DNA-Damaging, Mutagenic, and Clastogenic Activities of Gentiopicroside Isolated from *Cephalaria kotschyi* Roots

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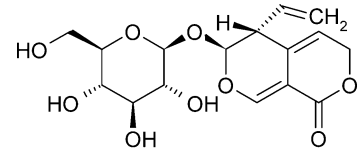
Gentiopicroside (**1**) is the major secoiridoid glucoside constituent of *Cephalaria kotschyi* roots. The mutagenicity, DNA-damaging capacities, and clastogenicity of this molecule were evaluated by the *Salmonella typhimurium* mutagenicity assay (Ames test) on tester strains TA97a, TA98, TA100, and TA102, the alkaline comet assay, and the micronucleus assay on CHO cells. All tests were performed with and without the metabolization mixture, S9 mix. In the Ames test, the mutagenicity of **1** was limited to TA102 without S9 mix (2.3 rev μg^{-1}). The genotoxicity was more evident without S9 mix (0.78 OTMχ^2 units μg^{-1} mL) than with the metabolic mixture (0.16 OTMχ^2 units μg^{-1} mL) with the comet assay. Similarly, the clastogenicity without S9 mix was 0.99 MNC μg^{-1} mL and 0.38 MNC μg^{-1} mL with S9 mix in the micronucleus assay. The interaction of **1** with DNA is probably through the involvement of oxidative DNA lesions.

For centuries, plants have provided important therapeutic agents for the treatment of illnesses among different ethnic groups. Currently, a large part of the human population still relies on medicinal plants for primary health care and alternative therapy in both developing and industrialized countries. Since traditional herbal remedies refer to a long historical knowledge accumulated over years and to a long-term use in extended populations, plants or natural compounds that are frequently employed in traditional medicine are assumed to be efficient and safe.[1] Consequently, their possible adverse effects and their long-term toxicity have been poorly studied and documented.

In order to preserve plant populations and ancestral knowledge, the World Health Organization (WHO) has focused on the need to promote traditional medicine for health care. In this ethnobiological context, the confirmation that plant extracts and natural products are safe has become a determinant element of development for traditional drugs.[1] Unfortunately, various studies that have been conducted to evaluate the potential genotoxic effects of medicinal plants have revealed positive results.[2,3] They have raised concerns about the carcinogenic hazards resulting from the long-term use of several plants and underlined the necessity to investigate the possible genotoxic activity of natural products present in all the most commonly used traditional remedies.

As a result of a bilateral research program between France and Azerbaijan, the phytochemical analysis of *Cephalaria kotschyi* Boiss. & Hoghen. (Dipsacaceae) has led to the identification of gentiopicroside (**1**) as the major secoiridoid glucoside present in the roots.[4] On the basis of the wide occurrence of this natural compound in the well-known medicinal plant gentian[5,6] and Chinese medicinal plants such as "Long-dan-tan",[7] we evaluated the possible genotoxic, mutagenic, and clastogenic effects of **1** by the *Salmonella typhimurium* mutagenicity assay (Ames test), comet assay, and the in vitro micronucleus assay.



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Results and Discussion

Compound **1** was isolated from the roots of *C. kotschyi*, an endemic flowering plant of the Caucasus region, as previously reported.[4]

The capacity of **1** to induce mutations was evaluated by the *S. typhimurium* mutagenicity assay. This short-term assay, also called the Ames test, has been extensively used to survey a great variety of environmental substances for mutagenic activity. It has been shown to detect over 80% of the known organic carcinogens,[8] and it is now widely accepted for identifying substances that can produce genetic damage leading to gene mutations.[9] Four tester strains, with different preexisting mutations in the histidine operon, were used in the present study: *Salmonella typhimurium* TA97a, TA98, TA100, and TA102. Moreover, an exogenous activation system (S9 mix) was added to the *S. typhimurium* cultures during the assay[9] to mimic the possible in vivo transformation of **1** by the cytochrome-based P450 oxidation systems located in the liver of mammals and to estimate the potential mutagenic activity of its electrophilic metabolites.

Complete results of the *S. typhimurium* mutagenicity test on **1** are reported in Table 1. The results of regression analyses are included in Table 2 and displayed in Figure 3a. Revertant scores significantly increased with the *S. typhimurium* strain TA102 without S9 mix only. The corresponding dose-dependent activity was determined by a nonlinear regression analysis according to the mathematical model proposed by Kim and Margolin.[10] The mutagenic activity (MA) calculated for TA102 was MA = 2.3 rev μg^{-1} ($r^2 = 0.85$, $p < 10^{-5}$, $p_E = 0.80$). This indicates that **1** can induce DNA damage directly and can lead to gene mutation in prokaryotic cells.

The TA102 tester strain used in this study contained an ochre type of mutation characterized by the presence of A−T base pairs at the *hisG428* mutant site carried on a multicopy of the pAQ1 plasmid.[11] This ochre mutation can be reverted by all the possible base-pair changes, both transitions and transversions.[9] It has been

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Table 1. Mutagenic Activity of **1** in the *Salmonella typhimurium* Mutagenicity Assay

compound		dose (μg/plate)	number of revertants by plate[a] TA97a	TA98	TA100	TA102
without S9 mix	DMSO[b]	10 μL	146 ± 8	22 ± 4	108 ± 5	290 ± 10
	ICR 191[c]	0.002	1287 ± 52	−	−	−
	TNF one[d]	0.002	−[h]	727 ± 13	−	−
	NaN_3[e]	5.0	−	−	1880 ± 27	−
	MitC[f]	0.002	−	−	−	3499 ± 25
	1	40.0	188 ± 9	19 ± 4	117 ± 5	*368 ± 30*[i]
		60.0	174 ± 18	22 ± 6	135 ± 23	*378 ± 21*
		80.0	180 ± 17	26 ± 5	138 ± 24	*395 ± 14*
		100.0	180 ± 7	26 ± 8	136 ± 3	*392 ± 25*
with S9 mix	DMSO[b]	10 μL	154 ± 18	34 ± 4	124 ± 13	369 ± 34
	BaP[g]	0.5	636 ± 54	667 ± 22	1477 ± 13	1412 ± 6
	1	40.0	274 ± 13	32 ± 9	119 ± 10	376 ± 33
		60.0	186 ± 13	29 ± 3	116 ± 4	385 ± 18
		80.0	178 ± 6	28 ± 3	140 ± 6	413 ± 24
		100.0	173 ± 10	31 ± 6	138 ± 14	385 ± 17

[a] Results are expressed as means ± SD from triplicate plates. [b] DMSO: solvent control. [c] ICR 191: 2-methoxy-6-chloro-9-(3-(2-chloroethyl)aminopropylamino)acridine. Two HCl (positive control for TA97a). [d] 2,4,7 TNFone: 2,4,7-trinitro-9-fluorenone (positive control for TA98). [e] NaN_3: sodium azide (positive control for TA100). [f] MitC: mitomycine C (positive control for TA102). [g] BaP: benzo[*a*]pyrene (positive control for S9 mix). [h] −: not tested. [i] *Italics*: Statistical difference ($p < 0.05$) between the numbers of induced revertants at a given tested dose and the number of spontaneous revertants by the Dunnett test.[10]

Table 2. Modeling of Experimental Responses of **1** in the *Salmonella typhimurium* Mutagenicity Assay, the Comet Assay, and the Micronucleus Test

genotoxicity bioassays		model[a]	r^{2b}	p^c	$p_E{}^d$	activity
mutagenicity assay (TA102)	− S9 mix[e]	MAR-2	0.85	$>10^{-5}$	0.85	2.3 rev μg^{-1}
	+ S9 mix[f]	MAR-2	0.17	0.29	0.34	NS[g]
comet assay	− S9 mix	MC-1	0.96	$>10^{-5}$	0.13	0.78 OTMχ^2 units μg^{-1} mL
	+ S9 mix	MC-1	0.85	$>10^{-4}$	0.17	0.16 OTMχ^2 units μg^{-1} mL
micronucleus assay	− S9 mix	MN-1	0.90	$>10^{-4}$	0.26	0.99 MNC μg^{-1} mL
	+ S9 mix	MN-1	0.86	$>10^{-4}$	0.17	0.38 MNC μg^{-1} mL

[a] Regressions were performed with the software TableCurve 2D using three arbitrary models as described in the Experimental Section. [b] r^2: coefficient of determination. [c] p: model probability. [d] p_E: error probability of the model. [e] − S9 mix: experiment conducted without the metabolic fraction S9 mix. [f] + S9 mix: experiment conducted with the metabolic fraction S9 mix. [g] NS: not significant.

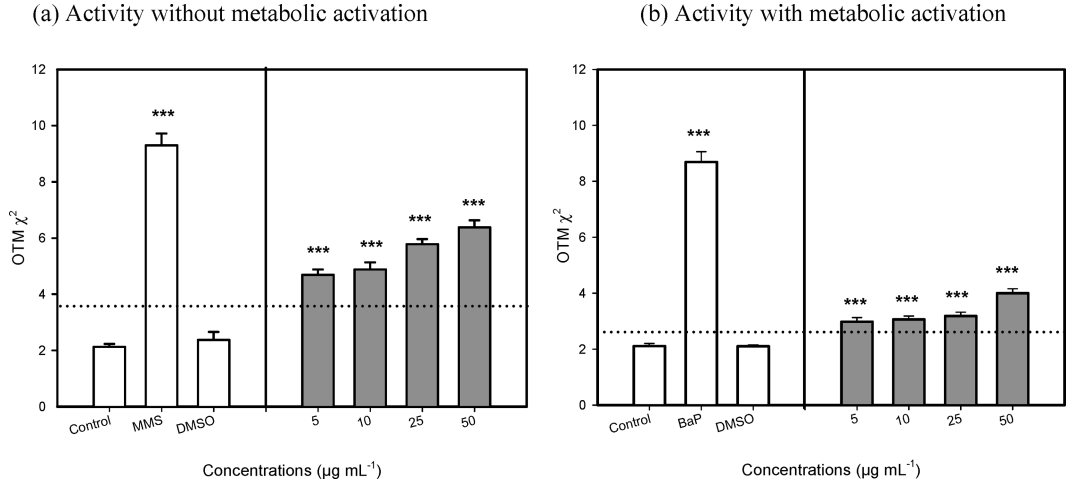

Figure 1. Evaluation of the DNA-damaging activity of **1** on CHO cells by the alkaline comet assay. The negative controls were DMSO (5 μL), and the positive controls were methylmethanesulfonate (MMS, 60 μM) without S9 mix and benzo[*a*]pyrene (BaP, 0.4 μM) with S9 mix. Dashed line: $p = 0.05$. ***: $p \leq 0.001$.

shown to be particularly sensitive to oxidizing mutagens that are not significantly mutagenic in other tester strains.[11] Therefore, the positive result obtained exclusively with TA102 without S9 mix suggested that DNA damage induced by **1** was of an oxidative type.

The capacity of **1** to induce DNA strand breaks was assessed by the comet assay on Chinese hamster ovary (CHO) cells. This microelectrophoretic method, which is also referred to as the single-cell gel electrophoresis assay (SCG or SCGE assay), is a rapid and quantitative technique by which visual evidence of DNA damage in eukaryotic cells may be measured.[12] It is based on the quantification of denatured DNA fragments migrating out of the cell nucleus during electrophoresis. The resulting image obtained with this technique is a "comet" with a distinct head consisting of intact DNA and a tail that contains damaged or broken pieces of DNA.

In the present study, Chinese hamster ovary cells were exposed for 2 h to a range of concentrations of **1** in the absence or in the presence of the metabolization mixture, S9 mix. At these concentrations, cell viability was more than 95%, as assessed by the WST1 test, and it did not decrease during a 24 h incubation period. DNA

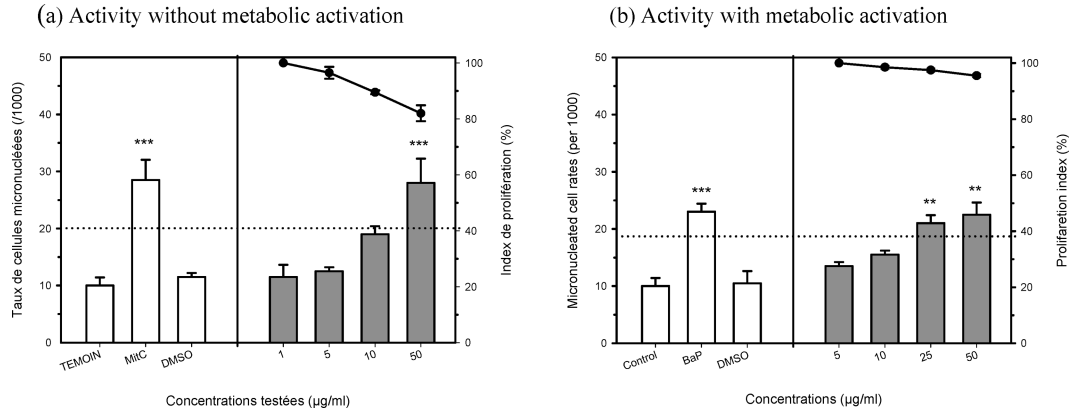

Figure 2. Evaluation of the clastogenic properties of **1** on CHO cells by the micronucleus assay. The negative controls were DMSO (5 μL), and the positive controls were mitomycin C (MitC, 0.06 μg mL^{-1}) without S9 mix and benzo[*a*]pyrene (BaP, 5 μg mL^{-1}) with S9 mix. Dashed line: $p = 0.05$. **: $p \leq 0.01$. ***: $p \leq 0.001$.

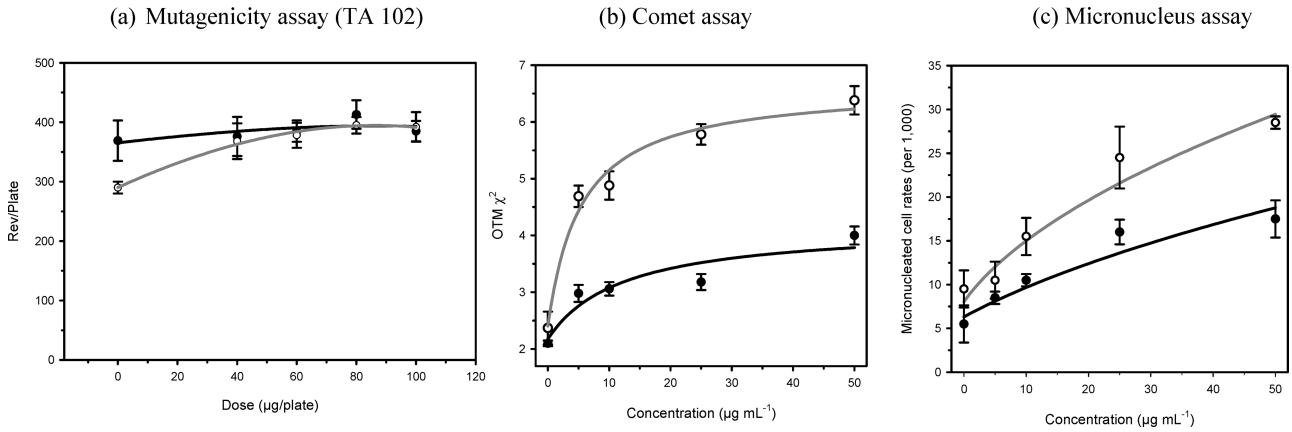

Figure 3. Modeling of the experimental dose−response relationships. Nonlinear regressions were performed with the software TableCurve 2D using arbitrary models as described in the Experimental Section. (Gray line) Nonlinear regression obtained without S9 mix. (○) Experimental data obtained without S9 mix. (Black line) Nonlinear regression obtained with S9 mix. (●) Experimental data obtained with S9 mix.

strand breaks were scored as compared to a negative control containing the solvent (DMSO) and to a positive control containing methylmethane sulfonate (MMS) without S9 mix, or benzo[*a*]pyrene (BaP) with S9 mix. The results of the comet assay are reported in Figure 1, and modeling of the dose−response curves is included in Table 2 and displayed in Figure 3b. Product **1** induced a significant increase of DNA strand breaks in CHO cells. The corresponding dose-dependent activity was determined by a nonlinear regression analysis according to the mathematical model proposed by Aouadene et al.[13] The induction activity (IA) was 0.78 OTMχ^2 units μg^{-}1 mL ($r^2 = 0.96$, $p < 10^{-5}$, $p_E = 0.13$) in the absence of the metabolic fraction S9 mix, and IA = 0.16 OTMχ^2 units μg^{-1} mL ($r^2 = 0.85$, $p < 10^{-4}$, $p_E = 0.17$) in the presence of the metabolic fraction. These results indicated that **1** can induce DNA lesions that evolve into DNA strand breaks. Various types of genotoxic mechanisms could be envisaged, since in the alkaline version of the assay increased DNA migration is associated with single-strand or double-strand breaks leading from direct DNA interaction, incomplete excision repair sites, and alkali-labile sites. The strong reduction of the DNA-damaging activity in the presence of the S9 mix was probably due to the antiradical properties of the proteins contained in the S9 fraction.

The capacity of **1** to induce chromosome mutations was assessed by the micronucleus assay performed on Chinese hamster ovary cells, according to the protocol described by Kirsch-Volders et al.[14] Micronuclei are defined as chromosome fragments or whole chromosomes that lag during cell division due to the lack of a centromere or to a defect in cytokinesis.[15] They may be produced by clastogenic or aneugenic compounds, according to a wide range of mechanisms that includes both genotoxic and epigenetic events.[15] The micronucleus assay allows the scoring of micronuclei in the cytoplasm of interphasic cells exposed in vitro or in vivo to clastogenic and/or aneugenic agents.[14]

In the present study, Chinese hamster ovary cells were exposed for 3 h to a range of concentrations of **1** in the absence or presence of the metabolization mixture, S9 mix. Then, micronucleated cell ratios (MNC μg^{-1} mL) were scored as compared to a negative control containing the solvent (DMSO) and to a positive control containing mitomycin C (without S9 mix) or benzo[*a*]pyrene (with S9 mix). The results of the micronucleus assay are reported in Figure 2. The proliferative index (PI), calculated as the ratio between dividing plurinucleated cells and nonproliferating mononucleated cells, gave an estimation of the antiproliferative activity of the compound. No significant decrease of the PI could be observed in this study, indicating that **1** did not exert a strong cytostatic activity against CHO cells. On the contrary, a significant increase of micronucleated cell rates could be observed both in the absence and in the presence of S9 mix. The clastogenic activities (CA), determined by nonlinear regression analysis according to the mathematical model proposed by Aouadene et al.,[13] were CA = 0.99 MNC μg^{-}1 mL ($r^2 = 0.90$, $p < 10^{-4}$, $p_E = 0.26$) in the absence of the metabolic fraction S9 mix and CA = 0.38 MNC μg^{-1} mL ($r^2 = 0.86$, $p < 10^{-4}$, $p_E = 0.17$) in the presence of the metabolic fraction (Table 2 and Figure 3c). These results established that **1** exhibits a significant clastogenic/aneugenic activity against CHO cells.

Micronuclei may be formed by two main mechanisms in mammalian cells.[16] The first mechanism, named clastogenicity, involves a direct interaction of exogenous agents with DNA. This results in an increased rate of DNA lesions that may undergo DNA repair and lead to double-strand breaks. These evolve into chromosomal mutations and produce chromosome fragments, which lag during cell division due to a missing centromere and form heritable micronuclei. The second mechanism, named aneugenicity, is based on the interaction of exogenous agents with cellular components involved in cell mitosis such as the mitotic spindle.[16] These initiate chromosome malsegregation and chromosome loss, leading to whole chromosome micronuclei. In the present study, the positive results obtained in the *S. typhimurium* and the comet assays suggested that micronuclei induced by **1** mainly originate from a clastogenic effect rather than from aneugenic events.

Compound **1** has been isolated from a variety of plants of the Gentianaceae.[17−19] It is a major constituent present in various traditional remedies used to treat chronic liver diseases, acute and chronic dysentery, digestive dysfunctions, and cancer.[20] Its biological activity has been established by both in vivo and in vitro experiments, which have revealed choleretic, antihepatotoxic, adaptogenic, anti-inflammatory, and, more recently, analgesic properties.[21−23] However, in the absence of acute toxicity, the possible long-term adverse effects of this secoiridoid glycoside have not been studied.

The results observed in this study have presented evidence that gentiopicroside exerts a genotoxic activity against both prokaryotic and eukaryotic cells. It has been demonstrated that **1** generates DNA lesions that could undergo DNA repair systems and transform into heritable gene and chromosome mutations. Therefore, the data obtained raise concern about the possible mutagenic hazards resulting from the extensive use of this natural compound.

The mechanism by which gentiopicroside (**1**) may interact with DNA appears difficult to envisage, since comparison between its molecular structure with predictive models in computational databases[24,25] revealed a weak structural similarity to established genotoxic and mutagenic agents. However, the specific positive response obtained with the TA102 tester strain suggested the involvement of oxidative DNA lesions, probably due to the presence of hydroxy groups that may produce oxygen singlets.

The long-term and transmissible risks generated by the use of gentiopicroside (**1**) in traditional herbal remedies are not easy to estimate in the absence of anecdotal information or epidemiological data. Traditional remedies generally are aqueous extracts of complex mixtures in which the activity of each phytochemical component may be modulated by interactions due to synergism, antagonism, or additivity. For example, the bioavailability of **1** in rats has been shown to be markedly improved when administered as a decoction rather than as purified compound.[26] On this basis, interactions between mutagenic and protective compounds may lead to a different response pattern according to the plant material used, the mode of preparation of the medication, and the physiological status of the patient. However, since most of the genotoxic/mutagenic environmental molecules have been shown to exert both carcinogenic and teratogenic properties,[27] traditional remedies containing high concentrations of **1** should be prescribed with particular care, especially during pregnancy or when they are administrated for extended periods of time. At the same time, additional experiments should be performed to estimate the in vivo genotoxic activity of **1**.

Experimental Section

General Experimental Procedures. Gentiopicroside (**1**), isolated from the roots of *Cephalaria kotschyi*, was identified using spectroscopic methods.[4] The purity of **1** was performed by HPLC analysis on an Agilent series 1100 HPLC system equipped with a G1315B diode-array detector. A Symmetry C_{18}, 5 μm (4.6 mm × 250 mm), column was used for the analysis, with a flow rate of 1 mL/min and a detection wavelength of $\lambda = 235$ nm. The elution was carried out by using an isocratic solvent system, H_2O−MeOH (80:20). The sample (20 mg) was suspended in 10 mL of mobile phase and filtered through a 0.2 μm syringe filter. The purity of **1** was equal to 99.3%.

Metabolic Activation Mixtures (S9 Mix). The metabolic activation system was a 9000 g centrifuged supernatant of a liver homogenate (S9) and was prepared from male Sprague−Dawley rats treated with a single injection of Aroclor 1254 (500 mg kg^{-1} body weight). The protein concentration in the S9 homogenate was 26 mg mL^{-1} as determined by the method of Lowry et al.[28] In the *S. typhimurium* mutagenicity assay, the composition of the final metabolic mixture (S9 mix) included 4% S9, 10 mM glucose-6-phosphate (G6P), and 8 mM nicotinamide adenine dinucleotide phosphate (NADP).[29] In the micronucleus and comet assays, the S9 mix contained 10% S9, 5 mM G6P, 4 mM NADP, 33 mM KCl, and 8 mM $MgCl_2$, diluted in saline phosphate buffer.[13]

Cell Cultures. Micronucleus and comet assays were performed using Chinese hamster ovary cells (CHO-K1, ATCC) maintained in McCoy's 5A medium (Sigma, St Quentin-Fallavier, France) supplemented with 10% fetal calf serum, 1 mM glutamine, and penicillin−streptomycin (100 U mL^{-1} and 10 μg mL^{-1}) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

***Salmonella typhimurium* Mutagenicity Assay.** The mutagenicity of **1** was assessed by the microsuspension version of the *S. typhimurium* mutagenicity test[29] using four strains of *S. typhimurium* with and without S9 mix: TA97a, TA98, TA100, and TA102. The prepared plates were incubated at 37 °C for 48 h, and colonies were counted with an automatic laser counter (Spiral System Instrument Inc., Bethesda, MD). To interpret the data, a two-step analysis was performed. The Dunnett test[10] was primarily performed to determine a significant difference between the mean number of induced revertants and the mean number of spontaneous revertants. If the Dunnett test was positive for at least one sample concentration, a nonlinear regression analysis was carried out using an arbitrary model as described previously[10] with TableCurve 2D software (version 5.0, Jandel Scientific Software, San Rafael, CA):

$$\text{MAR-2: rev/plate} = (a + bD)e^{(-cD^2)}$$

where rev/plate is the number of revertants by plate, D is dose, and a, b, and c are calculated coefficients.

Model significance was based on two criteria: (i) model probability (p) being <0.05 and (ii) error probability (p_E) being >0.05. The mutagenic activity (MA, rev μg^{-1}) was defined as the maximal slope of the ascending part of the dose−response curve and was calculated as the first derivative at the origin.

WST1 Test. The cytotoxicity of **1** was assessed using the oxidation−reduction indicator WST1. Cells were seeded in 96-well plates and incubated overnight at 37 °C with 5% CO_2 in a humidified incubator. A range of concentrations of **1** was incorporated in triplicate cultures (final DMSO concentration less than 0.2%), and cells were incubated at 37 °C for 2 and 24 h. At the end of the incubation period, cultures were submitted to three successive washes in PBS and incubated in 10% WST1 in culture medium for 30 min. Cell viability was evaluated by the assessment of WST1 absorbance at 450 nm in a MRX II microplate spectrophotometer (Dynex Technologies, Chantilly, VA).

Alkaline Comet Assay. The alkaline comet assay was performed as described by Tice et al.[12] with slight modifications.[30] A total of 50 000 Chinese hamster ovary cells were plated in chamber slides and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Compound **1** was diluted with DMSO and added in duplicate to the cell cultures.

Following a 2 h contact period, the cells were imbedded in low melting point agarose. Lysis, DNA unwinding, and electrophoresis were performed as already described.[30] Following the electrophoretic run, the slides were neutralized with 0.4 M Tris-HCl pH 7.5, rinsed with ultrapure water, dipped into 100% methanol (HPLC grade purity solvent), and dried at room temperature.

Staining was performed with ethidium bromide solution (2 μg mL^{-1}), and the slides were examined at 250× magnification using a BH2-RFL fluorescence microscope (Olympus Optical Co. Tokyo, Japan). Image analysis was performed using the Komet software (version 5.5 Kinetics Imaging, Nottingham, UK) on 100 randomly selected cells (50 cells for each of two replicate slides). DNA damage was expressed as Olive tail moment (OTM, arbitrary units).[31] The calculated OTM values were distributed into 40 classes between the minimal and the

maximal OTM values. A nonlinear regression analysis was performed on the normalized distribution frequencies using a χ^2 function with TableCurve 2D. The calculated degree of freedom (n) for the function, named OTM χ^2, was assumed to be a quantitative measure of the level of DNA damage in the sample.[13] The test was considered positive when a dose−response relationship could be established between the OTMχ^2 and the concentrations of **1** and when one concentration at least induced a significant increase of OTMχ^2 by the χ^2 statistical test ($p < 0.05$). The induction activity was calculated by nonlinear regression analysis with TableCurve 2D using an arbitrary model:

$$\text{MC-1: IA} = \frac{aC}{b + C} + d$$

where IA is the induction activity (OTM χ^2 units μg^{-1} mL), a, b, and d are calculated coefficients, and C is the tested concentration (μg mL^{-1}).

Model significance was based on three criteria: (i) correlation coefficient r^2, (ii) model probability (p) being <0.05, and (iii) error probability (p_E) being >0.05. The induction activity (OTM χ^2 AU μg^{-1} mL) was defined as the maximal slope of the ascending part of the concentration−response curve and was calculated as the first derivative at the origin.

Micronucleus Assay on CHO-K1 Cells. Altogether, 50 000 Chinese hamster ovary cells were plated in chamber slides and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. Compound **1** was diluted in DMSO, and four concentrations of the original solution were added to duplicate cell cultures as already described.[13] Cytochalasin B was incorporated into each chamber slide to arrest cytokinesis. Air-dried slides were stained with 5% Giemsa.

The PI was considered as a measure of antiproliferative activity.[14] It was determined by scoring the number of mononucleated (M1), binucleated (M2), and trinucleated (M3) cells among 500 Giemsa-stained cells with well-preserved cytoplasm: PI = (M1 + M2 + M3)/500.

The micronucleated cell rates were determined for concentrations inducing less than 50% decrease of the PI: 2000 binucleated cells were examined and micronuclei were identified according to the morphological criteria previously defined by Kirsch-Volders et al.[14] Statistical differences between negative controls and treated samples were performed using the χ^2 test. The assay was considered positive when a dose−response relationship could be established between the numbers of micronucleated cells and the concentrations of **1** and when one concentration at least induced a significant increase of micronuclei.

The clastogenic activity (MNC μg^{-1} mL) was calculated by nonlinear regression analysis with TableCurve 2D using the following arbitrary model:

$$\text{MN-1: MNC}^2 = a + bC$$

where MNC is the micronucleus frequency (%), a and b are calculated coefficients, and C is the tested concentration (μg mL^{-1}).

Model significance was based on three criteria: (i) correlation coefficient r^2, (ii) model probability (p) being <0.05, and (iii) error probability (p_E) being >0.05. The clastogenic activity (MNC μg^{-1} mL) was defined as the maximal slope of the ascending part of the concentration−response curve and was calculated as the first derivative at the origin.

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