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DNA-damaging activity and mutagenicity of 16 newly synthesized thiazolo[5,4-*a*]acridine derivatives with high photo-inducible cytotoxicity

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Abstract

The discovery of the potent anticancer properties of natural alkaloids in the pyrido-thiazolo-acridine series has suggested that thiazolo-acridine derivatives could be of great interest. In a continuous attempt to develop DNA-binding molecules and DNA photo-cleavers, 16 new thiazolo[5,4-*a*]acridines were synthesized and studied for their photo-inducible DNA-intercalative, cytotoxic and mutagenic activities, by use of the DNA methyl-green bioassay, the Alamar Blue® viability assay and the *Salmonella* mutagenicity test using strains TA97a and TA98 with and without metabolic activation and photo-activation. Without photo-activation, one compound showed a DNA-intercalative activity in the DNA major groove while three compounds displayed intercalating properties after photo-activation. In the dark, four molecules possessed cytotoxic activities against a THP1 acute monocytic leukemia cell line while 15 derivatives displayed photo-inducible cytotoxic activity against this cell line. All compounds were mutagenic in strain TA97a with metabolic activation (+S9mix) and 15 molecules were mutagenic in strain TA98 without activation (−S9mix). Study of the quantitative structure–activity relationships (QSAR) from the *Salmonella* mutagenicity data revealed that several descriptors could describe cytotoxic and mutagenic activities after photo-activation. From the results of the mutagenicity test, four compounds with elevated mutagenic activities were selected for additional experiments. Their capacities to induce single-strand breaks (SSB) and chromosome-damaging effects were monitored by the comet and the micronucleus assays in normal human keratinocytes. Comparison of the minimal genotoxic concentrations showed that two compounds possessed higher capacities to induce SSB after photo-activation. In the micronucleus assay, three molecules were able to induce high numbers of micronuclei following photo-activation. Overall, the results of this study confirm that acridines are predominantly genotoxic via a DNA-intercalating mechanism in the dark, while DNA-adducts were probably induced following photo-activation.

Keywords: Thiazolo-acridines; DNA-binding activity; *Salmonella* mutagenicity test; Comet assay; *In vitro* micronuclei; Cytotoxicity; QSAR; Photoactivation; Simulated sunlight

1. Introduction

Since their first synthesis by Graebe and Caro in 1870, acridine chromophores have revealed a broad spectrum of anti-infectious activities [1,2]. In the mid 1960s, the discovery of the activity of acridine in preventing the growth of tumor cells led to the possibility of using such compounds in anticancer therapy and stimulated the synthesis of new derivatives [3]. Recently, the synthesis of acridines with additional rings, e.g., acromycine [4,5], has focused the interest on tetracyclic compounds. Furthermore, the discovery of natural alkaloids of the pyrido-thiazolo-acridine series with potent anticancer properties

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[6,7] has suggested that thiazolo-acridine derivatives could be of great interest.

The mechanisms by which acridines exert their pharmacological properties have been shown to be closely related to their capacity to reversibly bind with DNA [8], to interact with DNA regulatory enzymes [9] and to disrupt DNA functions in the cell. The concept of the intercalation of acridines between adjacent base pairs of the double-helical DNA, first proposed for proflavine [8], has been extensively demonstrated with various other derivatives [10]. Subsequently, acridine derivatives have revealed genotoxic properties leading to mutagenicity [11], reproductive toxicity and carcinogenicity [12]. However, while simple acridines were ascertained as intercalating agents and model frameshift mutagens in bacteriophages [13], bacteria [14,15] and mammalian cells [16], several derivatives revealed a large variety of mutagenic properties. Nitroacridines, capable of reacting as alkylating agents depending on the position of the nitro-group [17,18] and ICR compounds, in which the acridine ring was used as a carrier for targeting alkylating agents to DNA [19], produced both frameshift and base-pair substitutions [20,21]. Acridines with additional fused aromatic rings such as benzacridines were shown to interact covalently with DNA after metabolic activation to mainly produce base-pair substitution mutations [22,23]. This suggested that the DNA-binding properties of acridine-based compounds could substantially differ according to their chemical structure, the intracellular environment or the experimental conditions, and illustrated the multi-factorial aspect of acridine-induced mutagenic events.

Among the various factors capable of modulating the cellular effects of acridine, the involvement of oxidative stress by reactive oxygen species has been suggested since the 1980s [24]. This was supported by the fact that acridine-induced DNA damage was greatly enhanced with increasing oxidation potential of the compounds and demonstrated the importance of electron-transfer reactions during acridine–DNA interactions [24]. As a consequence, several members of the acridine series exhibited photo-inducible properties, mainly photo-toxicity, photo-bactericidal activity [2] and photo-enhanced genotoxicity [25].

In an attempt to discover new DNA photo-cleavers and to better understand the genotoxic consequences of their interactions with DNA, 16 thiazolo[5,4-*a*]acridines were synthesized and assessed for their photo-inducible DNA-binding activity [26], mutagenicity in *Salmonella typhimurium* [27], and cytoxicity against human monocytes. The most mutagenic compounds of this series were then tested for the ability to induce single-strands breaks and heritable chromosomal damages, by use of the comet assay [28] and the micronucleus assay in human keratinocytes [29].

2. Materials and methods

2.1. Chemicals

Two classical synthetic routes were used for the synthesis of acridine derivatives [30] as shown in Fig. 1. 2-Chloro-6-nitro-benzothiazole was first prepared by nitration of 2-chloro-benzothiazole (CAS No. 615-20-3, Sigma–Aldrich, Saint Quentin Fallavier, France) with HNO_3/H_2SO_4 and used as a starting material for the synthesis of all other compounds. The reduction of the nitro-group with iron powder in hydrochloric acid led to 2-chloro-amino-benzothiazole. Then, the Ullmann condensation [30] with orthobromobenzoic acid gave the corresponding *N*-6-aryl-anthranilic acid. The cyclation of *N*-6-aryl-anthranilic acid was done according to the methodology of Friedel-Crafts [30] with $POCl_3$ or H_2SO_4 to produce, respectively, compounds **3** and **11**. The thiation of compound **3** with P_4S_{10} produced compound **7**, and compound **5** after an additional *S*-alkylation. Compound **10** was obtained by substitution of the chloro-group with ammonium carbonate in phenol. In parallel, the thiation of 2,11-dichloro-thiazolo[5,4-*a*] acridine (**11**) with P_2S_5 provided compound **8**, while the substitution of chlorine in position 11 with phenol or alkylamines gave, respectively, compound **13**, and compounds **2**, **6**, **14** and **51**.

An additional synthetic pathway was developed to obtain acridine derivatives bearing a 2-substituted thiazole-ring: 2-chloro-6-nitro-benzothiazole was subjected to nucleophilic substitution by various alkylamines to give the corresponding 2-substituted 6-nitro-benzothiazoles and the equivalent 2-substituted 6-amino-benzothiazoles were then obtained by reduction of the nitro-groups according to the methodology of Katz, which finally yielded the thiazolo[5,4-*a*]acridine analogues **1**, **9**, **67**, **70** and **86** following Ullmann condensation with orthobromobenzoic acid and cyclization according to the protocol developed by Friedel-Crafts.

Chemical structures of acridine derivatives were characterized by 1D and 2D NMR analyses. TLC, microanalysis and HPLC confirmed that the purity of the compounds was >99.5%. In all assays, acridine orange (CAS No. 65-61-2, Sigma–Aldrich) was used as the reference compound. All compounds were dissolved in spectra-grade dimethyl sulfoxide (DMSO) at their solubility limit (5–10 mg/ml). The solutions were kept frozen at −80 °C until used.

2.2. Irradiation procedure

Irradiation was performed with a solar simulator Suntest CPS+ (Atlas Material Testing Technology BV, Moussy le Neuf, France) equipped with a xenon-arc lamp, a treated quartz filter to block infrared light and a glass filter to block UVC light and to reduce UVB light (UVA/UVB: 0.5/0.001 mW/cm^2, simulated sunlight 800 lx). Preliminary dose-finding experiments performed with *S. typhimurium* TA98 and various doses of acridine orange showed that the optimal incident dose of light was 2 mJ/cm^2 for a period of 1 min.

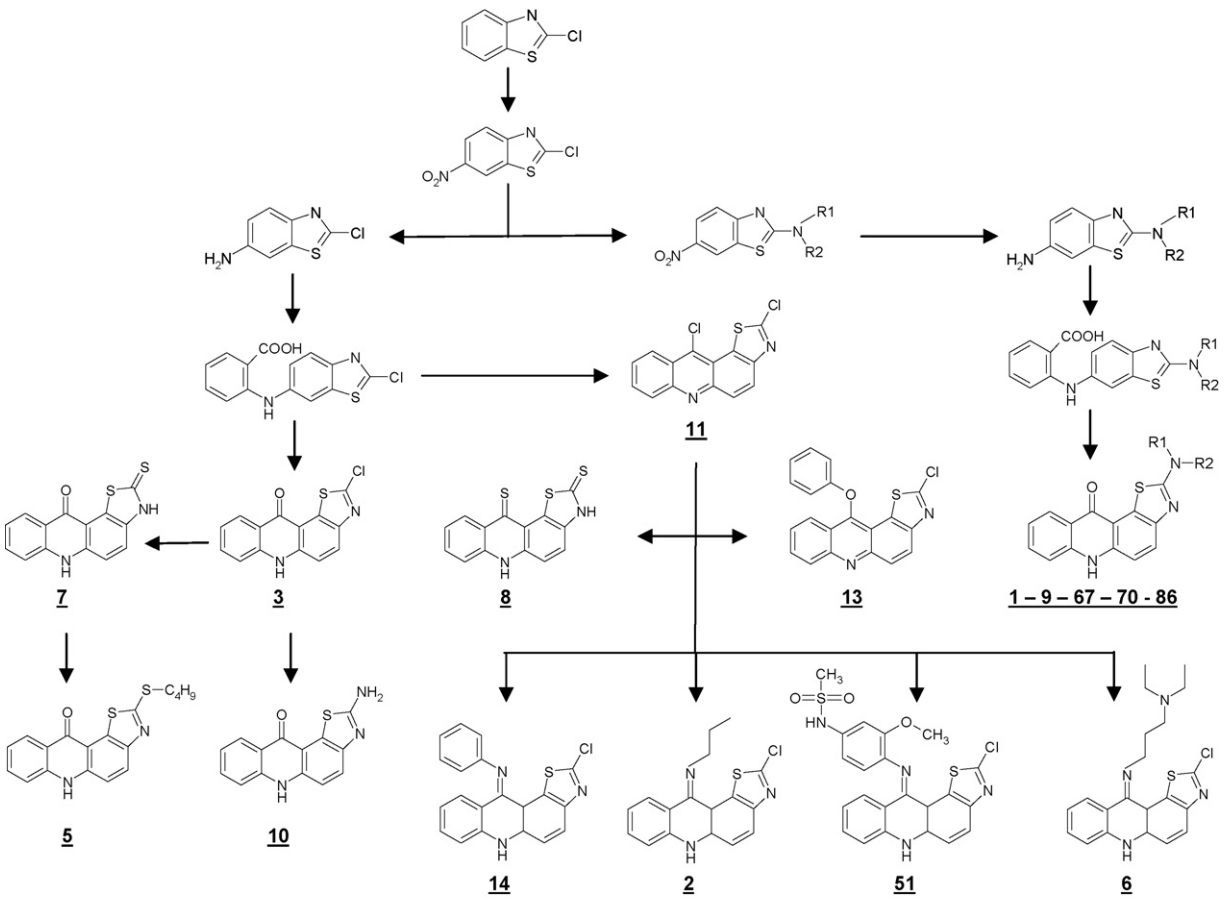
2.3. DNA–methyl green bioassay

The capacities of acridine derivatives to interact with the DNA major groove were assessed by the DNA–methyl green bioassay [26], using the triphenyl methane dye methyl green, a major groove binding agent which may reversibly interact with DNA according to a reaction that can be followed spectrophotometrically. Twenty milligrams DNA–methyl green (Sigma) were suspended in 100 ml of 0.05 M Tris–HCl buffer, pH 7.5, containing 7.5 mM of magnesium sulfate and stirred at 37 °C during 24 h. A volume of 200 μl of the above solution was put into each well of a multi-well plate and various concentrations of acridine derivatives were added (from 0.1 to 100 μM). The initial absorbance of each sample was measured at 630 nm. After an incubation period of 24 h in the dark at room temperature, the final absorbance of the samples was measured again. The readings were corrected for the initial absorbance and normalized as percentages of the untreated DNA–methyl green absorbance value. Dose–response curves were calculated by non-linear regression analysis (TableCurve 2.1), according to a log-normal mathematical model. $IC_{80\text{-DNA}}$ corresponded to the concentration of chemical compounds that induced a 20% decrease of absorbance.

2.4. Salmonella mutagenicity test

2.4.1. Salmonella tester strains and activation mixture

Salmonella tester strains TA97a, TA98, TA100 and TA102 were gifts from Prof. B.N. Ames (Berkeley, CA, USA). The strains were stored at −80 °C and regularly checked for genetic markers [33]. The liver homogenate used for metabolic activation (S9) was prepared from male Sprague–Dawley rats treated with Aroclor 1254 (500 mg/kg body weight). The protein concentration was 26 mg/ml as determined by the technique of Lowry [31]. The S9 mix was a mixture of 4% S9 and a solution of NADPH-generating factors (mix).



Molecules	R1	R2
1	CH_3	CH_3
9	C_2H_5	C_2H_5
67	H	C_2H_4OH
70	H	$(CH_2)_3$-N-$(C_2H_5)_2$
86	H	$C_5H_{10}N$

Fig. 1. Chemical structures and synthetic route of the 16 acridine derivatives.

2.4.2. Assay protocol

The assay was carried out according to [27] using a modified version of the liquid incubation technique [31]. *Salmonella* strains were grown in Oxoid Nutrient Broth No. 2 with ampicillin (25 μg/ml) for 12 h at 37 °C with gentle shaking. After the incubation period, the following ingredients were placed in 12-mm × 75-mm polystyrene tubes: (1) various volumes of the tested molecules (four tested doses per compound) not exceeding 10 μl (0.5%, v/v) to avoid DMSO toxicity, (2) 0.1 ml of S9 mix if needed; (3) 0.1 ml of the overnight culture. The mixtures were incubated either for 60 min in the dark or for 45 min in the dark followed by irradiation during one minute. Then, 2-ml volumes of melted top agar containing 0.045 mM histidine and biotin were added to the tubes and the mixtures were poured onto Vogel-Bonner (VB) minimal plates. For each series of experiments, the controls included: (i) 5 or 10 μl of DMSO without and with S9 mix, respectively, to determine the numbers of spontaneous revertants/plate; (ii) positive controls: 0.5 μg of acridine orange for quality control of S9 mix in the dark and 0.05 μg of acridine orange for quality control of irradiation. After a 48-h period of incubation, the revertants were counted with a laser colony-counter equipped with a bacterial enumeration program (Spiral System Instrument Inc., Bethesda, MD, USA). All experiments were conducted three times and the mutagenic activity was calculated by non-linear regression analysis using an arbitrary model on the ascending part of the dose–response curve, as described previously [31]. The minimal mutagenic concentration was defined as the lowest concentration (μM) inducing a doubling of the number of spontaneous revertants/plate, with all compounds being tested in a final volume of 0.2 ml during the contact period.

2.5. Cytotoxicity against human transformed monocytes

The cytotoxic activity of acridine derivatives was assessed on the THP1 human acute monocytic leukemia cell line (ATCC, Manassas VA, USA) by colorimetric determination of the number of viable cells using the oxidation–reduction indicator Alamar Blue® (Invitrogen, Cergy-Pontoise, France) [32]. Late log-phase human monocytes were incubated in RPMI 1640 medium without phenol red supplemented with 2 mM L-glutamine, 10% fetal calf serum and 10% Alamar Blue®. A range of acridine concentrations (from 0.001 to 25 μM) was incorporated in duplicate cultures (final DMSO concentration <1%). After a 48-h incubation period at 37 °C in a 5% CO_2 atmosphere, enzymatic reduction of Alamar Blue® from blue to pink in viable cells was measured by monitoring absorbance at 570 and 630 nm. Negative controls were treated with solvent (DMSO) and were included to each set of experiments. Dose–response curves were calculated by non-linear regression analysis (TableCurve 2.1 software, Systat Software Inc, USA) from duplicated experiments according to a log-normal mathematical model. The $IC_{50\text{-THP1}}$ corresponded to the concentration of acridine compound able to induce a 50% decrease of cell viability as compared with the control culture.

2.6. Induction of DNA-strand breaks (Comet assay) and micronuclei in normal human keratinocytes

Keratinocytes were used to study the photo-inducible DNA-damaging activity and clastogenic and/or aneugenic effects of the chemical compounds. These constitutive cells of the skin provide an efficient barrier against sunlight, which is essential for survival, and have developed resistance to the lethal effects of UVB light [33]. Since the 1990s they have been used to assess the phototoxicity of molecules included in cosmetics, to detect the photo-protective activity of sunscreens and to evaluate the genotoxic effects of radiation on normal human cells [34]. Recently, keratinocytes have been proposed as an alternative non-animal tool for ascertaining the safety of ingredients in cosmetics [35].

2.6.1. Isolation and culture of normal human keratinocytes

Normal human keratinocytes were isolated from infant foreskin obtained after circumcision according to the technique described by Decome et al. [28]. Tissues were cut into 5-mm × 5-mm pieces and incubated in 2.5 ml trypsin (2.5%, v/v) in saline at 37 °C with constant shaking. After a 90-min incubation period, the trypsin was inactivated by addition of 50 μl fetal calf serum. Dermis and epidermis were separated mechanically and epidermis fragments were transferred into sterile PBS. After pipetting to break-up the cell pellets, the cell suspension was centrifuged three times (1200 × *g*, 5 min). Cells were suspended in K-SFM medium containing 25 μg/ml BPE and 0.1–0.2 ng/ml rEGF, and seeded in flasks in complete K-SFM medium. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2, and the culture medium was changed every 3 days. Pure keratinocyte cultures were obtained within 2 weeks.

2.6.2. Comet assay

The Comet assay was performed under alkaline conditions [28]. Duplicate cultures of human keratinocytes were transferred to 35-mm Petri dishes and treated with various acridine concentrations (0.35, 0.7, 0.14, and 1.75 μM). The first set was directly incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2 h, while the second set was irradiated before incubation. At the end of the incubation period, cells were trypsinized (0.05% trypsin, 0.02% EDTA in PBS) for 3–5 min at 37 °C and centrifuged at 1,200 × *g* for 5 min. Cell pellets were resuspended in 75 μl of low melting-point agarose (LMP, 0.5% agarose in PBS), placed on pre-treated slides coated with 85 μl normal agarose gel (0.8% in PBS) and finally covered with 75 μl LMP. Slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% *N*-lauryl-sarcosinate, pH 10–12, 1% triton X-100 and 10% DMSO) during 90 min at 4 °C. After cell lysis, the microscope slides were placed in an electrophoresis tank and DNA was allowed to unwind in freshly prepared alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA, pH 13) for 20 min at room temperature. Then, electrophoresis was conducted for an additional 20 min period at 25 V and 300 mA. The slides were rinsed three times with cold neutralizing buffer (0.4 M Tris–HCl, pH 7.5) for 5 min, dehydrated in pure methanol and kept in a dried-atmosphere chamber until assessment. DNA damage was analyzed after staining with ethidium bromide (2 μg/ml in ultrapure water) and examination of slides was performed with a BH2-RFL fluorescence microscope (Olympus Optical Co, Tokyo, Japan) equipped with a 20BG-W2 dichroic mirror (band-passfilter, 515–560 nm; long-pass filter, 590 nm) and a D plan-Apo 20× UV objective (oil immersion). Images were analyzed with Fenestra Komet software (Kinetics Imaging, Liverpool, UK, version 3.1) on 100 randomly selected cells from duplicate slides. DNA damage was quantified as the increase of the Olive Tail Moment (OTM). Normalized distribution frequencies of OTM were calculated using 40 OTM classes between minimal and maximal values for each set of data. Non-linear regression analyses were performed with a χ^2-function model. The calculated degree of freedom (n) of the function has been previously shown to be a quantitative parameter of the level of DNA-damage in samples and was named OTM χ^2. The value of OTM χ^2, expressed in arbitrary units, was used as the only parameter for comparison purposes. The minimal DNA-damaging concentration was defined as the lowest acridine concentration showing a statistically significant DNA-damaging activity. The minimal DNA-damaging concentration was calculated from regression models, assuming that an OTM χ^2 of 2.4 ± 0.11 was statistically significant from the control value (2.11 ± 0.09).

2.6.3. Micronucleus assay

A total of 50,000 human keratinocytes were plated in chamber slides and incubated during 24 h at 37 °C in humidified atmosphere containing 5% CO_2. Solutions of acridine derivatives were thawed before use and added to the cell cultures at concentrations ranging from 0.01 to 25 μM. After a 24-h incubation period, cells were rinsed and incubated in fresh medium. Cytochalasin B (5 μg/ml) was added into each chamber slide to arrest cytokinesis and the cells were incubated for an additional 24-h period. At 72 h, cells were submitted to two successive washes with PBS and fixed with methanol/acetic acid (3/1, v/v). Staining of air-dried slides was performed with 5% Giemsa stain in Milli-Q water for 15 min. The binuclearity index (BN), considered as a measure of cytotoxicity, was determined by scoring the number of binucleated keratinocytes among 1000 Giemsa-stained cells with well-preserved cytoplasm. The micronucleated cell rates were determined for acridine concentrations inducing less than 50% toxicity [29]: 2000 binucleated keratinocytes were examined and micronuclei were identified according to the morphological criteria previously defined by Fenech [36]. To eliminate micronuclei resulting from apoptosis or necrosis, cells containing two well-defined cytoplasms and nuclei were scored only [29]. Statistical analyses of differences between controls and treated samples were performed by the contingency 2×2 chi-square test [37]. The minimal clastogenic concentration was defined as the lowest concentration (μM) inducing a statistically significant increase of micronucleated cell rates as compared with the control culture.

2.7. Quantitative structure-activity relationship (QSAR) and statistical analysis

Quantitative structure–activity relationship (QSAR) models were formulated using Cerius2® software (Version 4.2, Accelrys Inc., Cambridge, UK). Multiple linear regressions correlating the cytotoxic and the mutagenic activities to molecular descriptors were performed with respect to two putative tautomeric molecule series which could exist in aqueous solution, as shown in Fig. 2. A set of 32 constitutive, electronic, geometrical, topological and molecular descriptors was analyzed by the Cerius2® Descriptor + software [38,39]. Electronic and steric parameters were selected after a forward stepwise multiple-regression elimination procedure (Cerius2+). During the development of the best-fit equation correlation model, molecular descriptors were selected when F-ratios were statistically significant ($P < 0.05$). Thus, the selected parameters included Hydrogen Acceptor index (HA), Hydrogen Donor index (HD), energy gap (GAP) related to the difference between the energy of HOMO and LUMO, Hosoya index (log Z) related to the number of sets of non-adjacent bonds, Wiener's index (Wiener) related to the sum of the distances between all pairs of vertices and the $^3X_p^v$ descriptor involving ramification and steric effects. The statistical level of significance of each regression model was evaluated by the correlation coefficient r, the standard error s, the F-test value, the significance level of the model given by the leave-one-out cross-validation coefficient Q^2 and its standard deviation of predictive residual sum of squares (SPRESS).

3. Results

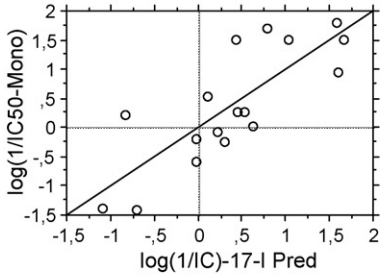
Complete data concerning the DNA-intercalating activity, the mutagenicity and the cytotoxicity of acridine compounds are reported in Table 1. Only a few acridine derivatives could competitively displace the DNA–methyl green complex. Compound **6** displayed interactions with DNA in the dark (IC_{80} = 12.5 μM), while compounds **3**, **5** and **7** interacted with DNA following irradiation (IC_{80} averaging 51.6, 69.2 and 8.4 μM, respectively).

Preliminary testing with the *Salmonella* mutagenicity test showed that the mutagenic activity of two compounds (**67** and acridine orange, AO) required the presence of metabolic activation in the dark. Both were mutagenic in tester strains TA97a, TA98, TA100 and TA102, with TA97a being the most sensitive. Following photo-activation, AO and **67** were mutagenic on all strains with and without S9mix. The presence of the

Set I Set II

(a) Regression obtained with the cytotoxic activity [log(1/IC_{50}-Mono)] after light irradiation

Set I and Set II gave the same results

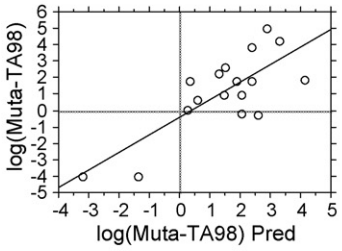


log(1/IC_{50} – Mono)=-0.004+ 1.001*log(1/IC_{50}-Mono) Pred. by Eq.1

$$\log(1/IC_{50}\text{Mono}) = -0.810*HD - 0.177*\log Z + 3.273 \quad \text{Eq. 1}$$

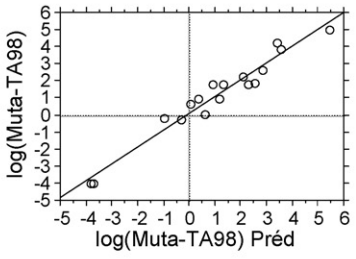
(n = 17, r = 0.80, s = 0.65, F = 12.75 Q^2 = 0.48 SPRESS = 0.78)

(b) Regression obtained with the mutagenic activity on TA98 [log(Muta-TA98] after light irradiation



log(Muta-TA98)=-0.407+1.072*log(Muta-Ta98) Pred.by Eq.2

Set I



log(Muta-TA98)=0.106+0.986*log(Muta-Ta98) Pred. by Eq. 3

Set II

Set I -> log(Muta-TA98)=0.234*HA – 0.683*HD + 0.276*GAP + 0.997* $^{3}\chi_{p}^{v}$ - 0.004*Wiener - 221.369 Eq. 2

(n = 17, r = 0.774, s =1.86, F=3,.75, $Q^2 < 0$)

Set II -> log(Muta-TA98)=0.636*HA + 1.219*HD + 2.,325*GAP + 4.656* $^{3}\chi_{p}^{v}$ - 0.009*Wiener – 221.369 Eq. 3

(n = 17, r = 0.98, s=0.63, F=46.02, Q^2 = 0.85 , SPRESS = 1.12)

Electronic descriptors	HA	Hydrogen acceptor index related to the number of hydrogen bond acceptors
	HD	Hydrogen donor index related to the number of hydrogen bond donors
	GAP	Energy gap related to the difference between the energy of the highest occupied orbital (HOMO) and those of lowest unoccupied orbital (LUMO)
Topological descriptor	Log Z	Hosoya index related to the number of sets of non adjacent bonds
	Wiener	Wiener's index related to the sum of the distances between all pairs of vertices
	$^{3}\chi_{p}^{v}$	Descriptor involving ramification and steric effects

Fig. 2. QSAR analysis with respect of molecule sets I and II.

metabolic fraction did not increase the mutagenicity and the most sensitive strain was TA98 (data not shown). Thereafter, the evaluation of the mutagenicity was conducted with these two strains. Results reported in Table 1 showed that in the dark, compound **6** had the strongest mutagenicity (mutagenic activity = 6401 rev/nmol), while after irradiation compounds **3**, **5** and **7** displayed the highest photo-enhanced mutagenicity in TA98. No significant correlation could be established between the mutagenic activities observed in TA97a and in TA98 ($P = 0.76$), suggesting two independent mechanisms of action.

Only four acridine derivatives produced a measurable cytotoxic activity against human monocytes in the dark: compounds **6** and **13** appeared the most active molecules with IC_{50}s averaging 0.71 and 0.029 μM, respectively, while compounds **11** and

Table 1
Cytotoxicity, DNA-intercalation and mutagenicity in *Salmonella typhimurium* of acridine derivatives

Compounds	DNA–methyl green IC_{90} (μM)		Mutagenic activity in *S. typhimurium* (rev/nmol)		Cytotoxicity against human transformed monocytes IC_{50} (μM)	
	Without photo-activation	With photo-activation	TA 97a + S9 mix	TA98 with photo-activation	Without photo-activation	With photo-activation
1	>100	>100	1.9	0.5	>25	0.93
2	>100	>100	4.1	59.1	>25	0.52
3	>100	51.59	4.4	16,934.8	>25	0.02
5	>100	8.39	4.1	98,712.0	>25	0.03
6	12.5	>100	6,401.6	65.0	0.71	0.29
7	>100	69.26	9.3	7,641.5	>25	3.67
8	>100	>100	1.3	1.2	>25	1.51
9	>100	>100	9.8	0.6	>25	0.52
10	>100	>100	29.6	9.1	>25	0.58
11	>100	>100	8.7	73.0	6.07	0.11
13	>100	>100	33.7	58.5	0.029	0.03
14	>100	>100	0.0	181.6	5.71	1.17
51	>100	>100	5.1	0.0	>25	0.03
67	>100	>100	2.9	4.3	>25	24.46
70	>100	>100	153.2	0.0	>25	>25
86	>100	>100	7.4	8.4	>25	1.75
Acridine Orange	>100	>100	560.9	903.6	2.21	0.016

>100: IC_{90} could not determined and was assumed to be above the highest concentration tested (100 μM). >25: IC_{50} could not determined and was assumed to be above the highest concentration tested (25 μM).

14 were far less cytotoxic (IC_{50} = 6.07 and 5.71 μM, respectively). On the contrary, almost all acridines showed strong cytotoxicity after irradiation, with compounds **3**, **5** and **51** displaying the strongest photo-inducible effects (IC_{50}s ranging from 0.02 to 0.04 μM).

Results of the QSAR analysis are shown in Fig. 2. The equation that best modeled the photo-inducible cytotoxicity of acridine derivatives did not differ according to their tautomeric form. This result established that the cytotoxicity mainly depended on the capacity of the molecules to act as hydrogen donors (HD descriptor) and on the number of sets of non-adjacent bonds ($\log Z$). In contrast, two different equations modeled the photo-inducible mutagenicity of the acridines, depending on their tautomeric forms. However, both equations concerned the capacity of the molecules to act as hydrogen donors (HD descriptor) or hydrogen acceptors (HA descriptor), the molecular stability (GAP descriptor) and the molecular topology (X and Wiener descriptors).

The effects of compounds **3**, **5**, **6** and **7** on human keratinocytes are illustrated in Figs. 3 and 4 and summarized in Table 2. Results observed with the comet assay showed that both compounds induced single-strand breaks in the dark, while only compounds **3** and **6** displayed photo-enhanced DNA-damaging activity. Results observed in the micronucleus assay indicated that irradiation with simulated sunlight produced a non-significant increase of micronuclei in human keratinocytes, since micronucleus levels in the control cultures were 16‰ in the dark and 28‰ after photo-irradiation. Compounds **3**, **5** and **6** induced a significant increase of micronucleated cells in the dark, whereas both compounds **3** and **5**, but not **6**, showed a significant photo-enhancement of their clastogenic and/or aneugenic activity. Compounds **3** and **5** displayed the strongest photo-inducible

Table 2
Minimal genotoxic concentrations of the acridine derivatives **3**, **5**, **6** and **7** in the *Salmonella* mutagenicity test, the comet assay and the micronucleus assay

Compounds	Minimal mutagenic concentration *Salmonella* mutagenicity test (μM)		Minimal DNA-damaging concentration Comet assay (μM)		Minimal clastogenic concentration micronuclei (μM)	
	TA97a + S9 mix Without photo-activation	TA98 − S9 mix With photo-activation	Without photo-activation	With photo-activation	Without photo-activation	With photo-activation
3	337.27	1.24×10^{-2}	1.06	0.44	3.84	0.03
5	404.88	2.13×10^{-3}	0.22	0.36	2.06	0.02
6	0.26	3.23	0.70	0.09	0.07	0.19
7	178.49	2.75×10^{-2}	0.02	0.81	NS	1.23
Acridine Orange	2.96	0.51	NS	0.30	0.05	0.009

Salmonella mutagenicity test: the minimal mutagenic concentrations were calculated from the regression models assuming that the doublings of the numbers of spontaneous revertants were statistically significant responses for each tester strain (166 ± 21 and 21 ± 11 for TA97a + S9 mix and TA 98-S9 mix, respectively). Comet assay: the minimal DNA-damaging concentrations were calculated from regression models assuming that an OTM χ^2 of 2.4 ± 0.11 was statistically significantly different from the control value (2.11 ± 0.09). Micronucleus assay: the minimal clastogenic concentrations were calculated from the regression models assuming that 38 ± 1‰ micronucleated cells was statistically significantly different from the control value (19 ± 1).

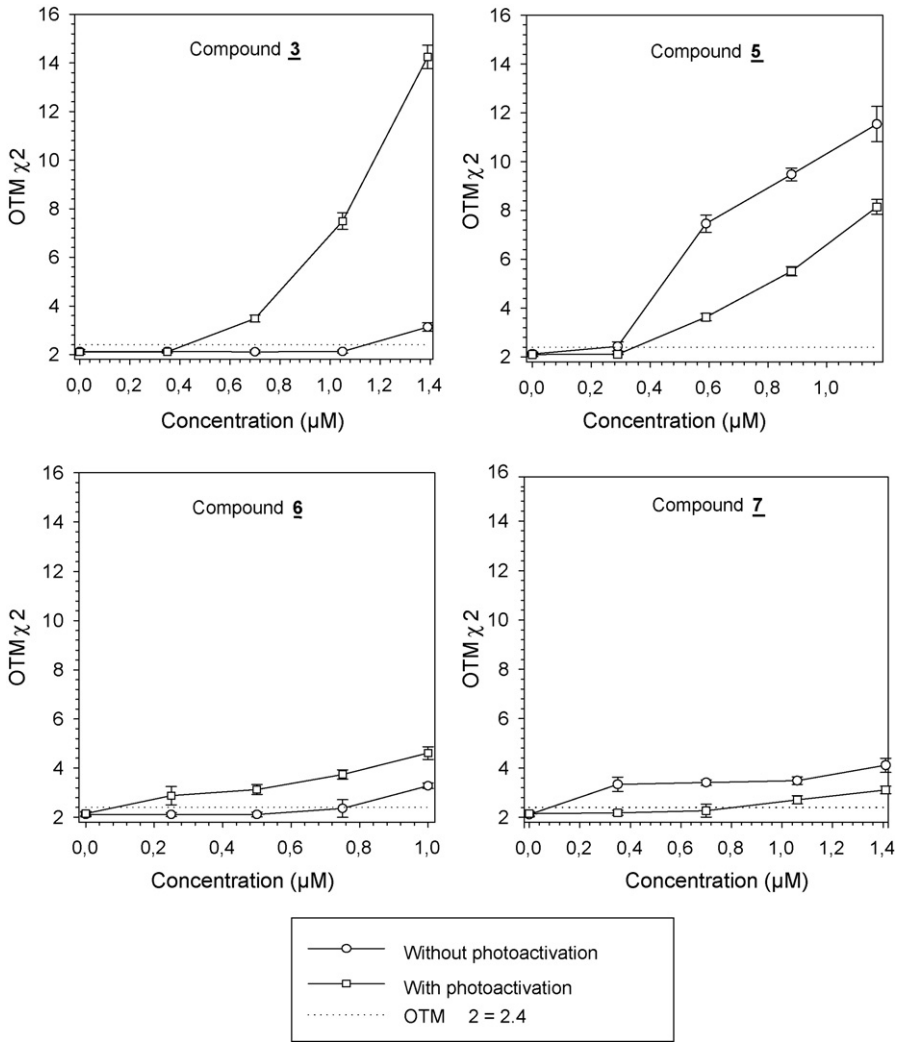


Fig. 3. Induction of single-strand breaks by the acridine derivatives **3**, **5**, **6** and **7** in keratinocytes using the comet assay without and with photo-activation, after a 2-h incubation period. The dotted line indicates an OTM $\chi^2 = 2.4$, significantly different from the control value (2.11 ± 0.09).

clastogenic and/or aneugenic activities with MCD averaging 0.02–0.03 μM, which corresponds to a 100-fold increase of their initial values.

4. Discussion

Acridines are typically considered as frameshift mutagens through a reversible intercalation of the planar aromatic rings within the macromolecules [19–21]. However, unlike simple intercalators the reactive acridine derivatives have been shown capable of forming covalent adducts with DNA [16,17].

Results observed in the present study demonstrate that thiazolo[5,4-*a*]acridines are predominantly mutagenic in the *Salmonella* tester strain TA97a after metabolic activation in the dark. This strain includes the *his*D6610 allele characterized by an insertion of one cytosine in a run of six [21]. Such a reverse mutation (−1 base frameshift), shown to be induced by various mutagens including 2-aminoacridine, ICR191 and dexon [40], indicates that thiazolo[5,4-*a*]acridines preferentially interact with DNA through a simple intercalation mechanism. This hypothesis was confirmed by data obtained with the highly mutagenic N1-(2-chloro-thiazolo[5,4-*a*]acridin-11(6*H*)-ylidene)-*N*,*N*-diethyl-1,3-propanediamine (compound **6**) which revealed a potent intercalative activity in the DNA major groove [8]. Furthermore, the presence of the diethylamino-propylamine side chain in position 11 of the acridine ring conferred to the compound a chemical structure similar to that of the well-known simple intercalator quinacrine [41], in which the aliphatic side chain tended to bind non-covalently to complementary DNA strands in the major groove [42]. In the dark, the necessity of metabolic activation for a maximal mutagenic response in the *Salmonella* mutagenicity test supposed that redox reactions could potentiate the DNA-intercalation mechanism: the metabolic fraction (S9 mix) could initiate electron-transfer events and form reactive oxygen species capable of maintaining the DNA of living cells in a continuous state of electron-transfer reactions [24]. As a consequence, charge-transfer mechanisms between DNA and small intercalative molecules could be favored and could contribute to a stronger DNA-binding activity [24].

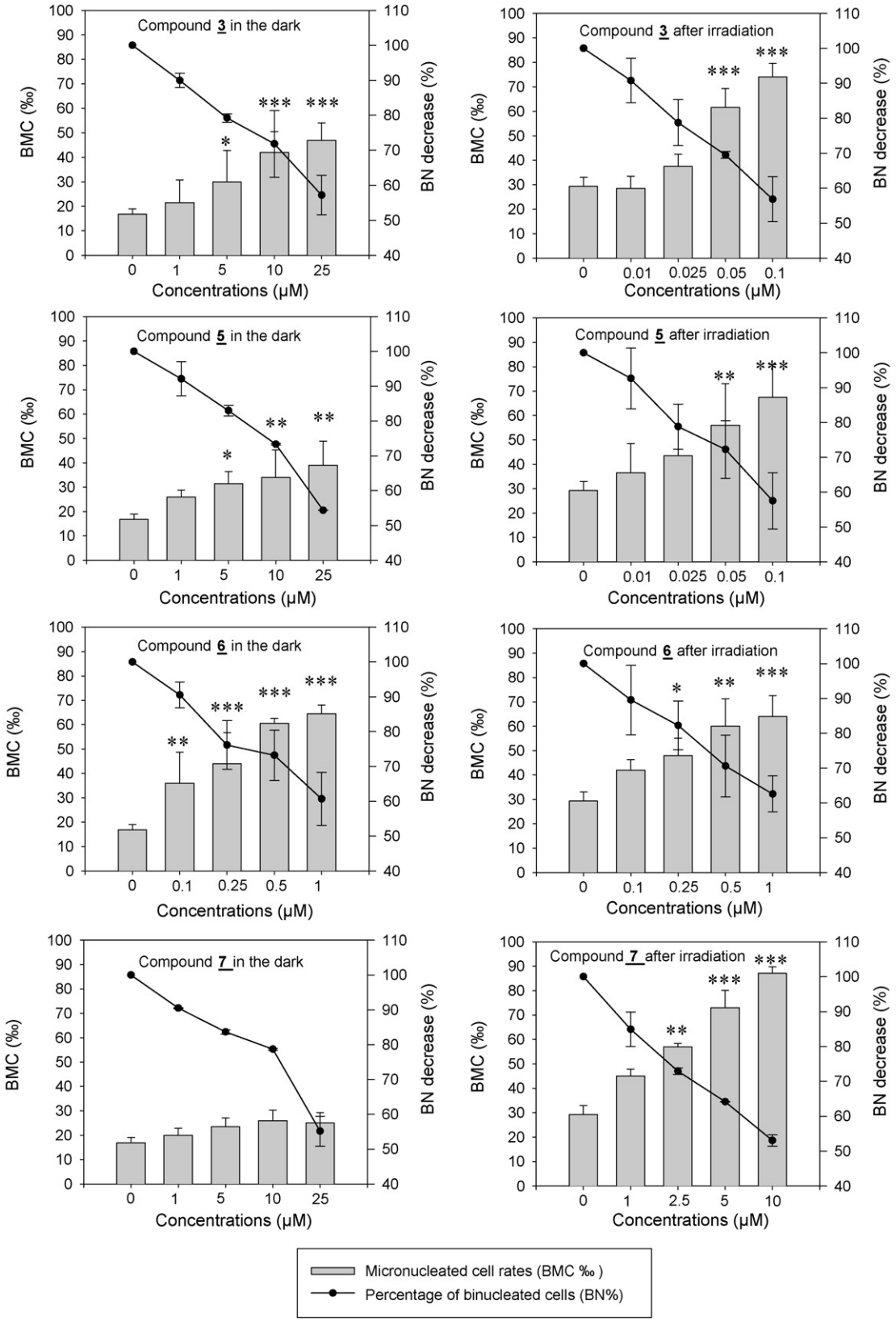


Fig. 4. Induction of micronuclei by the acridine derivatives **3**, **5**, **6** and **7** in keratinocytes without and with photo-activation.

After photo-activation, acridine derivatives were directly mutagenic in strain TA98. This strain carries the *his*D3052 allele, a −1 frameshift mutation which affects the reading frame of a repetitive $(C\text{-}G)_4$ sequence. Reversion of *hisD3052* back to the wild type is possible through a one-base insertion [21]. Such a mutation is induced by various frameshift mutagens such as nitroaromatics, aromatic amines [43] and polycyclic aromatic hydrocarbons [44], which bind covalently to DNA and generate bulky adducts. These results are consistent with previously published data, which suggested that the photolytic enhancement of acridine-induced mutagenesis in *Salmonella* appeared to be due to repairable lesions following covalent attachment to DNA [45,46]. Moreover, previous results indicated that irradiation could generate the formation of acridinium salts expected to be strong electron acceptors [47] and capable of forming hydrogen bonds. This hypothesis was supported by the QSAR analysis since the equations that best described the photo-inducible mutagenicity of acridines involved electronic descriptors such as HA (Hydrogen Acceptor index) and HB (Hydrogen Donor index). Moreover, it is likely that forms of the set II could have different behaviors than those of set I, especially when hydrogen bonding was involved. For the tautomeric forms, the electronic behavior of the substituted-groups in the thiazole-ring could be modified possibly to favor the formation of reactive species capable to generate DNA adducts. Three derivatives (**3**, **5** and **7**) revealed high photo-enhanced mutagenic activities in TA98. Compounds **5** and **7**, bearing a butylsulfanyl-group and a sulfur in position 2 of the thiazole-ring, respectively, were highly susceptible of forming DNA adducts after *S*-protonation. Compound **3**, bearing a chloro-group in position 2 of the thiazole-ring could react in the same way as acridine mustards by spontaneous loss of the chloro-group after aromatic nucleophilic substitution and formation of positively charged intermediates [15]. Unexpectedly, these three compounds also exhibited a positive result in the DNA methyl–green complex assay, indicating that a concomitant non-covalent DNA-intercalation could occur. It confirmed that an additional intercalation of acridines into DNA could be due to oxidative mechanisms and suggested that the boundary between reversible intercalation and DNA-adduct formation was not sharp for these compounds [15].

In mammalian cells, acridine derivatives have been shown to induce single-strand breaks [48], sister-chromatid exchange [49] and chromosome rearrangements [50]. Results observed in the comet assay are in line with these data. Compounds **3**, **5** and **7**, poorly active in *Salmonella typhimurium* TA97a, exhibited a significant DNA-damaging activity in the dark. On the other hand, the DNA-intercalating compound **6**, demonstrating a high mutagenicity in *Salmonella typhimurium* TA97a, did not show a potent DNA-damaging activity in human keratinocytes. This result is consistent with the genotoxic profile of quinacrine, which exhibited a decreased mutagenic activity in mammalian cells compared with that in bacteria [42]. This could be explained by the capacity of mammalian cells to transform quinacrine, and probably its analogues, into the less active mono-desethyl quinacrine intermediate [51]. As a consequence, the production of reactive metabolites by enzymatic oxidation could be hypothesized, suggesting that a covalent attachment of the thiazolo[5,4-*a*]acridines **3**, **5** and **7** to DNA could occur in the dark, according to a mode of action already described for antitumor acridines such as ledacrine [52,53]. Results observed after irradiation also confirmed the large differences between mammalian cells and bacteria. Compound **5**, shown to exert a strong photo-enhanced mutagenicity in *Salmonella typhimurium*, exhibited a decreased DNA-damaging activity, while compound **6** was deprived of mutagenicity in prokaryotic cells, but displayed strong photo-inducible DNA-damaging properties.

The consequences of DNA-damage induced by thiazolo[5,4-*a*]acridines on chromosomes have been evaluated by the scoring of micronucleated keratinocytes after a 48-h incubation period. Micronuclei can be defined as acentric chromosome fragments or whole chromosomes clearly visible in the cytoplasm of interphase cells. They originate from chromosome rearrangements or non-disjunctions induced by clastogenic or aneugenic agents, respectively [54]. Results observed in the present study confirmed that irradiation with simulated sunlight induced micronuclei in human keratinocytes and demonstrated that some thiazolo[5,4-*a*]acridines induced photo-inducible micronuclei. Compound **6**, characterized by a slight DNA-damaging activity, appeared as the most potent clastogenic and/or aneugenic derivative in the dark. In contrast, after irradiation the activity of compound **6** was lower while that of compounds **3**, **5** and **7** greatly increased. These results suggest that micronuclei could originate through various mechanisms. The first one comprises direct DNA-interaction that leads to the formation of double-strand breaks inaccurately repaired [54,55]. This mechanism could reasonably be envisaged for most of the thiazolo[5,4-*a*]acridines derivatives. In this case, the clastogenic potential of the compounds depended on both the type and the number of lesions in the DNA and the capacity of the cells to correctly repair DNA-damage. After irradiation, additional chromosome rearrangements could appear due to the excitation of endogenous photosensitizers, leading to oxidative DNA modifications [24,56] and to the formation of reactive acridine intermediates. The second mechanism assumes the possible interaction of acridines with enzymes involved in the regulation of conformational changes in DNA [57]. Among these, DNA topoisomerases I and II have been shown to play an important role in the relaxation of DNA supercoils generated by cellular processes such as transcription, recombination and replication, and the topoisomerases appeared essential for the condensation of chromosomes and their segregation during mitosis [57]. The stabilization of covalent DNA-topoisomerase complexes and the inhibition of their activity by exogenous agents have been identified as different sources of double-strand breaks in mammalian DNA [48]. Compound **6**, which demonstrated a slight DNA-damaging activity and a strong clastogenic effect, may induce chromosome rearrangements by inhibiting topoisomerase activity according to the same mechanism as proposed for its anticancer analogue quinacrine [57]. This mechanism of action, coupled with the possible interaction of acridines with various other cellular pathways, could be at the origin of the strong antiproliferative activity observed in the micronucleus assay.

As a consequence of their interaction with DNA, acridine compounds have demonstrated strong anticancer properties [1]. Structure–activity studies on diverse classes of acridines have shown that intercalation into DNA leading to the formation of ternary acridine/DNA/topoisomerase enzyme complexes, also named cleavable complexes, was a requirement for a maximal anticancer property [57]. Unfortunately, this pathway generated genotoxic events and increased the risk for secondary malignancies following anticancer chemotherapy. Results observed in the present study revealed that only compounds **6** and **13** displayed a significant cytotoxic activity in human monocytes in the dark. Concerning compound **6**, results obtained with *Salmonella typhimurium* and human keratinocytes suggested that genotoxic and cytotoxic activities were generated by the same mechanism as proposed for the anticancer drug quinacrine, namely intercalation between DNA strands and formation of topoisomerase II cleavable complexes [57]. However, results observed with compound **13** clearly indicate that the cytotoxicity of acridines could involve mechanisms other than DNA-interactions. After irradiation by simulated sunlight, almost all the thiazolo[5,4-*a*]acridines displayed strong cytotoxic activity. Since it has been demonstrated that the formation of DNA adducts could more efficiently inhibit DNA replication and transcription than intercalation [45,46], one could suppose that the covalent attachment of reactive acridine intermediates to DNA was at the origin of cell apoptosis. Nevertheless, the absence of a correlation between cytotoxic and mutagenic activities implied that other targets could be involved in the interaction between acridines and mammalian cells, depending on their lipophilicity, their degree of ionization and their capacity to migrate to different organelles. This was illustrated by the strong antiproliferative activity observed in CHO cells during the assessment of binucleated cell counts and by the presence of high levels of apoptotic cells.

Overall, the results of this study confirmed that acridines were predominantly genotoxic by a DNA-intercalating mechanism in the dark while DNA-adducts were probably induced following photo-activation. These photo-inducible properties demonstrated the necessity to consider the mechanisms of photoactivation when the pharmacological activities of acridines are evaluated.

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