharmacological actions, side effects and metabolism, with the first observation by Pasteur about (+)- but not (-)-tartrate being metabolized by *Penicillium glaucum*, and first proofs evidenced by Puitti in 1886 on asparagine, the (+)- or (-)-enantiomers of which present sweet taste or not, respectively. 1) Each of the enantiomers could have similar effects with nearly identical qualitative and quantitative effect. 2) Each of the enantiomers could have similar pharmacological effects but different concentration-response efficacies [-(-)-verapamil is 4-fold more active than (+)-verapamil at inhibiting MDR (Ellison et al., 1990)]. 3) One could be active whereas the other is inactive [(-)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene presents an important tumorigenicity, whereas the other three optically pure isomers of the benzo[a]pyrene 7,8-diol-9,10-epoxides shows little or no activity (Buening et al., 1978)]. 4) One is active whereas the other is toxic [e.g., thalidomide, the S form of which is assumed to be responsible for phocomelia in children born from thalidomide-treated mothers in the 1960s, whereas the R-isomer was considered responsible for the anti-inflammatory and sedative effects (Melchert and List, 2007)]. The present study establishes the relevance of the absolute configuration of the di-acetylated form of benzo[b]acronycine on its cytotoxic and antitumor activities in correlation with its DNA alkylation and locally induced DNA opening. The comparison of the cytotoxic and antitumor effects of both cis-enantiomers of S23906-1 indicates that the 1S-isomer is slightly more potent than the 1R-isomer. However, regarding the small difference, the resulting eudismic index is very close in terms of DNA alkylation and cytotoxic activities, and we could not distinguish a real active eutomer form from an inactive distomer one. S23906-1 might belong to one of the first two groups defined above (i.e., 1 and 2).

To understand why the 1S-isomer was slightly more potent than the 1R-isomer, we conducted several biochemical experiments to evaluate in vitro DNA alkylation, kinetics of reac-

---

**Fig. 5.** Kinetic measurements of the four diastereoisomers of the diacetylated compound. A, electrophoretic mobility shift assays of the four diastereoisomers (50 µM each) incubated with the radiolabeled DNA for the indicated time (min) before be subjected to electrophoresis. Free (f) and alkylated (bound, b) DNAs were resolved on a 10% polyacrylamide gel. B, quantification of the percentage of migration ΔL = Lbound / Lalk as described in Fig. 5.
tion as well as genomic DNA alkylation efficiencies. All experiments provide surprising evidence that the 1R-isomer as more potent than the 1S-isomer in alkyling the DNA (Figs. 4, 5 and 7). This contrasts with cytotoxic and in vivo data (Table 1 and Fig. 2). Previous studies indicated a satisfactory correlation between (in vitro and in cellulo) DNA alkylation and the cytotoxic activities of different benzo[b]acronycine series (David-Cordonnier et al., 2002, 2004a; Thi Mai et al., 2003; Nguyen et al., 2006). But all previous data were obtained using the racemate but not the pure enantiomers. Identifying why the most potent DNA-alkylating isomers are the worst cytotoxic isomers is an important point to understand how those compounds act within cells and therefore for drug design and selection. We did not evidenced stereo-selective detoxification using GSH as an explanation for the DNA alkylation/cytotoxicity opposite correlation (Supplemental Fig. S2). However, a clear difference between the R- and S-isomers is seen regarding their DNA destabilization potency (Fig. 9). It is therefore postulated that different orientations of the fused ring relative to the DNA axis, and dependent on the enantiomerism of the adduct, could modulate the electronic distribution in the bases thus resulting in a change in the DNA local opening properties.

At the molecular level, another interesting conclusion of this work is that the trans-enantiomers form covalent binding to DNA with a slower kinetic compared with the cis-enantiomers (Fig. 5). This is consistent with the hypothesis of the formation of an intermediate carbocation occurring after the release of the C1-acetate group. The release occurs faster in the presence of another acetate group in the vicinity of the leaving acetate. C1- and C2-acetate groups are positioned on the same side of the molecule relative to the planar organization of the core acronycine in the various cis orientations thus facilitating the acetate departure. By contrast, the C2-acetate in both trans configurations is spatially much more faraway from the C1-acetate, in correlation with a slower

![HPLC separation and mass spectra analysis of the adduct formed upon bonding of S23906-1 or its two pure cis-enantiomers on GSH. A, HPLC separation profile for 50 μM S23906-1 incubated for 24 h alone (top) or with an equal concentration of GSH (bottom) is measured using fluorescence properties of the benzo[b]acronycine core of S23906-1 (λex = 354 nm; λem = 510 nm). Open arrows localized the diacetylated, monoacetylated, and diol forms. Two series of double peaks that appear after incubation with GSH are exemplified with double full arrows. HPLC-EI/MS was performed using GSH incubated for 24 H with S23906-1 (B), S27589-1 (C), or S27590-1 (D). The retention time for adducts at m/z [M+H] = 737 or at m/z [M+H] = 695 are presented in each panels.](https://molpharm.aspetjournals.org/doi/10.1124/mol.117.105470)
kinetic of carboxylation formation through the release of the C1-acetate.

Overall, this study reinforces our idea that it is useful to study and compare the pure isomers of a racemate bioactive compound to evaluate their specific mode of action and identify potential consequences in terms of cellular activity and drug design. Even if no huge differences are observed using the two pure cis-isomers S27589-1 and S27590-1 defining an active and an inactive stereoisomer, the isomer inducing the wider local opening of the DNA helix is the one that presents the highest antitumor activity. Up to now, the potential correlation between antitumor activity and the opening of the DNA helix is not easy to explain, but it is definitely a key point that must be controlled for future drug development and must be further evaluated. Even if, at this stage, we cannot deny the assumption that the stereoisomeric differences could also arise from a distinct metabolism of the various enantiomers in animal models, different DNA destabilization potencies seem to be a crucial DNA structural change that may affect protein/DNA recognition by the cellular machineries as, for example, DNA repair (for recent

**Fig. 7.** Detection of DNA adducts from the cis- or trans-racemate or pure enantiomers of the diacetate in genomic DNA. KB3-1 cells were treated using 1, 2.5, 5, 7.5, 10, and 15 μM concentrations of the various tested drugs for 24 h. The fluorescence (λex = 300 nm; λem = 510 nm) of identical concentrations of extracted genomic DNA is plotted over drug concentration. This graph is a representative quantification of two independent experiments.

---

**Fig. 8.** Gel shift analysis of the DNA strand scission in the presence of the various racemate or pure enantiomers of the diacetate compound. Either cis (A) or trans (B) compounds were incubated at the concentrations ranging from 1 to 50 μM, as specified on the top of the lanes, with a radiolabeled double-stranded XH-24-bp oligonucleotide for 2 h at 37°C. Electrophoretic migration reveals different radiolabeled bands identified using arrows: ds-f, free double-stranded oligonucleotide; ds-b, alkylated (bound) double-stranded oligonucleotide; ss-f, free single-stranded oligonucleotide; ss-b, alkylated (bound) single-stranded oligonucleotide.

---

**Fig. 9.** Drug induced DNA-destabilization. A, DNA alkylation within a short hairpin oligonucleotide. EMSA was performed by incubating the short 22-mer oligonucleotide with a 9-bp stem containing a single guanine (•) surrounded by two adenine bases (HP-AGA) with 50 μM concentrations of various racemate or enantiomers of cis- or trans-diacetate compound or the racemate cis-dicarbamate S29385-1 as a control. B, nuclease S1 digestion of single-stranded DNA generated after benzoacryonic alkylation of the DNA. The various di-acetylated forms of the benzo[g]acryonic core (μM) were incubated for 1H with the radiolabeled 117-bp DNA fragment before digestion of the DNA at single-stranded positions by addition of nuclease S1 and subsequent incubation. The generated DNA fragments were resolved on a 10% native polyacrylamide gel under denaturing conditions. Free and bound DNA fractions are localized as "f" and "b" forms. Localization of the drug-induced S1 nuclease cleavage sites ("NS1") are exemplified by arrows. Nuclease S1 sites generated a position similar to that obtained in the presence of the pure enantiomers bearing a reactive acetate at the 1′ position (S27589-1 and S70256-1) are localized using a dashed arrow, whereas that obtained in the presence of their respective isomers bearing the acetate leaving group at position 1′R are presented using a plain shaft arrow.
review, see Lenglet and David-Cordonnier, 2009). Such stereospecific protein/DNA recognition was previously evidenced for platinated DNA adducts. In this case, cisplatin adducts, but not transplatin adducts, are recognized by HMG proteins as part of the drug-induced DNA bent (Gelasco and Lippard, 1998) that perfectly fit the L-shaped structure of HMG DNA binding domain (HMG-box) (Chow et al., 1994).

Likewise, the Mut-Sc (MSH2/MSH6 heterodimer) mismatch repair enzyme recognizes cisplatin but not transplatin cross-links, probably as part of poor base stacking and base flipping generated by the cisplatin-induced DNA bent (Fourrier et al., 2003). Indeed, such distortions and constraints of the DNA are known to play a role in the initiation of the DNA lesions recognition by repair proteins (Fuxreiter et al., 2002; Yang, 2006). This stereoselective binding of DNA repair proteins to a bulky DNA adduct was also recently found for benzo(a)pyrene DNA adducts. BaP lesions are recognized by XPC, the "sensor" protein of the nucleotide excision repair machinery that associates with HR23B to initiate DNA repair (Jung et al., 2007). Accordingly, an anti-convulsant drug: pharmacological profile in animals. 

Acknowledgments

M.-H.D.-C. thanks the Service de Spectrométrie de Masses de l’Université de Lille 2 (Dr. Mostafa Kouch and Gabrielle Briand) for access to and expertise in HPLC-coupled simple-quadrupole mass spectrometer API 3000 equipment, and Nicole Wattez for expert technical advice in HPLC separation.

References


Cai Y, Patel DJ, Geacintov NE, and Broyde S (2009) Differential nucleotide excision repair enzyme recognizes cisplatin but not transplatin cross-links, probably as part of poor base stacking and base flipping generated by the cisplatin-induced DNA bent (Fourrier et al., 2003). Indeed, such distortions and constraints of the DNA are known to play a role in the initiation of the DNA lesions recognition by repair proteins (Fuxreiter et al., 2002; Yang, 2006). This stereoselective binding of DNA repair proteins to a bulky DNA adduct was also recently found for benzo(a)pyrene DNA adducts. BaP lesions are recognized by XPC, the "sensor" protein of the nucleotide excision repair machinery that associates with HR23B to initiate DNA repair (Jung et al., 2007). Accordingly, an anti-convulsant drug: pharmacological profile in animals. 

Acknowledgments

M.-H.D.-C. thanks the Service de Spectrométrie de Masses de l’Université de Lille 2 (Dr. Mostafa Kouch and Gabrielle Briand) for access to and expertise in HPLC-coupled simple-quadrupole mass spectrometer API 3000 equipment, and Nicole Wattez for expert technical advice in HPLC separation.

References


Cai Y, Patel DJ, Geacintov NE, and Broyde S (2009) Differential nucleotide excision repair enzyme recognizes cisplatin but not transplatin cross-links, probably as part of poor base stacking and base flipping generated by the cisplatin-induced DNA bent (Fourrier et al., 2003). Indeed, such distortions and constraints of the DNA are known to play a role in the initiation of the DNA lesions recognition by repair proteins (Fuxreiter et al., 2002; Yang, 2006). This stereoselective binding of DNA repair proteins to a bulky DNA adduct was also recently found for benzo(a)pyrene DNA adducts. BaP lesions are recognized by XPC, the "sensor" protein of the nucleotide excision repair machinery that associates with HR23B to initiate DNA repair (Jung et al., 2007). Accordingly, an anti-convulsant drug: pharmacological profile in animals. 

Acknowledgments

M.-H.D.-C. thanks the Service de Spectrométrie de Masses de l’Université de Lille 2 (Dr. Mostafa Kouch and Gabrielle Briand) for access to and expertise in HPLC-coupled simple-quadrupole mass spectrometer API 3000 equipment, and Nicole Wattez for expert technical advice in HPLC separation.

References


Cai Y, Patel DJ, Geacintov NE, and Broyde S (2009) Differential nucleotide excision repair enzyme recognizes cisplatin but not transplatin cross-links, probably as part of poor base stacking and base flipping generated by the cisplatin-induced DNA bent (Fourrier et al., 2003). Indeed, such distortions and constraints of the DNA are known to play a role in the initiation of the DNA lesions recognition by repair proteins (Fuxreiter et al., 2002; Yang, 2006). This stereoselective binding of DNA repair proteins to a bulky DNA adduct was also recently found for benzo(a)pyrene DNA adducts. BaP lesions are recognized by XPC, the "sensor" protein of the nucleotide excision repair machinery that associates with HR23B to initiate DNA repair (Jung et al., 2007). Accordingly, an anti-convulsant drug: pharmacological profile in animals. 

Acknowledgments

M.-H.D.-C. thanks the Service de Spectrométrie de Masses de l’Université de Lille 2 (Dr. Mostafa Kouch and Gabrielle Briand) for access to and expertise in HPLC-coupled simple-quadrupole mass spectrometer API 3000 equipment, and Nicole Wattez for expert technical advice in HPLC separation.

References


Cai Y, Patel DJ, Geacintov NE, and Broyde S (2009) Differential nucleotide excision repair enzyme recognizes cisplatin but not transplatin cross-links, probably as part of poor base stacking and base flipping generated by the cisplatin-induced DNA bent (Fourrier et al., 2003). Indeed, such distortions and constraints of the DNA are known to play a role in the initiation of the DNA lesions recognition by repair proteins (Fuxreiter et al., 2002; Yang, 2006). This stereoselective binding of DNA repair proteins to a bulky DNA adduct was also recently found for benzo(a)pyrene DNA adducts. BaP lesions are recognized by XPC, the "sensor" protein of the nucleotide excision repair machinery that associates with HR23B to initiate DNA repair (Jung et al., 2007). Accordingly, an anti-convulsant drug: pharmacological profile in animals. 

Acknowledgments

M.-H.D.-C. thanks the Service de Spectrométrie de Masses de l’Université de Lille 2 (Dr. Mostafa Kouch and Gabrielle Briand) for access to and expertise in HPLC-coupled simple-quadrupole mass spectrometer API 3000 equipment, and Nicole Wattez for expert technical advice in HPLC separation.


**Address correspondence to:** Dr Marie-Hélène David-Cordonnier, INSERM-U837, Centre de Recherches Jean-Pierre Aubert (JPARC), Team-4 “Molecular and Cellular Targeting for Cancer Treatment,” Institut pour la Recherche sur le Cancer de Lille, Place de Verdun, F-59045 Lille, France. E-mail: marie-helene.david@inserm.fr