

Synthesis and Preliminary Biological Evaluation of a New Pyridocarbazole Derivative Covalently Linked to a Thymidine Nucleoside as a Potential Targeted Antitumoral Agent. I

Carmela SATURNINO,^a Mariafrancesca BUONERBA,^a Gianpiero BOATTO,^b Maria PASCALE,^a Ornella MOLTEDO,^a Lorenzo De NAPOLI,^c Daniela MONTESARCHIO,^c Jean-Charles LANCELOT,^d and Paolo de CAPRARIIS*^a

^aDipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno; Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy; ^bDipartimento Farmaco Chimico Tossicologico, Università degli Studi di Sassari; Via Muroni n° 23, 07100 Sassari, Italy; ^cDipartimento di Chimica e Biochimica, Università degli Studi di Napoli "Federico II"; via Cynthia 4, 80126 Napoli, Italy; and ^dCentre d'Etudes et de Recherche sur le Medicament de Normandie (C.E.R.M.N.), UFR des Sciences Pharmaceutiques; 1 rue Vaubenard, 14032 Caen Cedex, France.

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The therapy of human cancer is one of the more pursued goals by medicinal chemistry research. Most of the compounds clinically used as a treatment owe their efficacy to their cytotoxic interaction (direct or indirect) with nuclear DNA. This interaction results in the inhibition of DNA synthesis and the degradation of nucleic strands. Ellipticine is a naturally occurring 6*H*-pyrido[4,3-*b*]carbazole alkaloid endowed with antitumor activity, and several ellipticine derivatives have been used in clinical trials. We previously reported some 1,4-dimethyl-9*H*-carbazole derivatives structurally related to ellipticine. The purpose of our research was to transform the pyridocarbazole in a prodrug so that it would have more penetration in the tumor cells and block their replication. Our prodrug is slowly hydrolyzed in human plasma in the corresponding acid. From these preliminary results, we deduce that our compound can block cellular replication. Our hypothesis is that the antitumoral activity is probably related to the induction of damage to DNA, without cellular lysis in the short term.

Key words ellipticine; anticancer; cytotoxicity

Ellipticine is a naturally occurring alkaloid of the 6*H*-pyridocarbazole family; this compound and its derivatives are endowed with antitumor properties. They are DNA intercalating molecules and their high DNA binding affinity is thought to be responsible in part for these pharmacological properties.^{1,2} 5,11-Dimethyl-6*H*-pyrido[3,2-*b*]carbazole derivatives, which are very similar in their structure to ellipticine (Fig. 1), bearing either a fluorine, a bromine, or a chlorine atom in the 9 position and differently substituted on the pyridine ring, have been synthesized in our laboratories and its antitumoral activity was tested in *in vitro* and *in vivo*.^{3,6} With the aim of improving its pharmacokinetic properties, mainly enhancing cellular uptake in tissues where nucleoside receptors are present, we have designed and synthesized a new pyridocarbazole derivative in which the aromatic polycyclic moiety is linked to the 5 position of a thymidine nucleoside through an amido linkage (Charts 1, 2), thus exploiting the nucleosidic residue as molecular carrier.^{7,11} The synthesis of target compound **5** is illustrated in Chart 2. As the first step, ester **1** was treated with a stoichiometric amount of NaOH in pyridine, and after 12 h at room temperature, was transformed carboxylic acid **2** in 70% yield after chromatography. It was not possible to force this reaction to completeness, since prolonged basic treatment, higher temperatures, or the addition of more base in all cases led to a complex mixture of very polar compounds, which, according to ¹H- and ¹³C-NMR analysis, were the result of the opening of the heterocyclic system in **1**. However, under the cited conditions for the basic hydrolysis of **1**, unreacted starting material could be recovered in 10% yield and reused. Compound **2** was then reacted with 5-deoxy, 5-amino, 3'-*O*-*t*-butyldimethylsilyl (TBDMS)-thymidine **3**, which was synthesized from thymi-

dine in four steps in 75% overall yield (Chart 1), according to published procedures.⁴ The best coupling yields were obtained using the system benzotriazol-1-(yloxy)tris(pyrolidino)phosphoniumhexafluorophosphate/1-hydroxybenzotriazol (PyBop/HOBt) as the condensing agent in anhydrous *N,N*-dimethylformamide/*N,N*-diisopropylethylamine (DMF/DIEA), leading to compound **4** in 75% yield after purification. The successive treatment of **4** with the classic desilylating reagent TBAF in tetrahydrofuran (THF) to remove the TBDMS group led only to degradation products; however, the 3-OH group could be successful deprotected, but in low yield (32% after chromatography) with the addition of Et₃N·3HF in anhydrous THF. The purity and structure of all synthesized compounds were ascertained by ¹H- and ¹³C-NMR analysis as well as by mass spectral data.

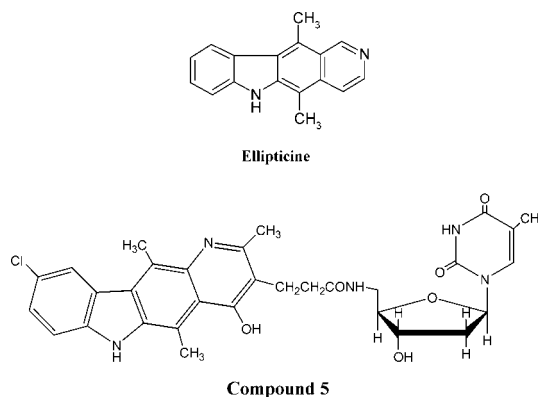


Fig. 1. Structures of Ellipticine and Compound **5**

* To whom correspondence should be addressed. e-mail: saturnino@unisa.it

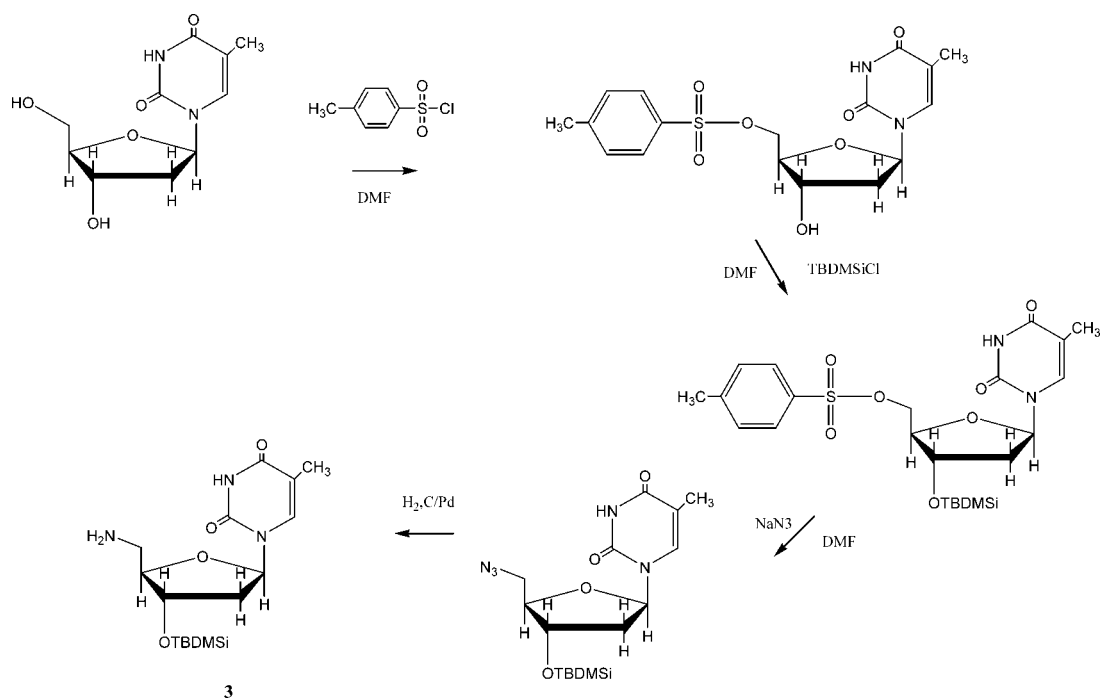


Chart 1

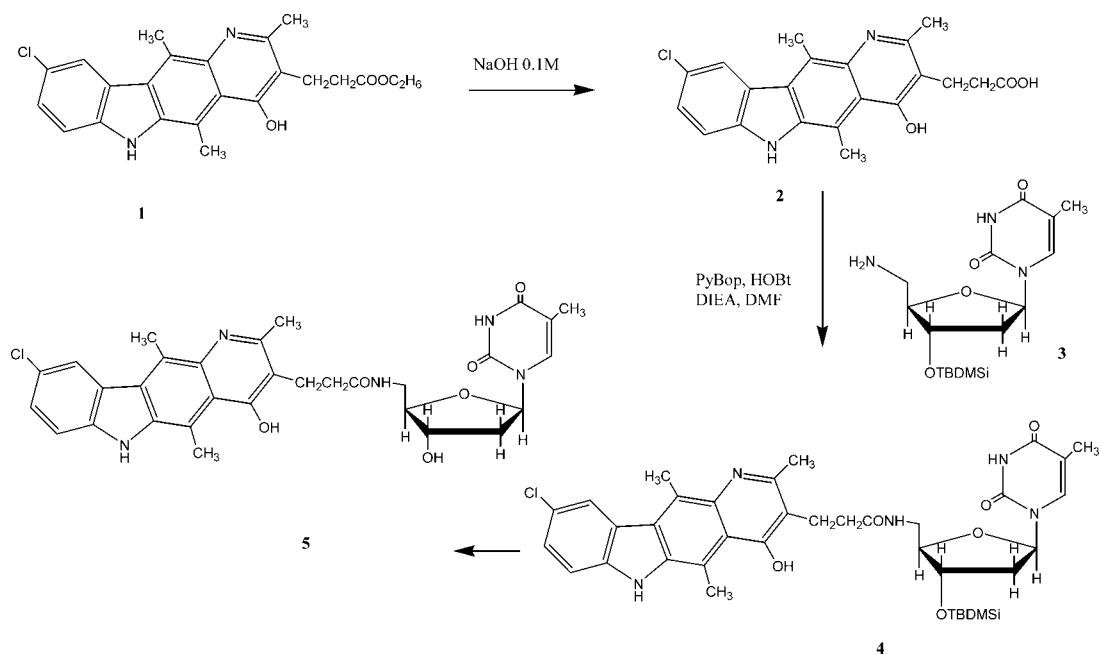


Chart 2

Results

Detection of Cytotoxicity, Apoptosis Induction, and Stability Assay

The effects of compound **5** on cytotoxic activity were studied *in vitro* in SAOS cell line, and cell vitality was evaluated after different periods of continuous exposure to compound **5** 50 μM . A SAOS parallel culture were treated with methanol only as an experimental control. Figure 2 shows the results obtained in triplicate cultures for each treatment. Cytotoxicity induced by **5** was time dependent; treatment with compound **5** 50 μM reduced cell viability by 40% at 4 d and by 58% at 5 d of exposure, whereas no effect

was found in the methanol-treated cell line. These results clearly indicate that compound **5** is highly cytotoxic in these conditions. To investigate whether the cytotoxic effect was due to apoptosis or another mechanism, nuclear morphology studies were performed using DAPI staining. After 4 d of treatment with the **5** SAOS cell line and parallel untreated culture 50 μM were stained as described in the Materials and Methods, and analyzed using an Axiophot fluorescence microscope with a 63 \times 1.4 NA plane Apochromat objective. No significant presence of apoptosis, in the form of condensed or fragmented nuclei, could be detected in response to treat-

ment, as shown in Fig. 3. Thus the lack of increase in apoptosis between treated and untreated cells may depend on p53 deficiency, as demonstrated in a previous report.¹² The present results indicate that compound **5** is potently cytotoxic in SAOS cells. Since these cells are p53 deficient, the mechanism(s) underlying cytotoxicity do not involve p53 activity. Topoisomerase inhibitors have been reported to induce the generation of hydrogen peroxide in target cells.¹³ An analogous activity might be displayed by compound **5**, leading to either cell necrosis or apoptosis-induced mitochondrial damage.¹⁴ However, further investigations will be necessary to identify the interesting mechanism(s) through which compound **5** activates cytotoxicity and to determine *in vivo* how many prodrugs (or drugs derived from enzymatic hydrolysis)¹⁵ are present in several organs of the guinea pig. The stability assay results demonstrated that compound **5** is stable at pH 7.4 and was hydrolyzed slowly in human plasma and transformed in the active compound. In this way, through the carrier, it is not hydrolyzed at the peripheral level, but shows greater activity in specific tumor cells. The enzyme cathepsin hydrolyzed the carbamidic bond, giving the active compound.

Experimental

General Column chromatography was performed on silica gel (Merck, Kieselgel 40, 0.063, 0.200 mm). TLC analysis was carried out on Merck Kieselgel 60 F254 0.25-mm plates, visualized with UV light.

For the electrospray ionization (ESI)-MS analysis, a Waters Micromass ZQ instrument equipped with an electrospray source was used in the positive and/or negative mode. NMR spectra were recorded on Bruker WM-400 and

on Varian-Gemini 300 spectrophotometers. All chemical shifts are expressed in ppm with respect to the residual solvent signal. *J* values are given in Hz. UV measurements were performed on a Perkin.

Synthesis of 2 Compound **1** (30 mg, 0.073 mmol) was left in contact with 730 μ l of a 0.1 M solution of NaOH in pyridine and stirred for 12 h at room temperature. The reaction was then quenched by the addition of 3.8 mg of NH₄Cl, and the resulting mixture was concentrated under reduced pressure and purified on two silica gel plates eluted with CHCl₃/CH₃OH 9:1 (v/v). The band at *R*_f=0.2 was scratched off the plates and eluted from silica with CHCl₃/CH₃OH 8:2 (v/v). After drying, the eluate afforded 19 mg (0.051 mmol, 70% yield) of pure **2**.

Compound **2**: Yellow powder. *R*_f=0.35, eluent CHCl₃/CH₃OH 8:2 (v/v). δ _H (DMSO-*d*₆, 300 MHz): 11.32 (1H, bs, NH); 8.29 (1H, s, H-10); 7.52 (2H, overlapping signals, H-7 and H-8); 3.13, 2.98, and 2.60 (3H each, s, three CH₃ groups); 2.72 (2H, t, *J*=6.8, CH₂CH₂COOH); 2.39 (2H, t, *J*=6—8 CH₂CH₂COOH). δ _C (DMSO-*d*₆, 125 MHz): 175.32 (COOH); 145.72, 140.84, 136.72, 132.96, 126.43, 123.69, 122.96, 128.80, 122.65, 122.34, 120.75, 116.31, 116.29, 116.08, 112.21 (aromatic carbons); 21.56 (CH₂CH₂COOH); 17.73 (CH₂CH₂COOH); 16.02, 13.98, and 11.32 (three CH₃ groups). ESI-MS, *m/z*: Found, 380.99 (M-H); Calcd for C₂₁H₁₈N₂O₃Cl, 381.101.

Synthesis of 5 Compound **2** 17 mg (0.044 mmol) was allowed to react at room temperature with 23 mg (0.066 mmol) of 5-deoxy, 5-amino, 3-*O*-TBDMS-thymidine, 34 mg (0.066 mmol) of PyBop, and 10 mg (0.066 mmol) of HOBt dissolved in 1 ml of anhydrous DMF containing 11 μ l (0.066 mmol) of DIEA. The reaction was monitored by TLC analysis (CHCl₃/CH₃OH 8:2, v/v) and after 2 h, **2** had completely consumed and a new product formed at *R*_f=0.9.

The mixture was then concentrated under reduced pressure and purified on three silica gel plates eluted with CHCl₃/CH₃OH 9:1 (v/v). The band at *R*_f=0.75 was scratched off the plates and eluted from silica with CHCl₃/CH₃OH 8:2 (v/v). After drying 23 mg (0.033 mmol, 75% yield) of **4** remained, and the purity was confirmed by TLC and NMR analysis. Compound **4** (23 mg, 0.033 mmol) was dissolved in anhydrous THF 500 μ l and treated with 25 μ l (0.16 mmol) of Et₃N·3HF overnight at room temperature. The crude reaction was diluted with THF, then dried, purified on two silica gel plates and eluted with CHCl₃/CH₃OH 85:15 (v/v). The band at *R*_f=0.6 was scratched off the plates, and eluted with CHCl₃/CH₃OH 8:2 (v/v).

The dried eluate afforded 6 mg (0.010 mmol, 32% yield) of **5**, and the purity was confirmed by TLC and NMR analysis.

Compound **4**: Yellow powder, *R*_f=0.9 (eluent CHCl₃/CH₃OH 8:2, v/v). δ _H (CD₃OD, 400 MHz): 8.03 (1H, s, H-10); 7.35 (2H, overlapping signals, H-7 and H-8); 7.31 (1H, s, H-6 thymine residue); 5.94 (1H, dd, *J*=6.4, 6.4 Hz, H-1); 4.30 (1H, m, H-3); 3.85 (1H, m, H-4); 3.41 (2H, m, H₂-5); 3.03 (3H, s, CH₃ group); 2.90 (2H, t, *J*=6.8 Hz, CH₂CH₂CONH); 2.77 and 2.56 (3H each, two CH₃ groups); 2.52 (2H, t, *J*=6.8 Hz, CH₂CH₂CONH); 2.07 (2H, m, H-2); 1.80 (3H, s, CH₃ thymine residue); 0.87 (9H, s, three CH₃ *t*-butyl group); 0.072 and 0.061 (3H each, s's, two CH₃-Si). δ _C (CD₃OD, 125 MHz): 176.79, 166.63 (C-4 thymine residue); 152.52 (C-2 thymine residue); 134.33 (C-6 thymine residue); 111.98 (C-5 thymine residue); 148.68, 142.96, 139.17, 138.23, 128.39, 125.55, 125.17, 124.40, 122.71, 118.19, 117.92, 117.58, 113.34 (aromatic carbons); 87.64 and 87.62 (C-1 and C-4); 74.97 (C-3); 42.95 (C-2); 41.36 (C-5); 36.71 (CH₂CH₂CONH); 26.71 (three CH₃ *t*-butyl group); 24.06 (CH₂CH₂CONH);

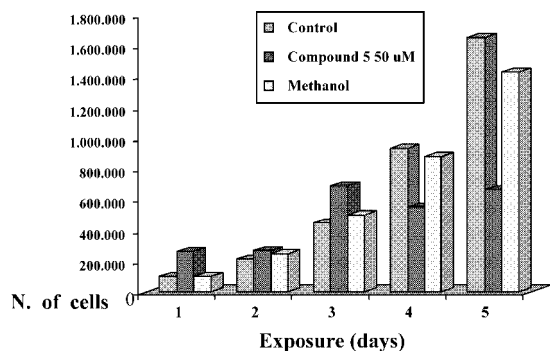


Fig. 2. Inhibition of Growth in SAOS Cells by Compound **5** and Methanol

The cells were incubated for different times in the presence of the drugs (50 μ M) or methanol or untreated as controls and their survival was determined by trypan blue dye staining.

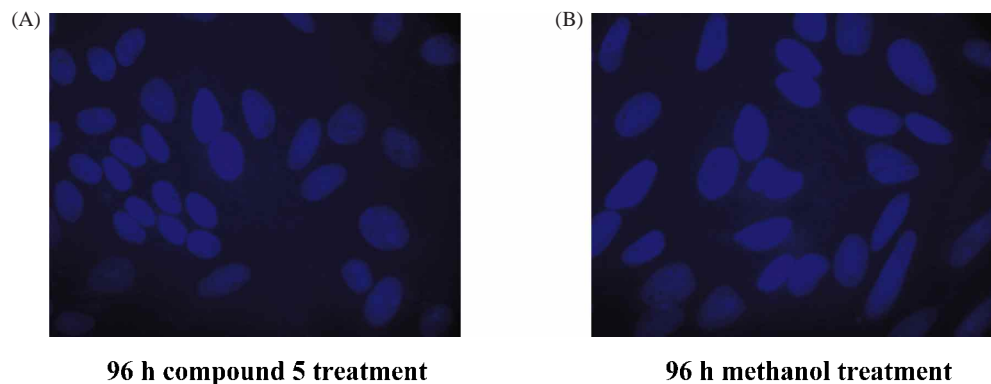


Fig. 3. Apoptosis Induction: DAPI Staining of SAOS Cells Treated (Panel A) and Untreated (Panel B) for 4 d with Compound **5** 50 μ M (A) or Methanol 50 μ M (B)

19.26 (quaternary C *t*-butyl groups); 18.78, 16.97 and 14.24 (three CH₃ groups); 12.28 (CH₃ thymine residue); 4.15 and 4.13 (two CH₃-Si). ESI-MS, *m/z*: Found, 720.31 (M+H)⁺; Calcd for C₃₇H₄₇N₅O₆ClSi, 720.298.

Compound **5**: Yellow powder, *R*_f=0.5 (eluent CHCl₃/CH₃OH 8:2, v/v). δ_H (CD₃OD, 400 MHz): 8.27 (1H, s, H-10); 7.48 (2H, overlapping signals, H-7 and H-8); 7.16 (1H, s, H-6 thymine residue); 5.86 (1H, dd, *J*=6.8, 6.8 Hz, H-1); 3.90 (1H, m, H-3); 3.45 (2H, m, H₂-5); 3.15, 3.04 and 2.64 (3H each, three CH₃ groups); 2.96 (2H, dd, *J*=7.5 Hz, CH₂CH₂CONH); 2.56 (2H, m, CH₂CH₂CONH); 2.01 (2H, m, H-2); 1.78 (3H, s, CH₃ thymine residue). ESI-MS, *m/z*: found for C₃₁H₃₃N₅O₆Cl, 606.27 (M+H)⁺. Elemental analyses was performed in our analytical labs and agreed with the theoretical values within ±0.4%.

Stability Tests. Materials and Reagents Phosphate buffer pH 7.4 (50 mM) was adjusted with phosphoric acid. Methanol was purchased from Carlo Erba (Italy) (HPLC grade). Human plasma was purchased from LAS Laboratory (Sassari, Italy). Deionized and distilled water was purified through a Milli Q system (Millipore, Milford, MA, U.S.A.).

Chemical Stability Compound **5** methanolic solution 2 ml (0.5 mg/ml) was added to 4 ml of pH 7.4 phosphate buffer. The solution was maintained at 37 °C and aliquots were withdrawn every 1 h for the initial 6 h of incubation. The possible disappearance of the prodrug was determined with HPLC analysis using the method reported below. The prodrug concentrations were determined from the calibration curve constructed by standard solutions with known concentrations (Table 1).

Enzymatic Stability A 4-ml volume of plasma was measured and a 3-ml volume of standard solution (1 mg/ml) was added with swirling. After standing for appropriate times (0, 1, 2, 3, 4, 6 h) 1-ml aliquots of the samples were extracted with 2 ml of acetonitrile; the obtained suspensions were vortex-mixed and centrifuged at 3000×*g* for 10 min. The supernatant was transferred to appropriate autosampler vials and analyzed by HPLC method reported below. The prodrug concentrations were determined from the calibration curve obtained by spiking the extracts of blank plasma samples with known concentrations (Table 2).

Analytical Procedures HPLC analysis was performed on a Lichrocart RP 18 column (particle size: 5 μm; 250×4 mm i.d.; Merck, Darmstadt Germany). Two isocratic elutions were used: mobile-phase 100% methanol for chemical stability and mobile-phase methanol/water 80/20 for enzymatic stability, respectively. The flow rate was set at 1.0 ml/min. Each sample containing the prodrug was filtered prior to injection using a Millex HV13 filter (Millipore) and an aliquot (20 μl) was injected into the HPLC apparatus (Hewlett-Packard chromatograph HP 1100, automatic volume injection, and DAD/UV detector) The prodrug was monitored at 260 nm. The retention time was 2.7 min. No peaks of **2** and **3** were obtained. The amounts of **2** and **3** were negligible in terms of background.

Pharmacology Ellipticine and derivative compounds are highly cytotoxic substances that kill mammalian cells at concentrations ranging from

10⁻⁸ to 10⁻⁶ M. These compounds form molecular complexes with DNA *in vitro* and are able to damage the DNA of mammalian cells by inducing double-strand breaks and DNA-protein-cross links.¹⁶⁾ Previous evidence suggested that ellipticine derivatives can interfere with the activity of topoisomerase II.¹⁷⁾ We studied the cytotoxic effects of an ellipticine derivative in human osteosarcoma SAOS cells, which lack p53, a tumor-suppressor gene product that causes cell cycle arrest in the late G₁ phase and induces apoptosis by upregulation of Waf 1 and Bax genes.¹⁸⁾

Materials and Methods. Cell Culture and Trypan Blue Staining Cells were grown in DMEM w/o glutamine medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum, penicillin (10⁵ UI/l), streptomycin (0.10 g/l) in a 5% CO₂ humidified atmosphere at 37 °C. The compound was dissolved in methanol, and added at various times to the cells in exponential phase of growth (0.15×10⁶ cells/plate). Nonclonogenic assay cytotoxicity was studied using the trypan blue dye exclusion test. Cells were incubated for different time in the presence of 50 μM of the compounds, and were incubated for 4 min with 0.25% trypan blue (Sigma Chemical Co.) and 5% fetal calf serum. Viable cells were identified by ability to exclude dye, whereas the dye diffuses into nonviable cells. Trypsin (0.25% Boehringer Mannheim) was routinely used for subculture. It was determined dose-response curves obtained from at least three independent experiments.

Nuclear Staining with DAPI To test abilities to induce apoptosis, SAOS cells were grown on glass coverslips, after treatment rinsed in phosphate buffered saline (PBS) fixed in paraformaldehyde for 20 min at room temperature, the coverslips were incubated at room temperature in a solution of diamino-2-phenyl-indole (DAPI), after 30 min, they were washed three times in water and put on a glass slide, using Mowiol.

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Table 1. Chemical Stability

Time (h)	Recovery (%)
0	100
1	100
2	100
3	100
4	100
6	100

Table 2. Enzymatic Human Plasma Stability

Time (h)	Recovery (%)
0	100
1	100
2	100
3	100
4	95
6	85