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Cytotoxicity assessment of three therapeutic agents, cyclosporin-A, cisplatin and doxorubicin, with the ciliated protozoan *Tetrahymena pyriformis*

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Abstract

Cyclosporin-A, a drug possessing potent immunosuppressive properties, is used to prevent allograft rejection. Cisplatin and doxorubicin are two of the pharmaceutical drugs most widely used in cancer chemotherapy. In this study, the cytotoxicological impact of these three therapeutic agents was determined using bioassays performed with a unicellular eukaryote, the ciliated protozoan *Tetrahymena pyriformis*. For this purpose we used the population growth impairment test and the non-specific esterase activities test. We also examined some morphological effects. The results show that these three agents are toxic towards *T. pyriformis*. A concentration-dependent inhibitory effect on the cell proliferation rate of *T. pyriformis* populations was found for the three drugs. The IC_{50} values were, respectively, 42.03 ± 4.64, 124.37 ± 7.47 and 74.62 ± 6.12 µM for cyclosporin-A, cisplatin and doxorubicin. Non-specific esterase activities were also modified compared with untreated cells. The IC_{50} values were, respectively, 88.32 ± 8.35 and 44.61 ± 3.33 µM for cisplatin and doxorubicin. Exposure of *T. pyriformis* to these drugs caused the prompt appearance of digestive vacuoles concentrating particulate elements. This phenomenon was more pronounced at higher concentrations. We also observed deformed cells with cisplatin. *T. pyriformis* bioassays can offer an alternative in vitro method to cell cultures for the risk assessment of potentially toxic drugs.

Keywords: *Tetrahymena pyriformis*; Cytotoxicity; Cyclosporin-A; Cisplatin; Doxorubicin; In vitro alternative test

1. Introduction

Cyclosporin-A, cisplatin and doxorubicin are three pharmaceutical agents with known toxicological issues in humans.

Cyclosporin-A is a cyclic peptide produced by soil fungi, first isolated from *Tolypocladium inflatum* Gams. It possesses a highly selective immunosuppressive activity, and is used in clinical practice in transplantations. Cyclosporin-A associates with a cytosolic receptor, cyclophilin, which belongs to the immunophilin family. The target of this complex is a Ca^{2+} calmodulin-dependent serine-threonine-specific protein phosphatase, calcineurin [20]. This drug inhibits signal transduction events required for T-cell activation following antigen presentation [8,38,40]. Suppression of allograft rejection in humans can usually be achieved by maintaining cyclosporin-A levels at 100–300 µg/l as measured by HPLC in whole blood [27].

Cisplatin is an anticancer agent similar to heavy metals, which is extensively used in cancer chemotherapy either alone or in combination with other drugs. It is a non-organic complex based on platinum. Its antitumor activity was first revealed by Rosenberg et al. [31]. Only the *cis*-form has significant clinical activity. Cisplatin acts mainly by the formation of adducts with both nuclear and mitochondrial DNA. It binds, after loss of two chloride ions, to two adjacent guanosines in DNA, causing a local conformational change in the molecule and the inhibition of DNA synthesis [28,41].

Doxorubicin was isolated at the HCl salt and not the free base from *Streptomyces peucetius* subsp. *caesius* Arcamone et al. It is an antineoplastic cytostatic antibiotic, belonging to

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the anthracycline group. Besides the intercalation of doxorubicin with DNA, the overall effects are complex, with chromatin condensation and inhibition of DNA and RNA synthesis. Doxorubicin also acts directly on the cell membrane. Maximum toxicity occurs during the S phase of the cell cycle, and chromosome aberrations are also observed. This drug is widely used in medical oncology (acute and chronic leukemia, Hodgkin's disease, lung cancer, breast carcinoma, ovary cancer, soft tissue sarcomas and osteogenic sarcoma, and pediatric tumors) [10].

To our knowledge, no in vitro investigation has been performed on the same model with these three pharmaceutical drugs, enabling assessment of their relative cellular toxicity.

The in vitro cellular model used in our work, *Tetrahymena pyriformis*, is a ubiquitous freshwater ciliated protozoan which has a number of characteristics offering advantages for toxicity studies. The physiology and biochemistry of this eukaryotic cell are well-known [11]. It can be easily cultured axenically in a small volume of a complex culture medium. This ciliate is characterized by a short generation time, approximately 3 h. Thus, the effects of test substances can be studied over a brief period through several generations.

Numerous studies have shown this ciliate to be a convenient model for the toxicological evaluation of various substances such as carcinogens, insecticides, fungicides, mycotoxins, organic chemicals, heavy metals and pharmaceutical drugs [3–5,16,25,36,39,48,49]. In two review articles, Nilsson [25] and Sauvant et al. [36] confirm that *T. pyriformis* is a choice test system for the assessment of xenobiotics and drug toxicity.

Several test methods for toxicity studies using this cell have been proposed [1,6,15,18,26,30,33]. Various criteria for cytotoxicity assessment (e.g., death, immobility, reproduction, growth, physiological functions, genotoxicity, use of biomarkers) can be employed.

In this study, the toxicological effects of three therapeutic targeting agents, cyclosporin-A, cisplatin and doxorubicin were evaluated using the *T. pyriformis* population growth impairment test (population test) and the *T. pyriformis* nonspecific esterase activities test (FDA esterase test). We also studied some morphological effects (phagocytosis and cell deformation) by photon microscopy.

The aims of the study were firstly to assess the relative toxicity of these three agents for the first time on the same in vitro cellular model (ciliated protozoan *T. pyriformis*), and secondly to show that *T. pyriformis* bioassays can offer an alternative in vitro method for the risk assessment of potentially toxic drugs.

This study is part of joint work carried out by several Cell Biology Departments in French Schools of Pharmacy, to assess the potential toxicity of some xenobiotics and other substances with therapeutic aim on various cellular models.

2. Materials and methods

2.1. Chemicals

Cyclosporin-A (Aldrich, Ref. 30024, purity >98.5%) was dissolved in DMSO (Sigma). Cisplatin (*cis*-platinum(II) diamine dichloride (Sigma, Ref. P4394)), a yellow freeze-dried powder, was dissolved in DMSO. Doxorubicin (Teva Pharma) was an orange-red injectable solution, with each flask containing 10 mg of doxorubicin hydrochloride in 5 ml (2 mg/ml). Excipients were sodium chloride, hydrochloric acid and water for injectable preparations.

For bioassays, final tested concentrations were 70, 60, 50, 40, 30, 20, 10 and 5 mg/l. The amount of solvent (DMSO) added was 0.5% (v/v).

2.2. Cell culture

The ciliated protozoan *T. pyriformis*, amicronucleated strain GL, was grown axenically at 28 °C in proteose-peptone yeast salts (PPYS) complex medium (pH 6.8–7.0) containing 0.75% proteose peptone (Difco, Detroit, MI, USA), 0.75% yeast extracts (Difco), and inorganic salts [29].

The cells were maintained in exponential growth phase by reseeding in PPYS liquid medium. During the growth, the cell density increases logarithmically up to around 130 000 cells/ml, and then a pre-stationary growth phase followed by a true stationary phase are reached [23].

2.3. Population growth rate test

Test cultures were prepared by inoculating *T. pyriformis* from a stock culture into 100 ml of PPYS in 500 ml conical flasks. Fifteen hours later (time $0 = T_0$), the chemicals were added to the culture during the exponential growth phase (10^4 cells/ml at time T_0). For cyclosporin-A and cisplatin, the tests were performed with the different dilutions from stock solutions (70 mg/l). For doxorubicin, different volumes from the injectable solution (2 mg/ml) were added in accordance with final desired concentrations.

Results were obtained with different concentrations of the agent and two controls (*T. pyriformis* in culture with and without DMSO 0.5%). The flasks were incubated at 28°C without shaking in darkness. One-milliliter aliquots were withdrawn from the cultures just before treatment with the substance (T_0) and then every hour for 9 h, corresponding approximately to three generations for control culture. The samples were fixed with 1 ml of 4% formaldehyde and diluted with 8 ml of Isoton II®. The cell density was determined electronically with a model ZM Coulter Counter® (Coultronics, Toronto, Canada).

The growth dynamics of the populations was evaluated by plotting time versus D_x/D_0 ratios, where D_x and D_0 are cell concentrations at time $T_0 + x$ hours and time T_0, respectively. The populations were characterized by their

generation time (GT), i.e., the time required for the D_x/D_0 ratio to increase from 1 to 2.

In the presence of toxic compounds, a decrease in cell growth was noted, inducing an increase in generation time. The generation times of the control population (GTC) and of the substance-treated populations (GTT) were determined. For each concentration, the relative generation time (RGT), expressed as percentage, was calculated using the ratio: $RGT = [(GTT - GTC)/GTC] \times 100$.

The relative toxicity of the tested substances was quantified by determining the median inhibitory concentration (IC_{50}), which is the concentration required to induce a 50% increase in RGT, i.e., 50% decrease in cell growth compared with the untreated cells. IC_{50} values were calculated by linear regression analysis, and the data were fitted to the following model: $RGT(\%) = a(\text{concentration}) + b$.

For each tested agent, we carried out three independent experiments to determine exact IC_{50} values using inhibition of population growth as a criterion.

2.4. Non-specific esterase activities

Esterases are ubiquitous enzymes present in the cells of all living organisms. Non-specific esterase activities were measured by the method described by Bogaerts et al. [6], which we modified. The experiment consists in the hydrolysis of fluorescein diacetate (FDA) and the spectrofluorimetric quantification of the free fluorescein. The non-polar FDA enters the cell where it is hydrolyzed by esterases to yield fluorescein, which is retained by the cell [32]. The degree of fluorescence depends on the physical and metabolic state of the cell and has been demonstrated to be a reliable indicator of the potential toxicity of a chemical [2].

A *T. pyriformis* culture in exponential growth phase was centrifuged for 10 min at 300 g, and the supernatant was discarded. The *T. pyriformis* pellet was suspended in Volvic mineral water to obtain a concentration of 4×10^3 cells/ml. One milliliter of this dilution was incubated with agents for 1 h (at 28°C, in darkness). After incubation, 1 ml of FDA (Sigma) stock solution (5 mg/ml in DMSO), diluted in Volvic water to obtain a solution at 4.8 µM, was added (final concentration of DMSO, 0.12% (v/v)). Each toxicity test included various agents concentrations and two controls, FDA in Volvic water to measure self degradation of this substrate and FDA with *T. pyriformis* (untreated cells). After 15, 30, 45 and 60 min, the amount of free fluorescein was measured by a spectrofluorimetric reader (Kontron SFM 25) using a 485-nm excitation filter and a 510-nm emission filter. Experiments were repeated three or four times.

The aim of this test was to determine the median inhibitory concentration (IC_{50}), concentration required to induce a 50% decrease in the fluorescence compared with the untreated cells. For each assay, IC_{50} value was determined by linear regression analysis. The data were fitted to the following model: decrease in fluorescence (%) = a (concentration) + b.

2.5. Morphological study

Morphological observations have been performed by photon microscopy (Leica). Living cells were withdrawn from the cultures and immediately observed at different magnitudes.

2.6. Statistical analyses

Linear regression analyses were performed with Microsoft Excel 2000 software.

A Student's t-test was used for the statistical evaluation of the data. A probability value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of the different agents on the T. pyriformis population growth rate

DMSO used to dissolve cyclosporin-A and cisplatin has no effect on the development of the *T. pyriformis* populations in the final concentration of 0.5% that we selected. Moreover, no cellular alterations were observed for concentrations <2.5% [21,22]. IC_{50} values with *T. pyriformis* were 33 000 ± 8 976 mg/l for population growth test and 24 520 ± 2 720 mg/l for esterase test [6]. The carrier solvents for doxorubicin have no effect on *Tetrahymena*.

Concerning cyclosporin-A, Fig. 1 reports the results of one experiment (experiment 3) showing the time course of D_x/D_0 ratios, the evaluation of the generation times (GT) and the IC_{50} value. Addition of cyclosporin-A to the culture medium produces a concentration-dependent decrease in population growth (Fig. 1a).

The plot of $\log(D_x/D_0)$ against time (Fig. 1b) yields the characteristic GT of the control culture and each treated population from the linear equations. The doubling of the cell densities ($D_x/D_0 = 2$) corresponds to $\log(D_x/D_0) = 0.301$. For example, from the linear equation of 9 h control

Table 1
Effects of cyclosporin-A, cisplatin and doxorubicin on population growth of *T. pyriformis*: evaluation of the average IC_{50}

	Cyclosporin-A		Cisplatin		Doxorubicin	
	mg/l	µM	mg/l	µM	mg/l	µM
Experiment 1	57.54	47.85	39.08	130.27	46.66	80.45
Experiment 2	50.21	41.75	34.16	113.87	44.80	77.24
Experiment 3	43.90	36.50	38.70	129.00	38.37	66.16
Mean	50.55^{A**}	42.03^{a}	37.31^{B2}	124.37^{b}	43.28^{2**}	74.62^{c}
SD	5.57	4.64	2.24	7.47	3.55	6.12
CV (%)	11.02	11.02	6.00	6.00	8.20	8.20

SD = standard deviation; CV = coefficient of variation; µM: different small letters for means indicate statistical differences ($P < 0.05$); mg/l: different capital letters for means indicate statistical differences ($P < 0.05$), identical number and ** indicate no statistical difference ($P < 0.05$).

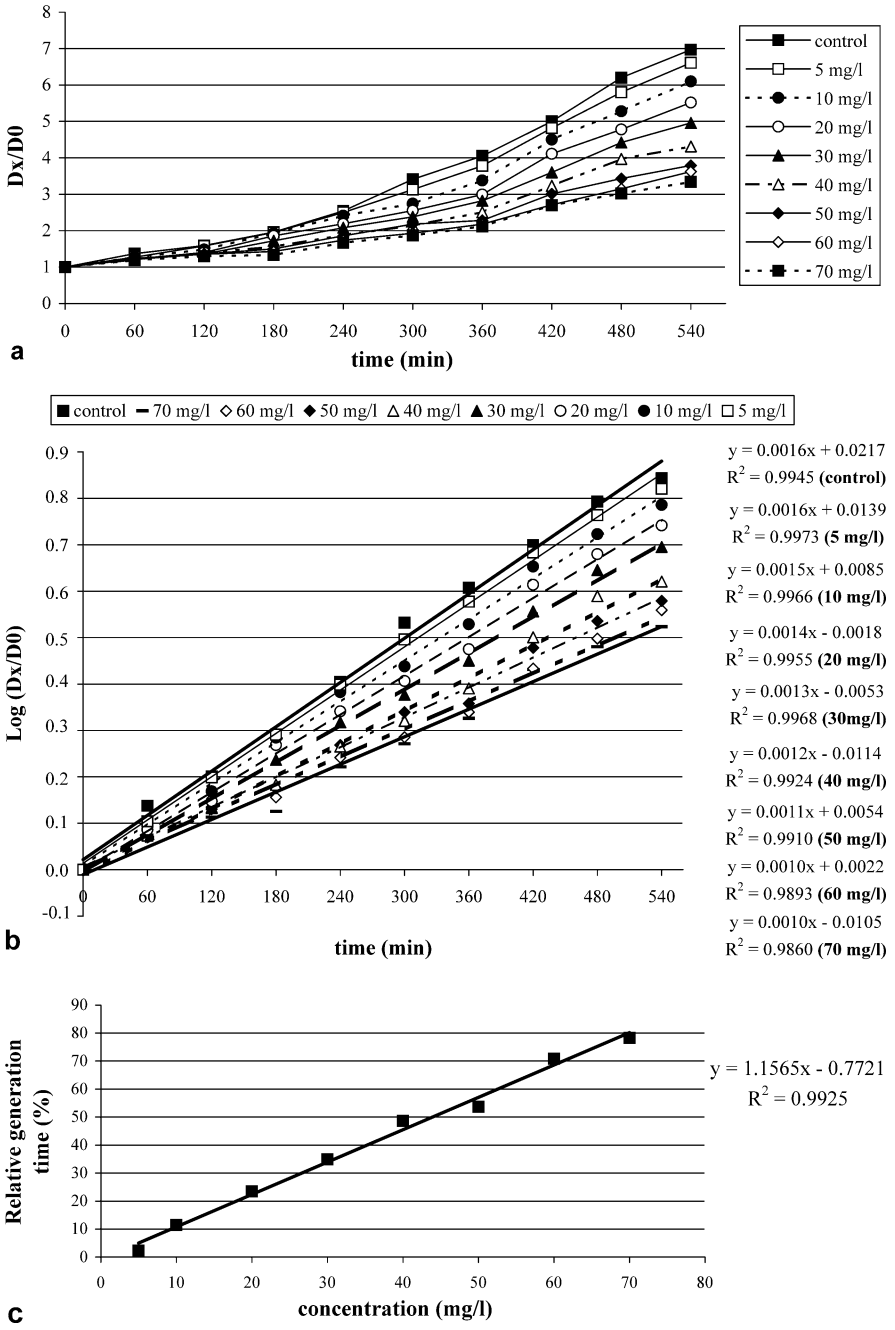

Fig. 1. Effects of cyclosporin-A on population growth of *T. pyriformis* (results of experiment 3). Time course of growth dynamics (a), evaluation of generation time (b) and IC_{50} (c). D_x and D_0 are cell concentrations at times $T_0 + x$ and T_0, respectively.

culture ($y = 0.0016x + 0.0217$), we deduce that the average GT is 175 minutes for the experiment length (9 h). We proceed in the same way for all the experimental series, with calculation of all relative generation times (RGT). The plot of RGT (%) versus concentrations is presented in Fig. 1c. The IC_{50} value can be determined mathematically from the linear regression equation, here 43.90 mg/l over a period corresponding to approximately three generations of the control population (i.e., 9 h).

The results of the three independent experiments (Table 1) show that cyclosporin-A exerts a concentration-dependent inhibitory effect on the growth of *T. pyriformis* populations with an average IC_{50} of 50.55 ± 5.57 mg/l equivalent to 42.03 ± 4.64 µM over a period corresponding to approximately three generations of the control population.

The same type of representation and analysis is applied to cisplatin. Results of one experiment (experiment 3) are reported in Fig. 2. Addition of cisplatin to the culture medium causes a decrease in the population growth (Fig. 2a). For all the concentrations higher than 20 mg/l, we observe an inhibition of the population growth during the first three hours. This inhibition is total throughout the experiment

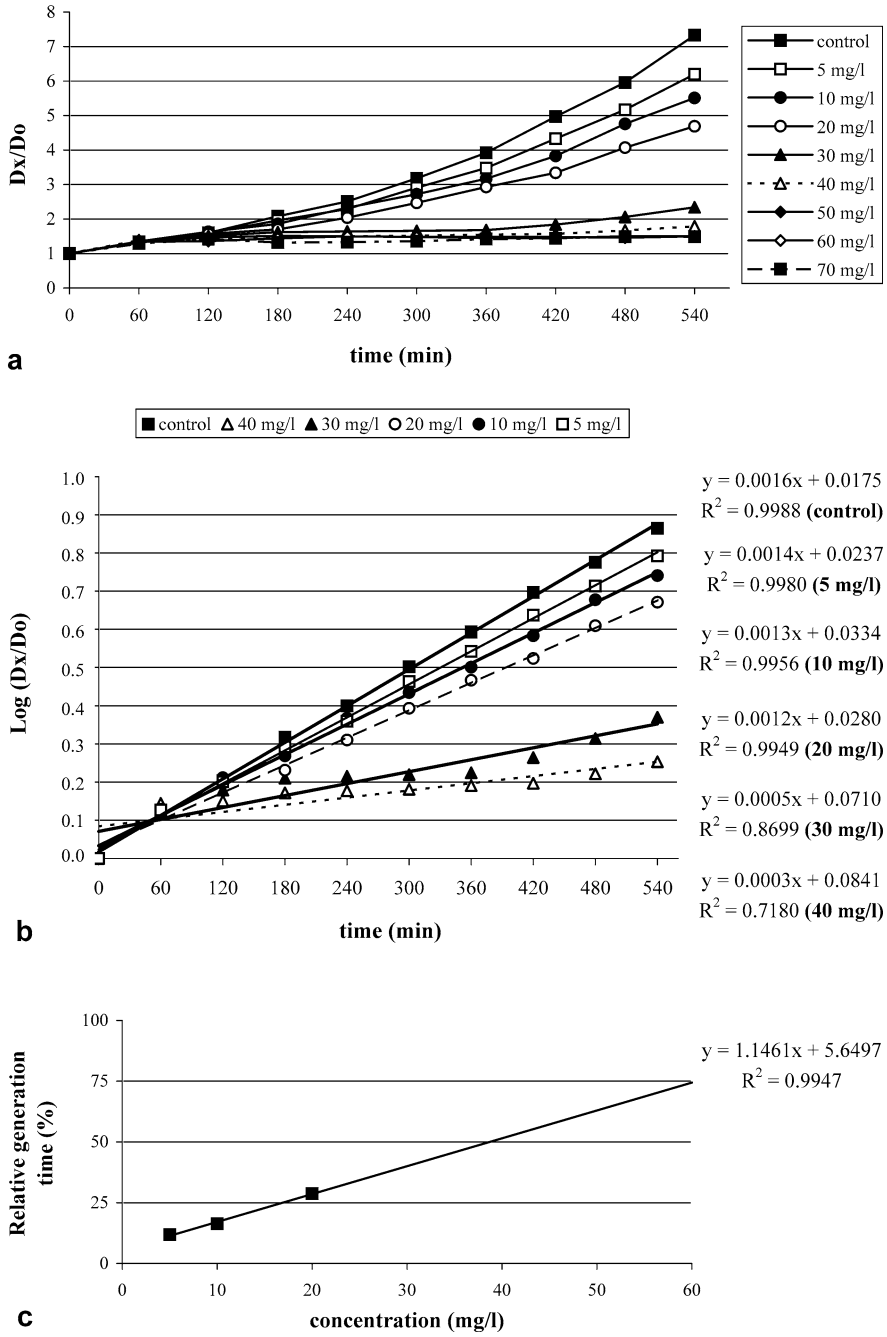

Fig. 2. Effects of cisplatin on population growth of *T. pyriformis* (results of experiment 3). Time course of growth dynamics (a), evaluation of generation time (b) and IC_{50} (c). D_x and D_0 are cell concentrations at times $T_0 + x$ and T_0, respectively.

(9 h) for the concentrations equal to or higher than 50 mg/l. The growth resumes at about $T_{8\,\mathrm{h}}$ for the culture treated with 40 mg/l and at about $T_{6\,\mathrm{h}}$ for the culture treated with 30 mg/l.

In this experiment, and by applying the same method as for cyclosporin-A, we determined the concentration of cisplatin that causes a 50% decrease in the *T. pyriformis* population growth. It was estimated that R^2 values from linear equations related to 30 and 40 mg/l were not in agreement with an optimal correlation (Fig. 2b). Thus, only concentrations equal to or lower than 20 mg/l were considered for the evaluation of the IC_{50} value: 38.70 mg/l in this experiment (Fig. 2c).

For each culture, a count was performed at time $T_{24\,\mathrm{h}}$ (Table 2) to evaluate whether all the populations had been either continuing (5–40 mg/l) or starting growth (50–70 mg/l). Control culture and populations treated with 5 to 40 mg/l of cisplatin continued their growth and reached practically the pre-stationary or true stationary phase. The populations treated with 50 to 70 mg/l of cisplatin which did not double after 9 h ($D_{9\,\mathrm{h}}/D_0 < 2$) had started their growth and we observed 3 to 4 generations after 24 h.

Table 2
Effects of cisplatin on population growth of *T. pyriformis*: number of cells/ml at times $T_{0\,h}$, $T_{9\,h}$ and $T_{24\,h}$; mean of 3 independent experiments

	$T_{0\,h}$	$T_{9\,h}$	$T_{24\,h}$
Control	6330 ± 110	45 510 ± 1663	283 138 ± 3063
Cisplatin 70 mg/l	5479 ± 569	8050 ± 970	46 413 ± 838
Cisplatin 60 mg/l	6288 ± 453	9305 ± 795	64 362 ± 4138
Cisplatin 50 mg/l	6028 ± 243	9047 ± 393	82 438 ± 8063
Cisplatin 40 mg/l	5395 ± 150	10 050 ± 110	95 763 ± 11 888
Cisplatin 30 mg/l	5604 ± 209	13 900 ± 1250	121 025 ± 11 875
Cisplatin 20 mg/l	5468 ± 643	22 475 ± 2385	218 050 ± 16 250
Cisplatin 10 mg/l	5873 ± 108	32 524 ± 397	248 575 ± 8800
Cisplatin 5 mg/l	5939 ± 212	37 247 ± 1194	272 275 ± 6475

The results of the three independent experiments (Table 1) indicated that the average IC_{50} for cisplatin was 37.31 ± 2.24 mg/l, equivalent to 124.37 ± 7.47 µM over a period corresponding to approximately three generations of the control population.

The addition of doxorubicin to the culture medium also affects the dynamics of the protozoan population development in a concentration-dependent way. Fig. 3 reports the results of one experiment (experiment 2) showing the time course of D_x/D_0 ratios (Fig. 3a), the evaluation of the generation times (Fig. 3b) and the IC_{50} value (Fig. 3c). In this experiment, the concentration of doxorubicin that causes a 50% decrease in cell growth is 44.80 mg/l. The average IC_{50} determined from three independent experiments (Table 1) was 43.28±3.55mg/l equivalent to 74.62±6.12 µM over a period corresponding to approximately three generations of the control population.

The coefficients of variation (CV) are reported in Table 1. The values lie between 6.00 and 11.02 % for the three tested substances. This indicates a good reproducibility for this test.

From a physiological point of view, it seems better to express the results of the relative toxicity of the three therapeutic targeting agents in µM rather than in mg/l.

Statistical analyses (Student's *t*-test) show that average IC_{50} values in µM were significantly different between cyclosporin-A and cisplatin, between cisplatin and doxorubicin and between cyclosporin-A and doxorubicin ($P < 0.05$).

In conclusion, the *T. pyriformis* population growth impairment test over a period of 9 h allows us to estimate the relative toxicity of the agents, with the rank order: cyclosporin-A (42.03 ±4.64 µM) > doxorubicin (74.62 ± 6.12 µM) > cisplatin (124.37 ± 7.47 µM).

3.2. Effects of the different agents on non-specific esterase activities of T. pyriformis

Optimal results were obtained when measurements of the free released fluorescein were considered at time 45 min after the addition of FDA. Indeed at times 15 and 30 min, released fluorescein (in fluorescence units) is sometimes insufficient, and after 60 min, measured fluorescein for control cells sometimes overflowed and IC_{50} values could not be calculated.

Table 3
Effects of cisplatin and doxorubicin on non-specific esterase activities of *T. pyriformis*: evaluation of the average IC_{50} at $T_{1\,h}$ + 45 min FDA

	Cisplatin		Doxorubicin	
	mg/l	µM	mg/l	µM
Experiment 1	30.49	101.63	25.70	44.31
Experiment 2	23.59	78.63	28.33	48.84
Experiment 3	26.16	87.20	23.60	40.69
Experiment 4	25.74	85.80	–	–
Mean	26.50^{A}	88.32^{b}	25.88^{A}	44.61^{a}
SD	2.50	8.35	1.94	3.33
CV (%)	9.43	9.45	7.50	7.46

SD = standard deviation; CV = coefficient of variation; µM: different small letters for means indicate statistical differences ($P < 0.05$); mg/l: identical capital letters for means indicate no statistical difference ($P < 0.05$).

With cyclosporin-A, the results did not allow us to accurately determine a representative IC_{50} because of self fluorescence of the compound in the wavelengths used.

For each of the two other agents, we present (Fig. 4) the results of one experimental series by plotting inhibition of fluorescence (%) against the concentration. For cisplatin (Fig. 4a) and doxorubicin (Fig. 4b), linear equations enable the determination of the IC_{50} values. We find 25.74 mg/l and 23.60 mg/l, respectively, for cisplatin (experiment 4) and doxorubicin (experiment 3).

All the results of this test are reported in Table 3. The average IC_{50} was 26.50 ±2.50 mg/l equivalent to 88.32 ± 8.35 µM for cisplatin and 25.88 ± 1.94 mg/l equivalent to 44.61 ± 3.33 µM for doxorubicin. The values of the coefficients of variation (CV) lie between 7.46 and 9.45%. This indicates a good reproducibility of the results for the esterase test.

Statistical analyses (Student's *t*-test) show that average IC_{50} values in µM were significantly different between doxorubicin and cisplatin (<0.05).

For this test, the rank order of the agents toxicity was the following: doxorubicin (44.61 ± 3.33 µM) > cisplatin (88.32 ± 8.35 µM). We cannot position cyclosporin-A.

3.3. Ingestion of the agents by T. pyriformis

As *T. pyriformis* is a unicellular phagotrophic organism, it allows us to visualize a possible ingestion of the substances, as it can occur in vivo for cells of the macrophage type.

Although no visible precipitate was observed in culture medium for the different solutions of the tested substances, even at the highest concentrations used, in every case the formation of digestive vacuoles concentrating particulate elements (non-soluble fraction) could be observed (Fig. 5). This phenomenon was prompt (from the first hour of contact) and was related to the concentrations of the agents. We also observed deformed cells in the presence of cisplatin (Fig. 5d–f).

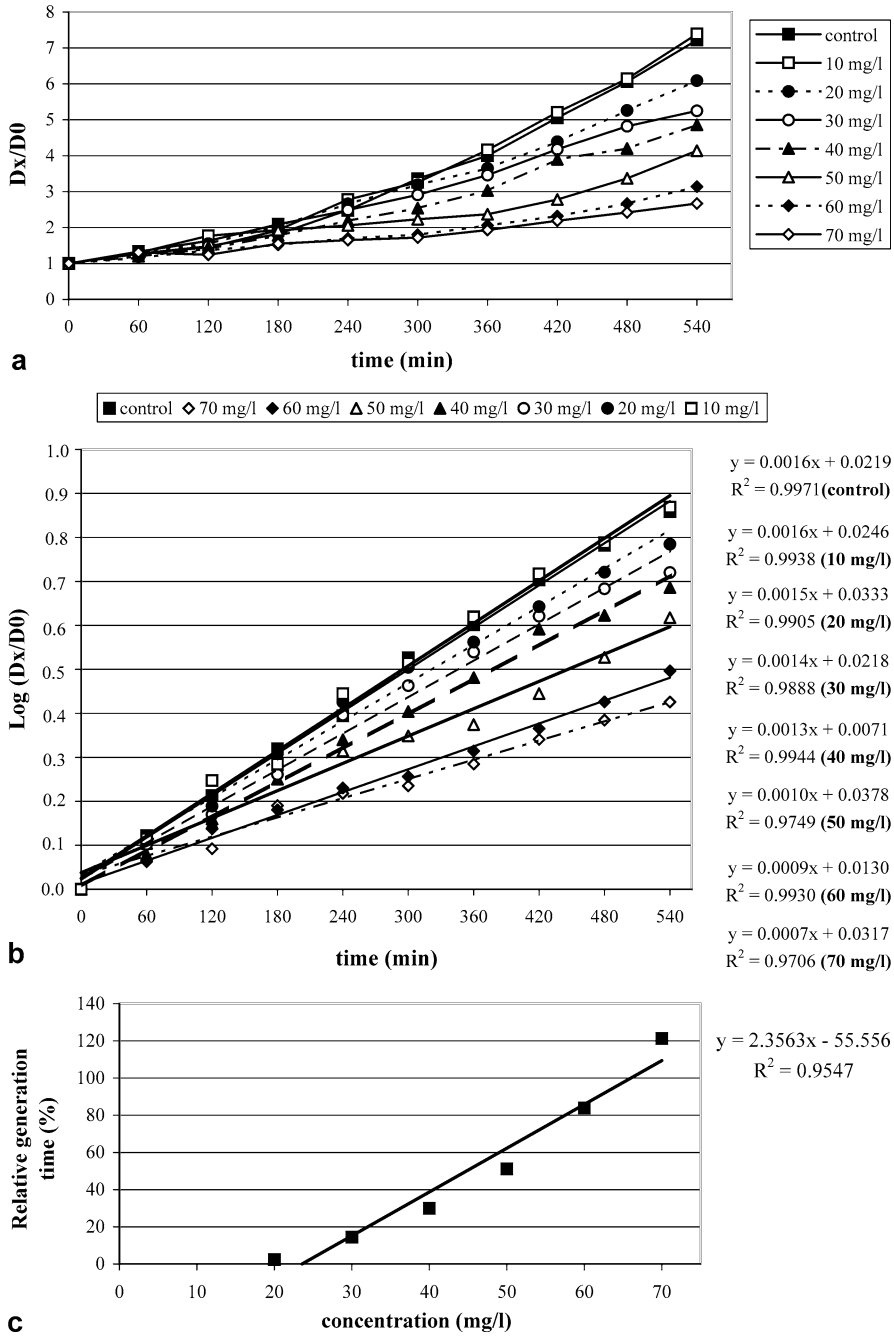

Fig. 3. Effects of doxorubicin on population growth of *T. pyriformis* (results of experiment 2). Time course of growth dynamics (a), evaluation of generation time (b) and IC_{50} (c). D_x and D_0 are cell concentrations at times $T_0 + x$ and T_0, respectively.

4. Discussion

To our knowledge, no in vitro investigation of cellular toxicity comparable to that presented here has been performed with these three pharmaceutical agents. Comparisons of our results with those of other studies are consequently very limited. However, there are some in vivo published toxicological data for these three substances in the literature, in The Merck Index [7] and in the technical literature of the suppliers (Caelyx® from Schering Plough for doxorubicin (1997), Neoral® and Sandimmun® from Novartis Pharma for cyclosporin-A (1996 and 1993), Cisplatyl® from Roger Bellon RPR for cisplatin (1989)). General information concerning these drugs could be obtained from the Hazardous Substances Data Bank of Toxnet (online). Some toxicological data are presented in Table 4.

Globally, no relevant rank order potency and toxicity can be determined from these in vivo published results.

In vivo toxicity based on tissue or plasma concentrations and in vitro mammalian cell cytotoxicity data would

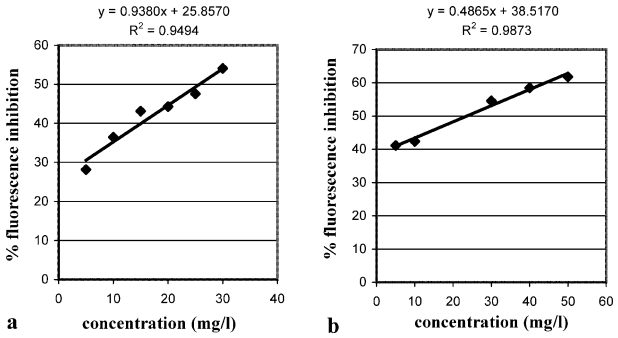

Fig. 4. Effects of cisplatin (a) (results of experiment 4) and doxorubicin (b) (results of experiment 3) on non-specific esterase activities of *T. pyriformis*: evaluation of IC_{50} at time $T_{1\,h} + 45$ min FDA.

have allowed a more pertinent discussion to compare our results.

We demonstrate a toxicity for these three drugs in an alternative cellular model to cell cultures, the ciliated protozoan *T. pyriformis*.

The advantages that make *T. pyriformis* a model of choice for rapid in vitro bioassay are summarized in part in the Introduction; one of the most important being that the effects of the tested substances can be studied over a short period through several generations. *T. pyriformis* is a true eukaryotic organism and therefore allowed the metabolism of organic substances to be studied.

Its performance and sensitivity has been shown to be very similar to those of other in vitro or in vivo mod-

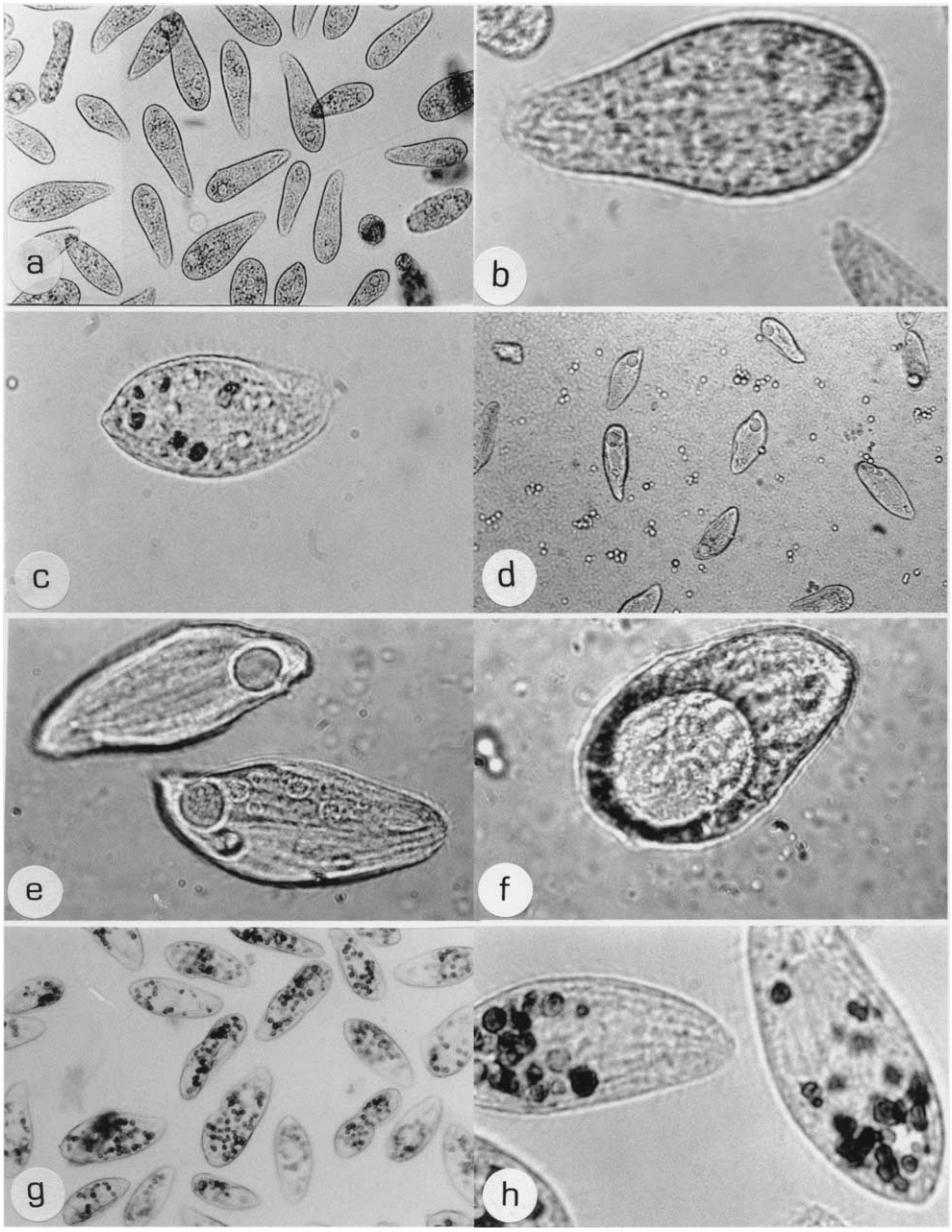

Fig. 5. *T. pyriformis*. Control culture: ×250 (a) and ×1000 (b); culture with cyclosporin-A (70 mg/l): ×1000 (c); culture with cisplatin (70 mg/l): ×250 (d) and ×1000 (e and f); and culture with doxorubicin (70 mg/l): ×250 (g) and ×1000 (h).

Table 4
In vivo published toxicological data for cyclosporin-A, cisplatin and doxorubicin

Therapeutic agents	Usual dose [10]	Single administration (LD_{50} mg/kg)		Repeated administration	Principal target organs and adverse reactions
Cyclosporin-A	10–15 mg/kg oral microemulsion 4–12 h prior to transplantation	Mouse:	107 iv [7] 2329 orally [7]	Kidney and liver toxicity (Neoral®) rat: 45 mg/kg/d monkey: 300 mg/kg/d dog: 45 mg/kg/d Embryotoxicity (Neoral®)* rat: 30–300 mg/kg/d rabbit: 100–300 mg/kg/d	Nephrotoxicity [19]* Hepatotoxicity [10]* Hypertension [10]* Convulsion [10] Neoplasia [10] Diabetogenic effect [14]*
		Rat:	25 iv [7] 1840 orally [7] 147 ip*		
		Rabbit:	>10 iv [7] >1000 orally [7]		
Cisplatin	50–100 mg m^{-2} iv injection every 3 or 4 weeks	Guinea pig:	9.7 ip [12]	Dog: 1.5–3.0 mg/kg/d iv [37] dead in 5–8 days 0.38 mg/kg/d reversible toxicity Monkey: 2.5 mg/kg/d [37] dead 5th day 0.16 mg/kg/d no toxicity	Disturbances in renal function [10]* Hypersensitivity [10] Vascular toxicity [10] Ototoxicity [12,43]* Neuropathy [10]* Disturbances in serum electrolytes [10] Hematological toxicity [45,50]*
		Rat:	7.7–12.0 ip [17] 20 orally*		
		Mouse:	17.0–18.0 ip [42] 12.0–13.0 iv		
Doxorubicin	60–75 mg m^{-2} iv every week as a single agent, 30–50 mg m^{-2} in combination with other drugs	Mouse:	35.0 ip [7] 21.1 iv [10]	Rat: 1 mg/kg/d, embryotoxicity [44] Rabbit: 0.5 mg/kg/d, embryotoxicity [44] Dog: 0.75–1.00 mg/kg/d every 3 weeks, cutaneous lesion and myelotoxicity [47] Dog: 0.25 mg/kg/d continuous infusion, thrombocytosis and leukocytosis [13]	Genotoxicity [46]* Bone marrow suppression [10] Cardiotoxicity (Caelyx®)* Nephrotoxicity (Caelyx®)* Myelotoxicity (Caelyx®)*
		Rat:	12.6 iv*		
		Rabbit:	6 iv*		

*Hazardous Substances Data Bank of Toxnet (online).

els (L-929 fibroblasts, lymphocytes, fishes, *Daphnia*, etc.) [16,25,34–36]. The large number of studies reported in the two review articles [25,36] confirm that *T. pyriformis* may be considered as a complement or alternative to animal and mammalian cell models in toxicant screening studies of xenobiotics or pharmaceutical substances, and in understanding cell toxicity mechanisms.

For the three drugs, our results show effects on the population growth rate and on non-specific esterase activities.

The population growth rate test enables the dynamics of the *T. pyriformis* population to be evaluated and the growth curve to be plotted. The toxicity of the molecules can be determined after one, two and/or several generations, and this process is more original than the end-point method generally used. With the end-point method, dynamics and lag phase could not be evaluated. The inhibitory effect of a toxic compound on population growth may be the consequence of (i) a lethal effect on some cells, (ii) a decrease of the division rate for all the cells, or (iii) the induction of a lag phase before the start of growth. We observed no lethality with the three drugs under the different concentrations used.

All three tested substances showed concentration-dependent inhibitory effects, but two different kinds of curve were obtained. During the 9 h exposure with cyclosporin-A or doxorubicin, rates fell gradually and regularly with the concentration, translating different proliferation rates directly related to the agent's concentrations. Another response pattern was observed with cisplatin. After exposure we noted a lag period for the high concentrations, followed by a marked reduction in the cell proliferation rate. According to Nilsson [25], this same event, observed in the presence of metal ions, might be explained by cell selection, related to an induced synthesis of methallothionein proteins, as a detoxification process. For *T. pyriformis* exposed to organic substances, it could be explained by the metabolism occurring concurrently with the detoxification process.

The main advantage of the non-specific esterase activities test is that toxicity information can be obtained within 2 h rather than the 9 h required for the standard growth rate test. This sublethal biotest also offers experimental simplicity and sensitivity. Nevertheless, it should be emphasized that cytotoxicity assessment is only possible for molecules that do not show a self fluorescence at 485 nm (excitation wavelength). We observed a gradual diminution of esterase activity with cisplatin and doxorubicin, indicating an impairment of the metabolic activity of the ciliated protozoan. A decreased fluorescence level in ciliates exposed to toxic compounds can be explained by a direct inhibition of enzyme activity and/or an indirect inhibition with alterations of the membrane properties (permeability, fluidity). In all cases, a decrease in the fluorescence level is a measure of the potential toxicity of a molecule.

We observed the following rank order for IC_{50} values expressed in µM for the population growth impairment test: cyclosporin-A > doxorubicin > cisplatin. The esterase activities test also showed a higher toxicity for doxorubicin than for cisplatin.

A further approach could consist in the determination of the intracellular concentration of the drugs in order to possibly relate their toxicity. One point could be the possible influence, in the population growth test, of the chemical composition of the culture medium on the relative toxicity of the

drugs, taking into account the different molecules properties. The organic culture medium for *T. pyriformis* (PPYS) is very complex and can interact with added toxicants. However, it is also the case for toxicity studies using other cell cultures.

Toxicology-based quantitative structure-activity relationships could also be used for the prediction of the toxic potency of chemicals and the interpretation of mechanisms of action [9]. The relative toxicity can be predicted for organic molecules by their structural properties (octanol/water partition coefficient for example), or for metals ions by their ionic characteristics (softness index σ_p and $\chi^2_m r$ value for example).

Morphological studies showed the formation of digestive vacuoles concentrating particulate elements with the three drugs and deformed cells in the presence of cisplatin. Those are not seen in untreated cells. These morphological alterations express some cell dysfunctioning. The formation of the small vacuoles is connected with the phagocytosis process; this process can lead to an intracellular accumulation of the compounds. Morphological abnormalities are probably connected to disturbances of the cytoskeletal elements which were not studied in this work.

Only cytotoxic effects of cisplatin (5 to 250 mg/l) have been previously studied on *T. pyriformis* [24]. The physiological effects observed were dose-dependent: inhibition of cell proliferation and endocytosis, accumulation of small refractive granules. A specific action on mitochondria was also observed. No experiment has been performed with cyclosporin-A and doxorubicin on this same cellular model.

Other criteria could be considered (electron microscopy study, DNA and RNA synthesis, perturbation of membrane fluidity, motility and swimming speed modification, cytoskeletal elements, etc.) to determine the cytotoxicity of these three molecules with this model and provide some explanations as to their cellular impact (mechanism).

With *Tetrahymena*, as with the other cellular models used to appreciate the toxicity of chemicals, the question arises as to the transposition of the results at the scale of the whole organism in which these molecules can be modified. In complementary experiments, we could consider the toxicity of doxorubicin and cyclosporin-A subjected to the action of the microsomal hepatic S9 fraction, thus accounting for possible modifications related to the presence of cytochromes P-450.

To our knowledge, our study is the only one carried out to date using the same cellular model, allowing in vitro relative toxicity assessment of these three therapeutic agents. Easy axenic culture conditions, experimental simplicity, sensitivity and rapidity confirm that *T. pyriformis* bioassays can offer alternative in vitro methods for the risk assessment of potentially toxic chemicals.

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