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Use of *Chlamydomonas reinhardtii* mutants for anticancer drug screening

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Abstract

We investigated the possibility of utilizing alga cells instead of mammalian cells for the screening of anticancer drugs. The alga cells grow in synthetic media whereas the mammalian cells require complex and more expensive media along with heavy investment and manpower. To assess the validity of this new approach, analysis of growth inhibition by antitumor agents was carried out jointly on a wall-less (*cw15*) mutant of *Chlamydomonas reinhardtii*, that obviates the problem of drug uptake, and the murine leukemic cell line L1210, commonly used for anticancer drug screening. The presence of the topoisomerases I and II (~97 and ~2 × 170 kDa, respectively) in the nuclear extracts of *C. reinhardtii* and their possible role as targets of the drugs was also investigated. Concentrated extracts were separated into >100 and <100 kDa fractions and their topoisomerase I and II activities were measured on relaxation of supercoiled plasmid DNA, decatenation of the catenated kinetoplast DNA and cleavage of plasmid DNA. Our results do not show significant difference in growth inhibition by antitumorals between the wall-less mutant of the alga and the murine leukemic cell line L1210. We noted that alga cells were inhibited by antibiotics that target gyrase, a bacterial variant of topoisomerase II which is also found in chloroplasts. At the molecular level, the alga nuclear fractions, >100 and <100 kDa, displayed the same activities as the mammalian enzymes topoisomerases I and II, respectively, and were blocked by the same poisons. We concluded that the wall-less *cw15* mutant of *C. reinhardtii* could advantageously replace mammalian cells in the screening of the anticancer drugs. The alga enzymes could also provide an opportunity to delineate the phylogeny of the topoisomerase superfamily.

Keywords: Algae; Antitumor; Drug screening assays; DNA gyrase; DNA topoisomerases; *Chlamydomonas*

1. Introduction

Screening models are essential for the detection of new anticancer drugs and still remain the corner stone of chemotherapy [1]. Experiments are carried out on a variety of mammalian cells in culture, among which, the widely used murine leukemic cell line L1210 [1–5]. However, mammalian cells require expensive investment in heavy equipment for the propagation, maintenance, and estimation of the cultures. For animal cell lines complex media and several passages under CO_2 atmosphere are needed for optimal growth and conservation. On the other hand, plant cells of diverse origins can be easily cultured and stored in synthetic solid or liquid media and largely eliminate the investment in heavy equipment required for animal cells [6,7]. The availability of various mutants of a particular genotype further permits precise selection of a phenotype ideally suited for the desired study [8]. In this context, the green alga *Chlamydomonas reinhardtii* exhibits a number of novel features that have rendered it very popular for studies in wide ranging domains such as the agroalimentary sector, cosmetology, pharmacology, water processing, production of biomass, etc. [9]. Indeed, this alga occupies a unique position at the cross roads of evolutionary dichotomy, being endowed with plant-like chloroplasts [10,11], protozoan flagella [8], rhodopsin contained in an 'eyespot' reminiscent of the mammalian photoreceptors [12], arrestin-like proteins for the regulation of the visual cascade seen in higher eukaryotes [13], putative receptor-like proteins for the transcription-modulation of genes by

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Abbreviations: ATP, adenosine triphosphate; TAP, Tris-acetate-phosphate; CMDB, carboxymethyl-dextran benzylamide; IC_{50}, drug concentration reducing by 50% the number of morphologically intact cells; DTT, dithiothreitol; EDTA, ethylenediaminetetra acetic acid; TBE, Tris-borate-EDTA; BET, ethidium bromide; NMHE, 2-*N*-methyl-2-hydroxy-9-ellipticinium; VP-16, etoposide (4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D glucopyranoside)); *m*-AMSA, amsacrine (4′-(9-acridinylamino) methanesulfon-*m*-anisilide).

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mammalian steroid hormones [14,15], and microtubules powered by adenosine triphosphate (ATP) analogous to the muscular contraction [16]. *C. reinhardtii* is particularly appropriate for a large scale production of the cytosolic extract for further analysis whereas the wall-less mutant obviates the problem of drug uptake. Since the general equipment and the growth medium, used for mammalian cell culture, are far more expensive than the simple Tris-acetate-phosphate (TAP) medium used for alga cell growth, anticancer drug screening on *C. reinhardtii* appeared particularly appealing.

The antitumor agents tested here on *C. reinhardtii* have been in clinical use for the treatment of various types of neoplasic invasions and have been commercialized under various trade names. Their molecular targets are located either in the nucleus or in the cytoplasm. Two antibacterial agents were also tested along with the antitumoral drugs to assess for possible action on the chloroplast. At the present time, a large number of anticancer drugs are targeted for nuclear DNA topoisomerases as these enzymes are ubiquitous and essential for DNA strand separation, chromatin compaction, and DNA decatenation during replication and recombination [17–21]. Actually, these enzymes play a crucial role in maintaining DNA topology [22–25]. The ATP-independent monomeric type I topoisomerase (EC 5.99.1.2) cuts and ligates the single stranded DNA and is essential for the cell replication [26,27]. Camptothecin, which inhibits the ligation of the DNA strand cleaved by the enzyme, is a potent poison of topoisomerase I [28]. The dimeric type II enzyme (EC 5.99.1.3) requires ATP to cut and ligate the double stranded DNA and is crucial for the survival of all eukaryotic organisms [29,30]. Topoisomerase II is the target of some of the most active and widely prescribed antitumoral drugs for the treatment of human cancer. This enzyme is inhibited by ellipticines [31,32] aminoacridines [31,33] and anthracyclines [34] which intercalate in DNA base pairs and interfere with the enzyme activity at these sites [35]. The chloroplastic or bacterial gyrase (EC 5.99.1.3), related to the mammalian type II enzyme [36], is inhibited by drugs belonging to the coumarin family [37], and by nalidixic acid [38]. Other potent anticancer drugs include cisplatin [39,40], melphalan [41], paclitaxel [42,43] and carboxymethyl-dextran benzylamide (CMDB) [44,45] which interact with various targets and interfere with different steps of the cell cycle.

We wished to assess whether the anticancer drug screening in the customary mammalian cell lines could be replaced by a model using the *C. reinhardtii* cells, where the enzymes topoisomerases I and II could be envisioned as potential nuclear targets. We also analyzed whether this alga could be targeted for screening antibacterial agents as its chloroplast contains an ATP-dependent topoisomerase activity. Like bacterial DNA gyrases (i.e., bacterial topoisomerase II), this enzyme is inhibited by novobiocin and nalidixic acid [46]. We found that the murine leukemic cell line L1210 can be easily replaced by the wall-less mutant *cw15* of *C. reinhardtii*. This could propel drug screening to unprecedented levels of technical simplicity, time effectiveness and economy. The identification in nuclear extracts of the *C. reinhardtii cw15* of topoisomerases type I and II sensitive to most of the known antitumoral drugs, could help the delineation of the practical and theoretical aspects of mammalian physiology in a significant manner. Furthermore, the availability of well characterized mutants [8] could form a new and novel tool to understand the action and distribution of the enzymes of the topoisomerase superfamily.

2. Materials and methods

2.1. Cell culture and growth inhibition

L1210 cells, derived from the chemically induced mouse DBA/2 tumor, were cultured in Roswell Park Memorial Institute (RPMI) medium [1–3], supplemented with 10% fetal serum, 2 mM L-glutamine, penicillin (100 U) and streptomycin (100 mg), and incubated at 37° in 5% CO_2. For growth inhibition they were dispensed into 24-well culture plates (10,000 cells per well) in 2 mL medium and exposed to the drug of choice for 72 hr at 37° under 5% CO_2. The cells were homogenized and counted with the aid of a Coulter counter ZM apparatus (Coultronics France SA). The results are expressed as IC_{50} drug concentration which reduces by 50% the number of morphologically intact cells after exposure for 3 days to the drug.

The wild type (WT) mating-type plus strain (isolate 11) and the wall-less *cw15* mutant mating-type plus of *C. reinhardtii*, kindly furnished by Dr. J. Girard-Bascou, were cultured in TAP continuously exposed to 4500 lx; *cw15* was also cultivated under alternate dark (12 hr) and light (12 hr) periods, at the room temperature [8]. For growth inhibition, the cells were distributed at 10,000 cells/culture tube in 2 mL TAP and exposed to the desired pharmacological agent for 72 hr at room temperature under continuous shaking and 4500 lx light source. Thereafter, the cells were counted under a phase contrast microscope after fixation with 1% glutaraldehyde and expressed as IC_{50} described above. To assess cell kinetics, the cells were exposed to the IC_{50} dose of the antitumor agent and processed as above.

2.2. Flow cytometry

The cells were washed with cold PBS and fixed at 10^6 in 70% ethanol at −20° for 1 hr. Thereafter, the cells were resuspended in PBS, incubated for 30 min at 37° in the presence of Hoechst 33342 at 20 μg/10^6 cells and counted in the Cytometer Coulter Coultronics (Coultronics France SA) using 346 nm (excitation) and 460 nm (emission) wavelengths. The fluorescent probe binds specifically with cell DNA and permits assessment of the cell cycle phase according to defined parameters.

2.3. Preparation of nuclear extracts

The *cw15* cells were lysed for 20 min at 4° in NP-40 (Nonidet® P40) 1% (v/v) buffered with Hepes pH 7.5 containing 20 mM KCl, 20 mM $MgCl_2$, 600 mM sucrose, 10% glycerol, 5 mM DTT (dithiothreitol), and rinsed in the same buffer without the detergent. Following centrifugation (650 *g*, 10 min), the cell extracts were frozen at −80° in 2.5% ficoll, 0.5 M sorbitol, 0.008% spermidine, 1 mM DTT, 5 mM $MgCl_2$, 10 mM Tris–HCl pH 7.5, 50% glycerol. The extracts were concentrated approximately 5-fold with the aid of Microcon 100 resulting in two fractions of either >100 kDa or <100 kDa.

2.4. Relaxation of supercoiled plasmid DNA

Two hundred nanograms pBR322 DNA was incubated with nuclear extracts (2 μL; 120 μg/μL) in 15 μL buffer (20 mM Tris–HCl, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$) in the presence or absence of 1.8 mM ATP. The reaction was terminated by the addition of 2 μL SDS (sodium dodecyl sulphate) (6%) and 2 μL proteinase K (1.5 mg/mL), the mixture was incubated at 50° for 30 min. After proteinase K digestion, 5 μL of loading buffer (0.25% bromophenol blue, 30% glycerol, 50 mM EDTA, pH 7) were added to each sample (15 μL). The products of reactions were separated on 1% agarose gel for about 15 hr (2.5 V/cm) in Tris-borate-EDTA (TBE) buffer. The gel was colored with ethidium bromide (BET; 0.5 mg/L), DNA bands were vizualized by transillumination with UV light (312 nm) and were photographed on Bio-profil using Bio-Capt program (Vilber Lourmat).

2.5. Decatenation of kinetoplast DNA

Decatenation was carried out as in relaxation experiment but pBR322 DNA was replaced by 200 ng trypanosoma cruzi kinetoplast DNA. The products of decatenation reactions were separated on 1.2% agarose gels in TBE buffer.

2.6. Topoisomerase II-mediated cleavage reaction

Two microliters aliquots of the nuclear extract (120 μg/μL) were incubated with 200 ng pBR322 DNA in the presence of varying amounts of poisons of topoisomerase II in 20 μL reaction buffer (40 mM Tris–HCl, pH 7.5, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 30 mg/mL BSA (bovine serum albumin), 1 mM neutralized ATP, 1 mM PMSF (phenylmethyl sulphonyl fluoride), 1 mM benzamidine, 0.01 mg/mL soybean trypsin inhibitor, 0.001 mg/mL aprotinin). The cleavage reaction was terminated by the addition of 2 μL 6% SDS plus 2 μL proteinase K (1.5 mg/mL) and the mixture was incubated for 30 min at 50°. After proteinase K digestion, 6.5 μL of loading buffer (0.25% bromophenol blue, pH 7, 40% sucrose) were added to each sample (20 μL). The products of reaction were fractionated on 0.8% agarose gels, for about 15 hr (2.5 V/cm), in TBE buffer containing 0.5 mg/mL of BET. DNA bands were vizualized by transillumination with UV light and quantitative densitometry was performed on Bioprofil using BIO-1D and BIO-CAPT programs (Vilber Lourmat). The peak areas of linearized DNA (Form III) were calculated.

3. Results and discussion

The wall-less mutant *cw15* of *C. reinhardtii* and the mammalian L1210 cells are equally sensitive to anticancer drug effects.

Initially, the cell cycle of *C. reinhardtii*, and especially that of the wall-less mutant *cw15*, was observed over the 24 hr period and the cell multiplication in cultures was synchronized by alternating dark and light phases every 12 hr. The cells remained in the G0/G1 phase during exposure to 4500 lx but reverted to the S phase within 2 hr after the beginning of the dark cycle. This was followed by the G2 + M phase for 1 hr and finally the G0/G1 phase (data not shown). Kinetics of growth inhibition, assessed by exposing the cell cultures to different drugs for several days, showed that 50% of the maximum response was obtained between 48 and 72 hr. Growth inhibition data for the three types of cells, L1210, *cw15* and WT(11), expressed as IC_{50}, after 72 hr of drug exposure, are presented in Table 1. The apparent lower sensitivity of WT cells (see Table 1) results primarily from the presence of a cell wall that can restrict internalization of the chemical and also prevent the cell lysis [8]. On the other hand, wall-less *cw15* mutant of *C. reinhardtii* was sensitive to growth inhibition by all of the materials tested in this study, and more particularly so to those that interfere with the activity of topoisomerase I and II viz. 2-*N*-methyl-2-hydroxy-9-ellipticinium (NMHE), etoposide (4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D glucopyranoside)) (VP-16), amsacrine (4′-(9-acridinylamino) methanesulfon-*m*-anisilide) (*m*-AMSA), CMDB, doxorubicin and camptothecin. No significant differences in growth inhibition were observed between the murine leukemic cell line L1210 and the alga mutant *cw15*, under the experimental conditions employed here, except for *m*-AMSA which was less effective on the latter. Novobiocin and nalidixic acid, both of which are known to antagonize the bacterial gyrase [38], were effective almost exclusively on *cw15* where they could interfere with the gyrase contained in the chloroplast of *C. reinhardtii* [36]. Furthermore, melphalan [41] and paclitaxel [42,43] that interact, respectively, with cell DNA and the tubulin subunits of microtubules, were much more effective in inhibiting the multiplication of L1210 than that of *cw15*. Interestingly, cisplatin, which also targets the cell DNA [39,40], was more effective on *cw15* than on the L1210 line. This

Table 1
Growth inhibition of L1210, *cw15* and WT(11) cells by chemicals agents

Drugs IC_{50}[a] (μM)	NMHE	VP-16	CMDB	*m*-AMSA	Doxorubicin	Cisplatin	Camptothecin	Novobiocin	Nalidixic acid	Paclitaxel	Melphalan
Cell lines											
L1210	0.024 ± 0.001	0.022 ± 0.011	5.340 ± 0.040	0.003 ± 0.000	0.007 ± 0.000	0.294 ± 0.011	0.010 ± 0.001	85 ± 1.730	704 ± 12	0.025 ± 0.001	0.003 ± 0.000
cw15	0.033 ± 0.001	0.002 ± 0.000	4.934 ± 0.274	0.147 ± 0.027	0.001 ± 0.000	0.014 ± 0.001	0.020 ± 0.000	0.035 ± 0.003	0.133 ± 0.005	0.113 ± 0.001	4.562 ± 0.217
WT(11)	0.116 ± 0.011	0.750 ± 0.004	22 ± 1.010	nd[b]	nd	nd	nd	nd	nd	nd	nd

[a] IC_{50} values as determined after 72 hr (Section 2).
[b] nd, not determined.

suggests that either the mechanism of action of some of these pharmacological agents involves accessory cellular sites or the DNA repair systems are less efficient in *C. reinhardtii*.

The technique of flow cytometry showed that almost all of the anticancer drugs arrested the *cw15* cells in the G2 + M phase (data not shown) thereby suggesting that they provoke cytotoxicity on the alga cells, similar to that previously observed with these chemicals in the mammalian cells [23]. The CMDB and the melphalan were the only compounds blocking the *cw15* in the G0/G1 phase and in the S phases, respectively, similar to the observation with mammalian cells [44,45]. Consequently, the *cw15* mutant of *C. reinhardtii* could very well replace L1210 cells to screen for the antitumor activity of various pharmacological agents.

3.1. The L1210 and C. reinhardtii cells have the same nuclear targets but C. reinhardtii has also targets in the chloroplast

Most of the chemicals exhibiting cytotoxicity against L1210 and *C. reinhardtii* cells are known also to be poisons of topoisomerases. Theses drugs convert the physiological enzyme into cellular poison in stabilizing a ''DNA cleavage complex'' in which the enzyme remains covalently attached at the DNA strand breaks. The DNA lesion interferes with the DNA transcription and replication machinery. Such poisons include the cytotoxic chemicals NMHE, VP-16, m-AMSA and camptothecin which all arrest L1210 and *cw15* cells in the G2 + M phase. In contrast, topoisomerase catalytic inhibitors block the DNA binding or the DNA cleavage site and are generally much less cytotoxic than topoisomerase poisons. A good example is provided in this study by CMDB which arrests the L1210 and *cw15* cells in the G0/G1 phase and displays cytostatic effects (data not shown).

The average molecular weight of topoisomerases I is ~97 while that of topoisomerase II is ~2 × 170. The presence of topoisomerases I and II activities in the nucleus of *cw15* cells was demonstrated by inhibition assays performed with concentrated nuclear extracts separated into >100 and <100 kDa. As shown in Fig. 1, both fractions were able to relax the supercoiled pBR322 DNA, similar to the action of the mammalian topoisomerases [23,35]. Moreover, the data obtained in the presence or the absence of ATP (only topoisomerase II needs ATP) prove that the alga nuclear extracts contain both the topoisomerase I and II activities. Data also show (Fig. 2) that only the high molecular weight fraction catalyses DNA decatenation, which is known as being a property of the topoisomerase II enzyme [25–27].

Additional proof for a topoisomerase activity in *C. reinhardtii* comes from the studies on the cleavage of a plasmid DNA. Both topoisomerases I and II provoke the linearisation of a plasmid DNA. Results are analyzed by

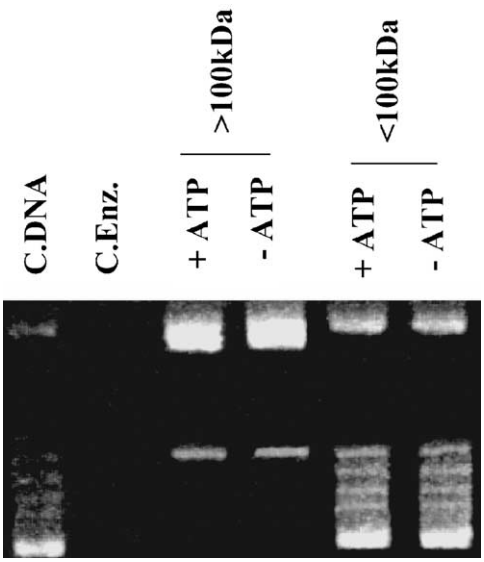

Fig. 1. Topoisomerase activity in *cw15* nuclear extracts: relaxation of supercoiled plasmid DNA. Two microliters of the respective nuclear extracts (120 μg/μL) from the <100 and >100 kDa fractions of *Chlamydomonas reinhardtii cw15* mutant were incubated with 200 ng pBR322 DNA in the presence or absence of 1.8 mM ATP. The relaxation was assessed after overnight migration on 1% agarose gel (2.5 V/cm). C.DNA: pBR322 DNA control; C.Enz: enzyme (nuclear extract) control. (>100 and <100 kDa): extracts with average molecular weights >100,000 and <100,000, respectively.

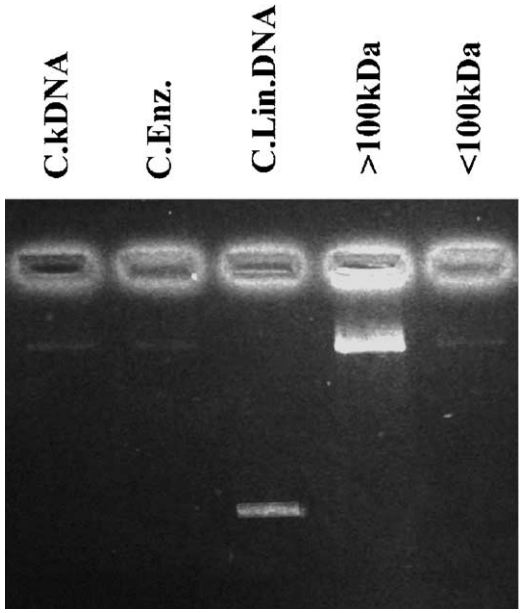

Fig. 2. Topoisomerase activity in *cw15* nuclear extracts: decatenation of kinetoplast DNA. Two microliters of the respective nuclear extracts (120 μg/μL) were incubated with 200 ng kDNA in a final volume of 15 μL. The decatenation was assessed after overnight migration on 1.2% agarose gel (2.5 V/cm). C.kDNA: kinetoplast DNA control; C.Enz: enzyme (nuclear extract) control; C.Lin.DNA: linear DNA control (also see legend to Fig. 1).

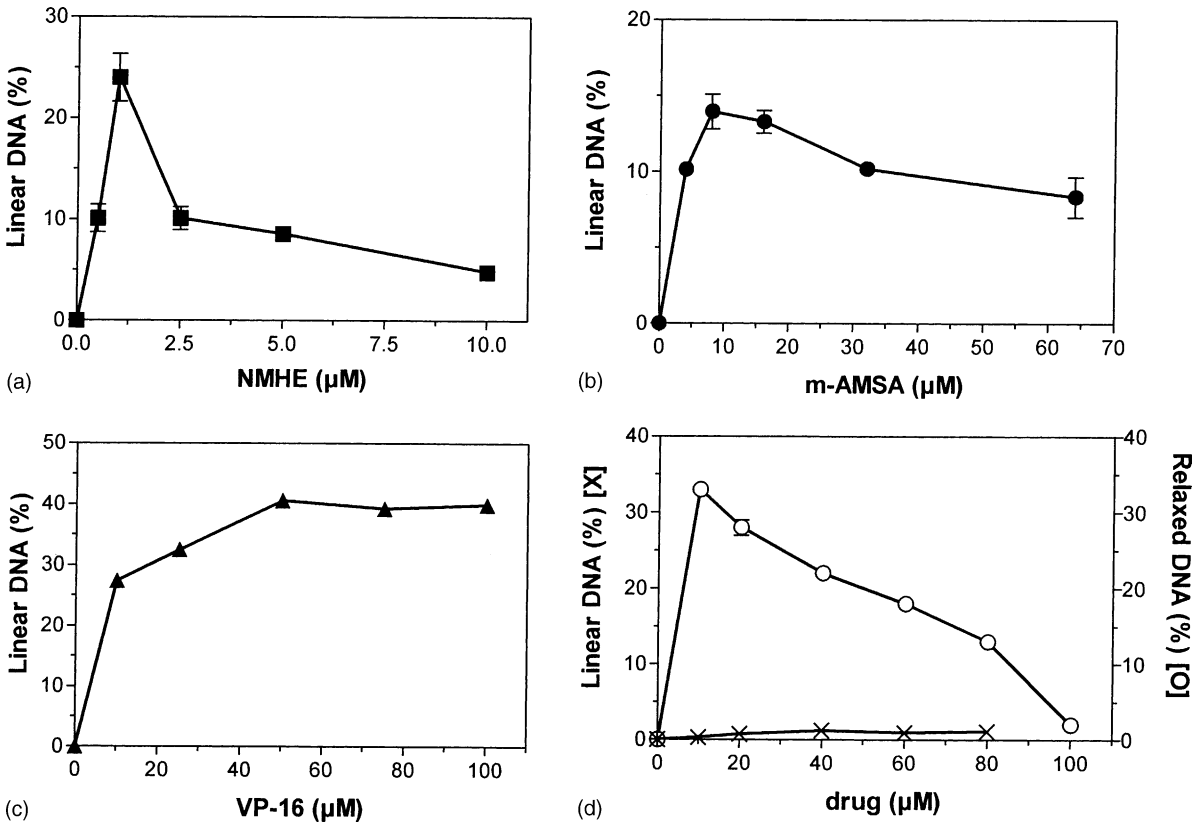

Fig. 3. Drug-stimulated cleavage of pBR322 DNA by *cw15* nuclear extracts. Two microliters aliquots of the >100 kDa nuclear extract (120 μg/μL) were incubated with 200 ng pBR322 DNA in a final volume of 15 μL containing the indicated concentrations of poisons of topoisomerase II: (a) NMHE; (b) *m*-AMSA; (c) VP16; (d) gyrase and topoisomerase I; X: nalidix acid; O: camptothecin, and processed as in Fig. 1. Quantitative densitometry was performed on Bioprofil using BIO-1D and BIO-CAPT programs (Vilber Lourmat). Controls (nuclear extracts without drugs) were subtracted. Bars (SE).

densitometric analysis of DNA on gel separation as presented in Fig. 3. The profiles are similar to those obtained with mammalian topoisomerase II, in the presence of NMHE, VP-16 or *m*-AMSA [47], and topoisomerase I, in the presence of camptothecin. As commonly observed, the amount of relaxed DNA increases proportionally with the drug dose. Yet, an excess of NMHE, *m*-AMSA or camptothecin inhibits the linearization process while no decrease in cleaved complexes is observed with an excess of VP-16. On the other hand, nalidixic acid which is specific for gyrase is without effect on the nuclear extracts.

4. Conclusion

From the foregoing, it is safe to conclude that *C. reinhardtii cw15* can replace mammalian cells cultures to screen molecules whose anticancer activity proceeds via the inhibition of cell topoisomerases I and II. The ease with which the algae can be handled and grown in relatively simple media would make it a very cost-effective model, indeed. The growth of algae can also be inhibited by drugs interacting with the microtubule tubulins and antibacterial drugs that interfere with the chloroplast gyrase. The latter effect is absent from the mammalian cell which is devoid of the gyrase enzyme. The presence of topoisomerase type I and II enzymes in *C. reinhardtii cw15*, similar to the mammalian proteins, suggests that the alga cells can form an appropriate model to delineate the structure and function of enzymes of the topoisomerase superfamily.

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