*Biochemical Pharmacology*, Vol. 39, No. 1, pp. 109–122, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
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# SELECTIVE ALTERATION OF MITOCHONDRIAL FUNCTION BY DITERCALINIUM (NSC 335153), A DNA BISINTERCALATING AGENT

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(*Received* 3 *March* 1989; *accepted* 1 *August* 1989)

**Abstract**—The bifunctional intercalator Ditercalinium (NSC 335153) demonstrates an anti-tumoral cytotoxicity markedly different from other intercalating agents. A delayed toxicity is observed in eucaryotic cells, both *in vitro* and *in vivo*, at drug concentrations far below those required to observe immediate toxic effects. Fluorescence microscopy demonstrates that Ditercalinium and the mitochondrial-staining fluorophore $DiOC_2(5)$ are concentrated in the same cellular organelles of L1210 cells. Electron microscopy of Ditercalinium-treated cells reveals extensive and progressive swelling of mitochondria, with no other ultrastructural changes observed. Ditercalinium uptake and toxicity are in part related to mitochondrial membrane potential. However, drug accumulation itself does not immediately alter the mitochondrial membrane potential. Cellular ATP pool levels and the rate of respiration fall progressively after drug treatment. Nucleotide pools in DC3F cells, measured between drug treatment and death, show marked drops in pyrimidine levels while purine nucleotide levels decline more slowly. Addition of uridine or cytidine partially rescues Ditercalinium-treated cells, while toxicity is increased in the presence of 2-deoxyglucose. The combined evidence indicates that the toxicity of Ditercalinium to murine leukemia cells (L1210) and Chinese Hamster lung cells (DC3F) is due to disruption of mitochondrial function.

In order to obtain molecules able to bind to DNA with high affinity, DNA bifunctional intercalators have been synthesized [1–3]. Among these molecules, ditercalinium (NSC 335153), a dimer derivative of 7H-pyridocarbazole was endowed with antitumor properties on a variety of animal tumors [2, 4]. Antitumor activity has also been demonstrated by naturally occurring bifunctional intercalating agents such as quinoxaline antibiotics and luzopeptin [5].

Ditercalinium bis-intercalates into DNA through the major groove [6, 7], and its binding to DNA is associated with a DNA conformational change which is specifically recognized in *Escherichia coli* by the Uvr ABC repair system. Since Ditercalinium is not covalently bound to DNA, an abortive DNA repair process is induced [8, 9].

Several observations suggest that the mechanism of action of Ditercalinium is different from that of other antitumor agents [10, 11]. Ditercalinium induces a delayed toxicity such that treated cells grow five to six generations after treatment and cells are not arrested in the G2+M phases of the cell cycle [10]. In contrast to monointercalating agents, Ditercalinium does not introduce Topoisomerase II mediated protein-associated DNA strand breaks both *in vitro* and *in vivo* [12]. Morphological alterations of the mitochondria are observed and are associated with a selective loss of mitochondrial DNA without measurable alteration of nuclear DNA [13]. In addition, the activity of cytochrome *c* oxidase decreases exponentially in Ditercalinium treated cells with a half-time of 24 hr, corresponding to the turnover of the enzyme [13]. Cytochrome oxidase is a multimeric enzyme whom subunits I, II and III are coded by the mouse mitochondrial DNA [14].

In order to determine whether the degradation of mitochondrial DNA is related to the antitumor action of Ditercalinium, the effect of this drug on mitochondrial function and cell metabolism were investigated in the murine leukemia L1210 cells and on the Chinese hamster lung fibroblasts DC3F. These observations were made during the rather long time

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‡‡ Abbreviations used: PBS, phosphate buffer saline (0.8% NaCl, 0.02% KCl, 0.115% disodium phosphate, 0.02% monopotassium phosphate, w/v); CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Rho 123, Rhodamine 123; $DiOC_2(5)$, 3,3′-diethyloxadicarbocyanide iodide; NRS, nucleotide releasing solution; PMSF, phenylmethylsulfonyl fluoride.

which separates the drug treatment from cell death (five to six generations). Ditercalinium doses were selected, therefore, which had a minimum immediate toxic effect but induced an almost complete arrest of cell growth after five to six generations. The true surviving cells which form normal size colonies in agar medium were distinguished from the death committed cells which form small and abortive colonies resulting from the delayed growth arrest. These treated cells were observed by fluorescence microscopy, electron microscopy and analysed for their mitochondrial membrane potential, ATP content, oxygen consumption and nucleotide pools. The potentiation of Ditercalinium by 2-deoxyglucose was also examined.

## MATERIALS AND METHODS

*Chemicals.* Ditercalinium chloride (NSC 335153) was from the Roger Bellon Laboratory. A 500 $\mu$M stock solution in water was sterilized by filtration through 0.22 $\mu$m Millex-GS membrane (Millipore Corp.), stored at −20° and used for the preparation of all solutions used in this study. Diluted solutions in water were checked for concentration by absorption spectroscopy ($\varepsilon M$ = 65,000 at 262 nm) and used for appropriate concentration in culture medium. Calf thymus DNA was purchased from Boehringer (Mannheim, F.R.G.) and bovine serum albumin fraction V from Sigma Chemical Co. (Poole, U.K.). Rhodamine 123 (Rho 123) and $DiOC_2(5)$ (both laser grade) were purchased from Eastman Kodak Co. (Rochester, NY) and CCCP from Sigma. Nucleotide Standards were purchased from Boehringer and uridine, cytidine and hypoxanthine from Sigma.

*Cell culture and drug treatment.* L1210 cells were grown in RPMI 1640 supplemented with 9% fetal calf serum (Gibco BRL, Cergy, France), penicillin (200 IU/ml), streptomycin (50 $\mu$g/ml) and $\beta$-mercaptoethanol (0.06 mM). In this medium, cells grew exponentially in non agitated suspension at 37° in 5% $CO_2$ at 100% humidity with a doubling time of about 13 hr. Exponentially growing cells ($1 \times 10^5$/ml) were incubated in medium containing different Ditercalinium concentrations for 24 hr, in culture conditions, and then resuspended in drug-free medium. For the determination of the colony-forming ability, 250 and 500 cells were diluted in 0.33% Noble agar containing culture medium and poured into a 35-mm diameter Petri dish. The plates were incubated at 37° in 5% $CO_2$ and colonies were counted 14 days later. When L1210 cells were incubated with 0.2 $\mu$M Ditercalinium during 24 hr and resuspended in drug-free medium, this treatment induced 25% inhibition of the growth during the time of treatment. After this Ditercalinium treatment, the number of abortive colonies observed was greater than 99%.

Chinese hamster lung cells DC3F maintained as monolayer cultures were routinely passaged in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, streptomycin (50 $\mu$g/ml) and penicillin (100 IU/ml). DC3F cells ($10^5$) were seeded in 16-mm wells of 24-well dishes and incubated overnight in similar conditions to L1210 culture. For Ditercalinium treatment, the medium was then replaced either with 1 ml of fresh medium or with 1 ml of medium containing the drug at the indicated concentrations. After 3 hr in the incubator, the drug was washed out by rinsing the wells twice with 1 ml of MEM and trypsinizated. For the determination of the colony forming ability, about 500 cells were plated in triplicate in 60-mm diameter culture plastic dishes. The colonies were counted 1 week later. In these conditions, the cloning efficiency of the control was approximately 70%. For the complementation experiments, uridine, cytidine or hypoxanthine was added both during the treatment and the cloning experiment.

*Electron microscopy.* Treated and control cells were fixed at room temperature by adding 1.5% glutaraldehyde to the culture medium and centrifuged. Pellets were then placed in new fixative (1.5% glutaraldehyde in 0.066 M Sorensen pH 7.4) for 1 hr at 4°. After 2 hr washing with buffer, cells were post-fixed in 2% $OsO_4$ in the same buffer. Dehydration in graded ethanol and propylene oxide, Epon embedding and uranyl-lead staining were performed using classical methods. Observations were made in a Philips EM 300 instrument.

*Determination of spectroscopic properties of free and bound Ditercalinium.* Spectroscopic properties were determined in sodium acetate buffer (100 mM, pH 5.45). Final concentration of Ditercalinium was 5 $\mu$M. A membrane fraction of A-431 cells has been prepared from exponentially growing cells and was a gift from Drs J. Markovits and J. Pierre in our laboratory. Briefly, the cells were lysed into a hypotonic buffer (20 mM Hepes, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, 0.01 mg/ml leupeptine, NaOH pH 7.2), centrifuged at 23,000 $g$ for 30 min. The pellet was resuspended in 20 mM Hepes pH 7.2, and the protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as reference standard [15].

Absorption spectra were recording in a Uvikon 860 spectrophotometer. Fluorescence emission spectra were recorded using a SLM 800 spectrofluorometer controlled by a Minc 11/23 Digital computer. $\Phi_b$ and $\Phi_f$ are defined as the fluorescence quantum yield of bound and free Ditercalinium respectively. In presence of DNA: $\Phi_b/\Phi_f = IF_b/IF_f$, where $IF_f$ and $IF_b$ are the fluorescence intensity of Ditercalinium in absence and presence of excess DNA, respectively, when fluorescence is excited at the isosbestic point (425 nm). With membranes,

$$\frac{1}{IF_b/IF_f + 1} = f(1/[\text{membrane}])$$

was drawn. $\Phi_b/\Phi_f$ was taken as a limit $_{(\text{membrane}\to\infty)}$ of $IF_b/IF_f$. The maximum final protein concentration in the mixture Ditercalinium-membranes was 20 $\mu$g/ml.

*Fluorescence microscopy.* Cells were incubated 3 hr with 1 and 4 $\mu$M Ditercalinium at 37° in culture medium. Then, the cells were centrifuged and suspended in PBS containing the fluorescent mitochondrial probe $DiOC_2(5)$ at 100 nM for 30 min in the incubator. The cells were washed and observed on a Zeiss photomicroscope III, with a high pressure mercury source (HBO 100 W). The combinations of

Table 1.

| | Free Ditercalinium | Ditercalinium + DNA | Ditercalinium + BSA | Ditercalinium + plasmic membranes | Free Ditercalinium in methanol |
|---|---|---|---|---|---|
| Absorption spectrophotometry | | | | | |
| $\lambda_{max}$ (nm) | 412 | 443 | 412 | 415 to 438 | 412 |
| Isosbestic point ($\lambda_{nm}$) | — | 425 | * | 425 | — |
| Fluorescence spectrophotometry | | | | | |
| $\lambda_{emission}$ (nm) | 600 | 595 | 600 | 580 | 575 |
| $\frac{\phi_b}{\phi_f}$† | — | 5.7 ± 0.6 | * | 10 | 17 |

Ditercalinium concentrations: 5 μM (O.D.$_{412}$ = 0.087) in 100 mM sodium acetate buffer, pH 5.45, or in methanol. Calf thymus DNA, concentration: 250 μM nucleotides. BSA: bovine serum albumin fraction V, concentration 10–100 μg/ml, in 100 mM sodium acetate buffer, pH 5.45. Crude preparation of membranes from A431 cells containing 5 mg/ml proteins: 5–40 μl of membranes in 1 ml of 5 μM Ditercalinium (100 mM sodium acetate buffer, pH 5.45).

* No evidence of binding.

† $\phi_b/\phi_f$ has been calculated as described in Materials and Methods.

filters (Zeiss) for Ditercalinium was 48 77 06 (λ exc = 430–440 nm, λ em > 470 nm) and for $DiOC_2(5)$ was 48 77 14 (λ exc = 510–560 nm, λ em > 590 nm). Zeiss 40× and 63× oil immersion objectives were used. Photomicrographs were made on Ilford HP5 (ASA 400) and developed at ASA 1600.

*Quantitative Rhodamine 123 and $DiOC_2(5)$ fluorescence measurements.* All reagents were stored in the dark at 4°. Stock solutions were prepared in DMSO for Rho 123 (5 mg/ml), in ethanol for $DiOC_2(5)$ (1 mM) and CCCP (1 mM). They were diluted in water and added to cell suspensions to a final concentration of 1 μg/ml for Rho 123, 100 nM for $DiOC_2(5)$ and 10 μM for CCCP. For comparison of retention of Rho 123 and $DiOC_2(5)$ by cells, L1210 cells were grown up to $9 \times 10^5$ cells/ml, and incubated as a function of Ditercalinium concentration during 3 hr at 37° in 5% $CO_2$. After centrifugation, cells were suspended in Ditercalinium-free buffer (5 mM KCl, 128 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1.6 mM $Na_2HPO_4$, 20 mM Hepes, 5.5 mM glucose, pH 7.3). Rho 123 or $DiOC_2(5)$ was added to 3 ml of cell suspension ($3 \times 10^6$ cells/ml) and incubated at 37° in a water bath for 2 hr in the case of Rho 123 and for 30 min in the case of $DiOC_2(5)$. Cells incubated with Ditercalinium in absence of CCCP, were then exposed to CCCP in order to abolish the mitochondrial membrane potential and stained with Rho 123 or $DiOC_2(5)$ at the same time and in the same conditions. Cells were centrifuged and suspended in 1 ml of PBS. An aliquot of 0.1 ml was used for cell counting and 0.9 ml was extracted with 2 ml of PBS-saturated *n*-butanol. After mixing, the two phases were separated by centrifugation and fluorescence of the *n*-butanol extract was measured in a Kontron SFM 23/B spectrofluorometer, with 496 nm for excitation and 535 nm for emission in the case of Rho 123 and with 580 nm for excitation and 600 nm for emission in the case of $DiOC_2(5)$. The fluorescence measurements were related to cell counts to normalize Rho 123 and $DiOC_2(5)$ retention per million of cells. Autofluorescence of control and Ditercalinium-treated cells was less than 1%. Ditercalinium was extracted in these conditions and had no quenching effect on Rho 123 fluorescence. The dye content of Ditercalinium-treated cells was expressed as a percentage of control cells.

*Flow cytometric analysis of Ditercalinium uptake.* The FACS 440 flow cytometer was used to measure the cellular fluorescence and light scatter. Ditercalinium fluorescence excited with the 457 nm line of the Argon laser was selected with the filter 580 BP for fluorescence emission (Oriel Co., Stratford, CT, U.S.A.). The forward light scatter was measured as a second parameter. Cells ($10^5$ cells/ml) were incubated first with 10 μM CCCP during 30 min, and then treated with 1 μM Ditercalinium during 3 hr in culture medium. From the fluorescence histogram of 5000 cells, the mean fluorescence channel was determined and was taken as a parameter proportional to Ditercalinium accumulation.

*Measurement of intracellular ATP.* The ATP quantity was measured from 2000 cells diluted in culture medium using the luciferin–luciferase method [16]. Reagents (Lumit) and photometer (Biocounter M 2010) were from Lumac 3M and used as indicated by the suppliers. A detergent solution (NRS) was added to cell suspension in order to release the ATP from the cells.

*Oxygen consumption.* Oxygen consumption rates were measured polarographically using a Clark-type electrode inserted into a water-jacketed sealed glass chamber. The oxygen electrode apparatus was connected to a chart recorded which was calibrated between 0 and 100% saturation with atmospheric oxygen at 37°. Rates of consumption in change in percentage/min were read directly from the chart recording and were converted to nanomoles of $O_2$/min using a conversion factor of 200 nanomoles of $O_2$/ml at 37°. Control and treated cells ($5–8 \times 10^5$/ml) were suspended in growth medium with magnetic stirring in a total volume of 1.8 ml. The oxygen consumption rate of treated cells was measured daily and compared to that of control cells (100%).

*High performance liquid chromatography of 5'-ribonucleotides pools.* Exponentially growing DC3F

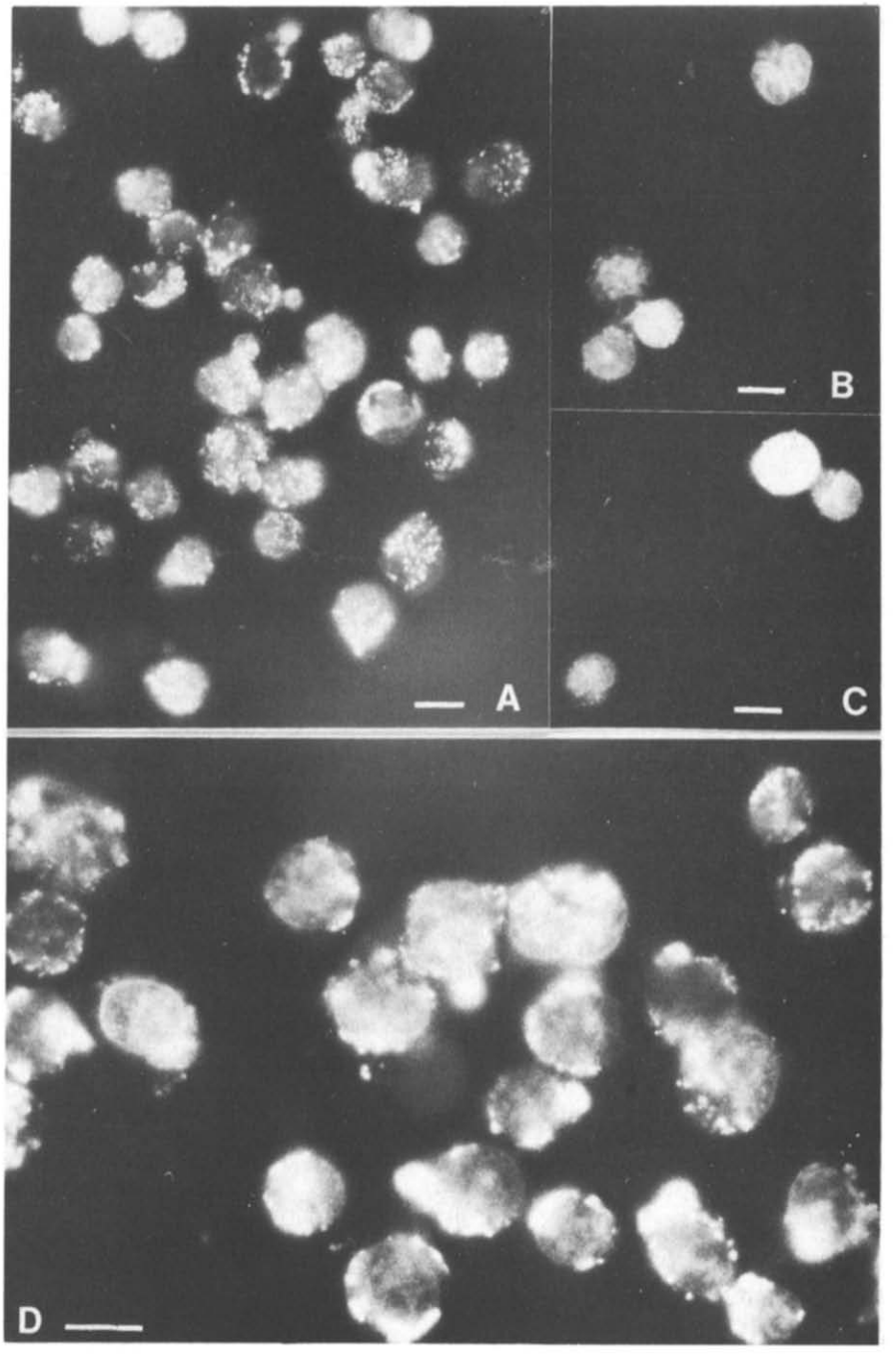


Fig. 1. Intracellular localization of Ditercalinium. L1210 cells were treated first for 3 hr with Ditercalinium and then with $DiOC_2(5)$ 100 nM for 30 min. Cells were observed by fluorescence microscopy before (A) or after (B–D) bleaching of $DiOC_2(5)$. (A–C): Ditercalinium 4 $\mu$M. (D): Ditercalinium 1 $\mu$M. In (A) the granular fluorescence was representative of the accumulation of the carbocyanine in the mitochondria, Ditercalinium fluorescence being very low in comparison with that of $DiOC_2(5)$. In (B–D) the fluorescence of Ditercalinium is observed after bleaching of $DiOC_2(5)$ and is located in granular structures identical to those characterized with $DiOC_2(5)$, and in nuclear structures such as membrane and network. In (C) is shown an intense fluorescent cell which is a dead one. Scale bars equal 10 $\mu$M.

cells ($10^7$/100-mm plastic culture dish) were incubated in 10 ml of MEM containing 1.6 $\mu$M Ditercalinium during 4.5 hr. The drug was removed from culture and the cells were plated at a density of $4 \times 10^6$ cells/60-mm dish. Duplicate dishes were used for cell number determination, and nucleotide extraction at 1, 2 or 3 days after Ditercalinium treatment. For nucleotide extraction, the dishes were

kept on ice. The cells (5–10 × $10^6$/60-mm dish) were washed with cold PBS and covered by 1 ml of cold formic acid (1.0 M, pH 2) for 30 min. The supernatant was frozen on dry ice and lyophilized [17]. The residue was dissolved in 0.2 ml water, and 0.05 ml was applied to an anion-exchange Whatman Partisil-10 SAX (250 × 4.6 mm) at room temperature. The buffer consisted of potassium phosphate (pH 6) and the column was eluted for 45 min with a linear gradient generated from 5–100 mM, at a flow rate of 1.0 ml/min. The HPLC was carried out using a Waters 440 liquid chromatograph equipped with a two-channel detector (254 and 313 nm). 5′-Ribonucleotides were identified by absolute and relative retention times, cochromatography with standards and mass spectrometry (FAB) of selected peaks. Nucleotide standards were: adenosine, AMP, ADP, ATP, GTP, GDP, GMP, CTP, CDP, CMP, UTP, UDP, UMP, NADPH, NADP, NADH, NAD, NMN. Standard solutions were prepared at a concentration corresponding to 1 O.D. (254 nm) unit. Nucleotide concentrations were converted to pmol/10 millions cells. The recovery of nucleotides during extraction was determined using a mixture of standards extracted in the same conditions.

## RESULTS

### *Visible and fluorescence spectroscopy of Ditercalinium interacting with macromolecules*

The spectroscopic properties of Ditercalinium were studied to provide a basis for the intracellular localization of the drug using fluorescence microscopy. Table 1 lists the visible and fluorescence spectroscopic properties of Ditercalinium interacting with different macromolecules or in different solvents. In the absorption spectra of free drug, there is a maximum at $\lambda = 412$ nm, which shifts to 443 nm when the drug is mixed with an excess of calf thymus DNA. The molar extinction coefficients of Ditercalinium, free or bound to DNA or membranes are similar and an isosbestic point is observed at 425 nm. When Ditercalinium is mixed with BSA, no change is observed in the absorption spectra. The maxima of fluorescence emission are at 600 nm for free drug and at 595 and 580 nm for DNA-bound and membrane-bound Ditercalinium, respectively. In the presence of DNA and membranes, the shape of the fluorescence emission spectra is similar to that of free drug but there is an increase in the Ditercalinium fluorescence. Ditercalinium is 17 times more fluorescent in methanol than in water. The interaction of Ditercalinium with membranes results in a 10-fold enhancement of fluorescence. When bound to DNA, Ditercalinium is 5.7 times more fluorescent than free in buffer. There is no change in either absorption or fluorescence spectra when Ditercalinium was mixed with bovine serum albumin. These results show that there is significant fluorescence enhancement of Ditercalinium with membranes and DNA but not in the presence of protein.

### *Intracellular localization of Ditercalinium*

Cells incubated with Ditercalinium were examined by fluorescence microscopy (Fig. 1) to determine the intracellular location of the drug. The Ditercalinium fluorescence in living cells is primarily centred in granular extranuclear structures, indicating an interaction between Ditercalinium and membranes. In addition to these extranuclear structures in living cells, Ditercalinium is also observed in the nucleus but weakly (1B and D). In dead cells, however, the major intracellular fluorescence resulting from Ditercalinium is observed in the nucleus (1C).

In order to identify the organelles stained by Ditercalinium, L1210 cells were incubated with $DiOC_2(5)$ which is concentrated specifically in mitochondria in response to the mitochondrial membrane potential [18]. The organelles found fluorescent with $DiOC_2(5)$ were identical to those labeled with Ditercalinium. The coincidence between the two stainings is observed in cells treated with both Ditercalinium and $DiOC_2(5)$. The $DiOC_2(5)$, however, is much more photosensitive than Ditercalinium and is rapidly bleached under the light microscope. The remaining fluorescence observed, therefore, after $DiOC_2(5)$ bleaching is from Ditercalinium. This Ditercalinium fluorescence is observed even after 15 min of illumination under the light microscope (1B–D).

### *Electron microscopy of mitochondrial alteration*

When compared to control cells (Fig. 2A), cells treated for 24 hr with 0.2 μM Ditercalinium (Fig. 2B) exhibit a swelling of their mitochondria. Practically all mitochondria appear enlarged and few show normally disposed cristae. In all mitochondria, the matrix is clearer than in the control cells even for mitochondria only slightly altered. In addition, lipidic inclusions are found in the cytoplasm of treated cells. No visible alteration is observed in the nucleus or other cytoplasmic organelles.

The irreversibility of the mitochondrial damage is illustrated in Fig. 2C and D which show cells cultured for 24 hr in the presence of 0.2 μM Ditercalinium, then another 24 hr in a drug-free medium. Mitochondria remain swollen and appear more damaged than after the first 24 hr in the presence of the drug. Original cristae, however, were unchanged in morphology but the global swelling sequestered these cristae at the periphery of the mitochondria as shown in the insert of Fig. 2D.

This enlargement of the mitochondrial volume is also correlated with the appearance of a clear matrix. Even 4 days after drug removal, the normal structure of the mitochondria is not recovered (data not shown).

### *Modification of Ditercalinium toxicity by co-treatment of the cells with a respiratory uncoupler*

Mitochondria are able to concentrate positively-charged dyes in relation to the membrane potential. Therefore, the measurement of the cellular uptake of these fluorescent dyes such as Rho 123 or $DiOC_2(5)$ indirectly estimates this potential [18]. The increased retention of these agent by mitochondria of some tumor cells has been attributed to an increased mitochondrial membrane potential in certain malignant epithelial cells [19, 20].

Ditercalinium is a lipophilic and cationic molecule with two quaternary ammonium moieties and two ionizable ternary amino groups with a pK between

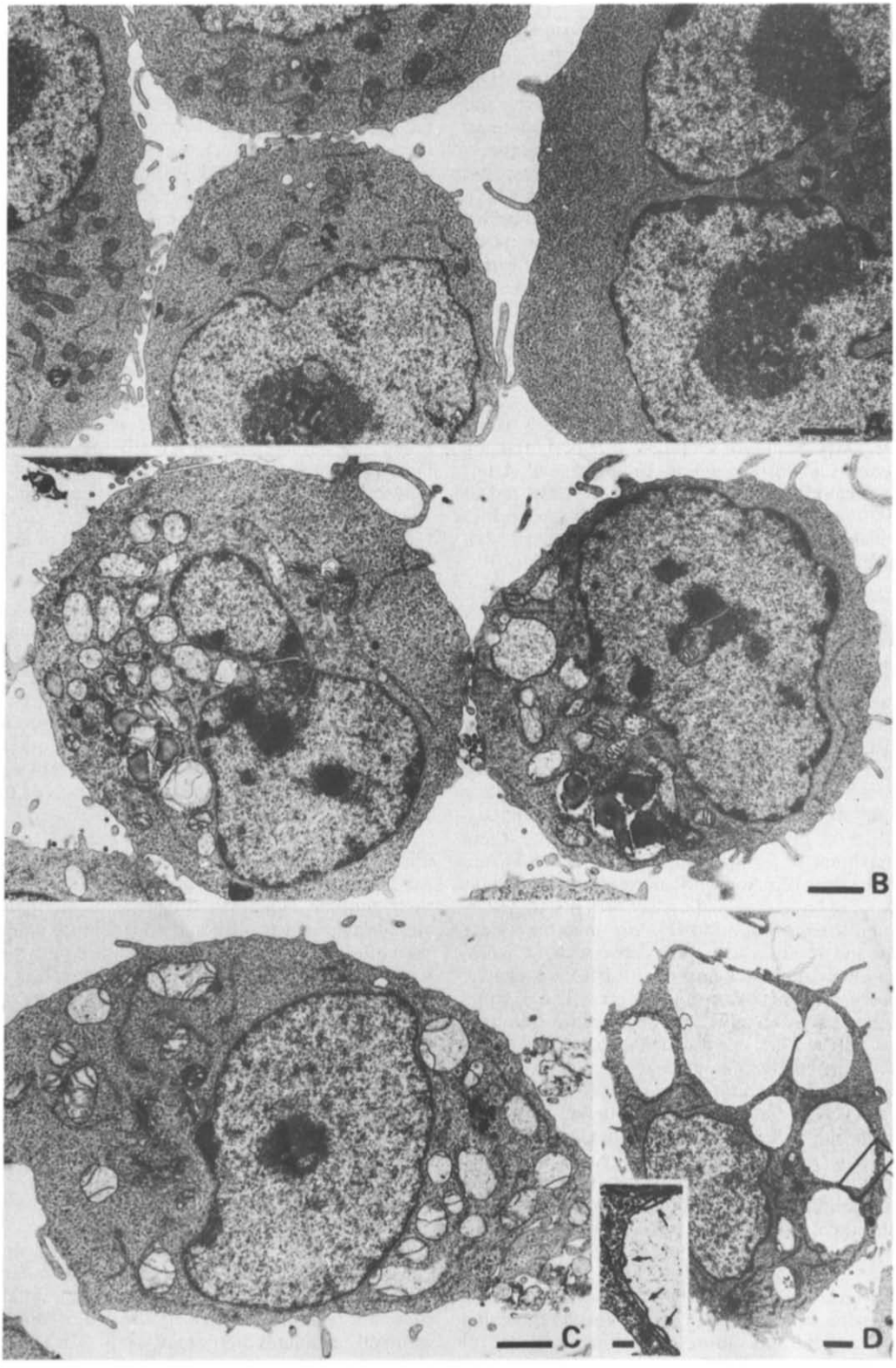


Fig. 2. Electron microscopy of Ditercalinium-treated cells. Electron microscopy of L1210 cell line: control cells (A); Ditercalinium treated cells (B–D). Cells grown 24 hr in the presence of 0.2 $\mu$M (B) and observed after an additional 24 hr culturing period in the presence of drug-free medium (C, D). In Ditercalinium-treated cells, the mitochondria are swollen. Cristae are well visible on the clear matrix but are segregated at the periphery of the organelles. Cells cultured in the presence of the drug, then with drug-free medium show even more damaged mitochondria. Scale bars equal 1 $\mu$M (A–D) and 0.5 $\mu$M for the insert of (D).

5 and 6 as determined by NMR [7]. Therefore, since Ditercalinium has a charge between +2 and +4, the concentration of Ditercalinium in mitochondria would be expected to be dependent on the membrane potential. To study this effect, we first examined the uptake of Ditercalinium in the presence and absence of the respiratory uncoupler CCCP which is known to dissipate the mitochondrial membrane potential [21]. As illustrated in Fig. 3A, a pretreatment of the cells with 10 $\mu$M CCCP for 40 min is sufficient to reduce the cellular accumulation of Rhodamine 123 by a factor of about 25. A similar result is observed when the carbocyanine $DiOC_2(5)$ (100 nM) is substituted for Rho 123 (data not shown). Therefore, such a CCCP treatment significantly reduces the mitochondrial membrane potential. The cellular ATP content is not altered by the treatment with CCCP under such conditions (4 ng ATP/2000 cells), and the main expected effect of CCCP is the diminution of the membrane potential. The uptake of Ditercalinium, evaluated by measurement of the fluorescence associated with cells by flow cytometry, is reduced by 25% in the presence of CCCP (Fig. 3B).

After this CCCP treatment which abolishes the mitochondrial membrane potential, Ditercalinium toxicity was determined by measuring the cloning efficiency. Figure 3C shows the cloning efficiency of cells following treatment with Ditercalinium alone or Ditercalinium + CCCP. The Ditercalinium concentration at which the cloning efficiency was reduced to 37% of the control cells was much higher for cells pre-incubated with both Ditercalinium and CCCP (2.25 $\mu$M) than for cells which were pre-incubated only with Ditercalinium (0.7 $\mu$M).

*Ditercalinium does not alter the membrane potential of mitochondria*

To study the effect of Ditercalinium on the membrane potential, two well-characterized dyes were selected, Rho 123 and $DiOC_2(5)$. In order to estimate the non-specific binding of the dye within cells, measurements were also performed in the presence of CCCP, which abolishes the membrane potential. Difference of dye uptake between native cells and CCCP treated cells, therefore is directly related to the membrane potential. Previously, cellular uptake of the two dyes was shown to establish an equilibrium after 3–4 hr for Rho 123 (1 $\mu$g/ml) and 10 min for $DiOC_2(5)$ (100 nM). On Ditercalinium-treated cells, however, equilibrium is established faster with Rho 123 (2–3 hr) than on control cells. Figure 4 shows the results of the uptake at equilibrium of $DiOC_2(5)$ and Rhodamine 123 by cells as a function of Ditercalinium concentration after a 4-hr treatment. $DiOC_2(5)$ uptake is not altered by Ditercalinium, whereas Rho 123 uptake is diminished in a concentration dependent manner. Since the Rho 123 concentration at equilibrium diminishes as a function of Ditercalinium concentration, these drugs may compete for identical targets in the cells. Therefore we selected $DiOC_2(5)$ for membrane potential studies since, for this compound, no interference with Ditercalinium was observed.

L1210 cells were treated for 24 hr with 1 $\mu$M Ditercalinium and resuspended in drug-free medium. The uptake of $DiOC_2(5)$ by cells was measured in the absence and presence of CCCP as a function of time with Ditercalinium. The relative uptake of $DiOC_2(5)$ determined as the ratio:

$$\frac{[DiOC_2(5)]_D - [DiOC_2(5)]_{D+CCCP}}{[DiOC_2(5)]_C - [DiOC_2(5)]_{C+CCCP}}$$

where $[DiOC_2(5)]_C$ and $[DiOC_2(5)]_{C+CCCP}$ are the $DiOC_2(5)$ amounts in control cells in absence and presence of CCCP respectively, and $[DiOC_2(5)]_D$ and $[DiOC_2(5)]_{D+CCCP}$ are the $DiOC_2(5)$ amounts in Ditercalinium-treated cells in the absence and presence of CCCP respectively. The relative $DiOC_2(5)$ uptake was measured early after the beginning of contact with Ditercalinium (30, 70, 140, 210 min) and later (24 hr and 24 hr after drug removal). To prevent interferences with dead cells, measurements were not performed more than 24 hr after drug removal. The value of the relative $DiOC_2(5)$ uptake was found constant, $1.2 \pm 0.2$ along time. To correlate the relative $DiOC_2(5)$ uptake with mitochondrial membrane potential, it should be necessary to correct for the mitochondrial volume increase because there was an enlargement of the mitochondria in treated cells. Nevertheless, these results show that there was no immediate and clear reduction of the mitochondrial membrane potential while obvious cytological modifications of mitochondria were observed.

*Cellular ATP levels and oxygen consumption are reduced after Ditercalinium treatment*

L1210 cells were treated with 0.2 $\mu$M Ditercalinium during 24 hr, then the cellular growth, the viability, the cellular ATP levels and oxygen consumption were measured daily during the time which separates the 24-hr treatment from the cellular death. The viability, as determined by Trypan blue exclusion by cells, and the cellular ATP levels show in Fig. 5A that the doubling time increases but the viability is not altered during the 120 hr following treatment. After 120 hr, however, cellular growth is arrested and the viability decreases. At the end of the 24-hr treatment, cells display a 40% increase in cellular ATP levels as compared to controls. In contrast to the viability, the cellular ATP levels significantly decreased 50 hr after drug removal. Between 50 and 120 hr, the ATP levels progressively decreased to 20% of the control cells. This low intracellular ATP level is concomitant with cell death. These results suggest that in treated cells a diminution of ATP content precedes the appearance of dead cells and is correlated with the increased doubling time.

The effects of Ditercalinium on oxygen consumption in Fig. 5B shows that at the end of the 24-hr treatment, the rate of oxygen consumption is significantly decreased (70% of that of control cells). After the removal of the drug, there was no recovery of the normal rate of oxygen consumption and between 50 and 100 hr after drug removal, a continuous decrease of oxygen consumption was observed with time.

*Ditercalinium treatment reduces the intracellular levels of pyrimidine 5'-ribonucleotides*

Since we have reported above a decrease of ATP

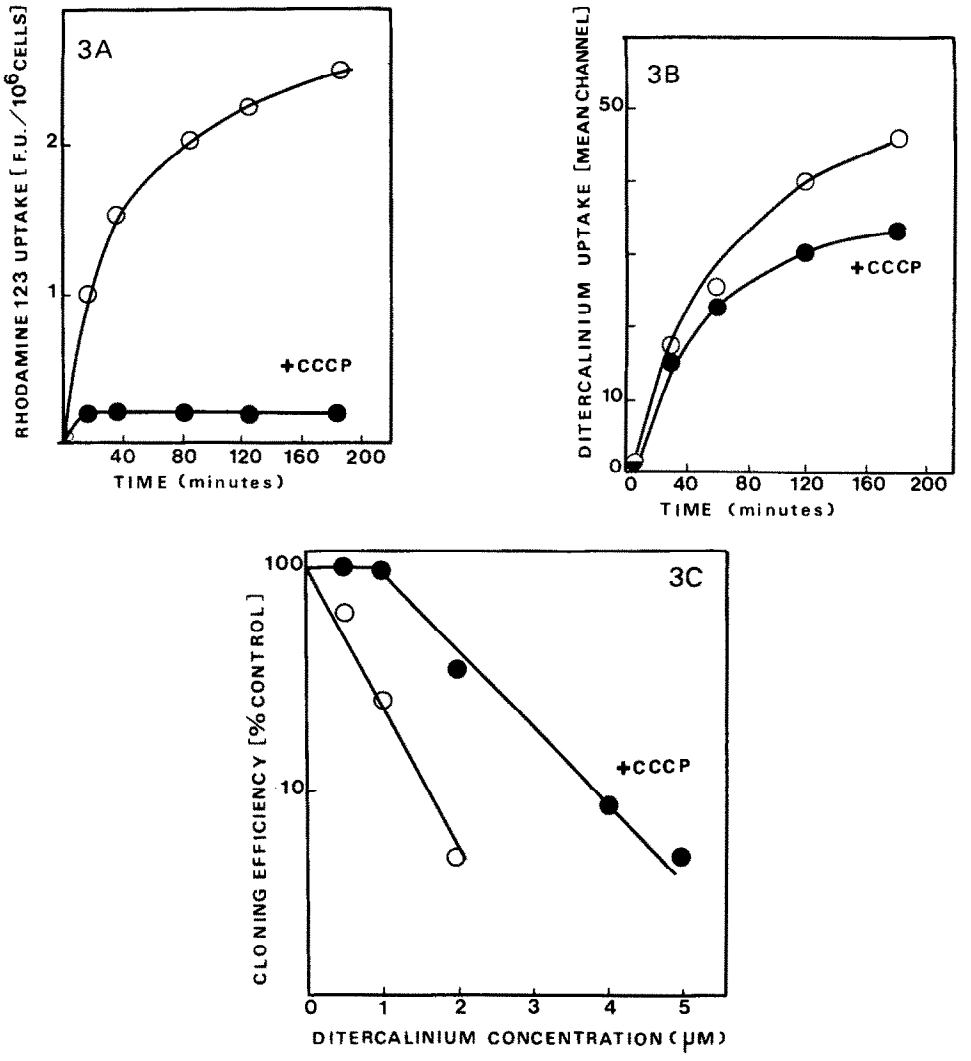


Fig. 3. Effect of a respiratory uncoupler on Ditercalinium uptake and toxicity. (A): Control of uncoupling of mitochondrial membrane potential by Rhodamine 123 uptake. Exponentially growing L1210 cells were pretreated with 10 μM CCCP in culture medium during 40 min, and then stained with Rho 123 (final concentration 1 μg/ml) in the presence of the uncoupler. At various times, Rho 123 was extracted with *n*-butanol and quantitated. Control cells (○), CCCP treated cells (●). (B): Effect of uncoupling on Ditercalinium uptake. Exponentially L1210 cells ($10^5$ cells/ml) were pretreated with 10 μM CCCP in culture medium, and then incubated with 1 μM Ditercalinium. At the indicated times, Ditercalinium content of the cells was evaluated by the measurement of the fluorescence of 5000 cells in a flow cytometer. Control cells (○), CCCP treated cells (●). (C): Effect of pretreatment with CCCP on Ditercalinium toxicity. Exponentially growing cells were pretreated with CCCP 10 μM in culture medium during 40 min, and then incubated for 4 hr with different concentrations of Ditercalinium in the presence of CCCP (●). Control cells were only treated with the same concentrations of Ditercalinium (○). After drug treatment, the cloning efficiency of these cells was tested in Ditercalinium-free soft Noble agar.

level in Ditercalinium-treated cells, we extended this study to the ribonucleotide content of these cells. We therefore analysed by HPLC the ribonucleotide pools in DC3F cells after treatment by Ditercalinium. The DC3F fibroblasts grow in monolayer which facilitates nucleotide extraction, and in addition the sensitivity of this cell line is Ditercalinium has previously been described [11]. The HPLC elution profiles of 260 nm absorbing material in Fig. 6 show that the pyrimidine ribonucleotide pool is depleted to a greater extent than the purine ribonucleotide pool two days after Ditercalinium treatment. The XTP content expressed in per cent of the control is shown in Table 2. The diminution of ATP in DC3F cells was similar to the decrease of ATP content in L1210 cells (Fig. 5A). In contrast, the UTP and CTP levels diminish earlier to 11 and 13%, respectively, 50 hr after Ditercalinium removal, whereas ATP and GTP represent 44 and 35%, respectively. Later, the fall in UTP and CTP is more pronounced after 71 hr (3 and 5%, respectively).

*Reversal of Ditercalinium cytotoxicity*

Morais and co-workers [22, 23] demonstrated previously that cultured chick embryo cells rendered

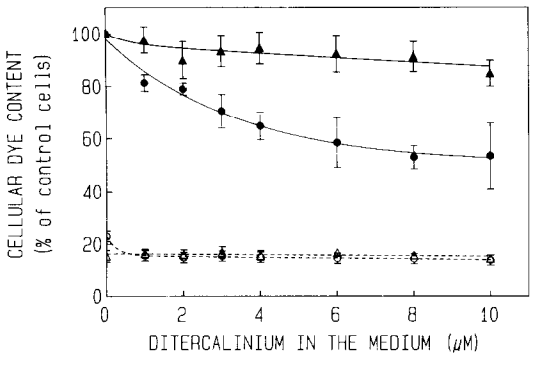


Fig. 4. Rho 123 and $DiOC_2(5)$ accumulation by L1210 cells treated with various Ditercalinium concentrations during 4 hr. After Ditercalinium treatment, the cells were resuspended in Ditercalinium-free medium containing either Rho 123 (1 μg/ml for 2 hr), or $DiOC_2(5)$ (100 nM for 30 min). CCCP (10 μM) was added simultaneously with the other dyes when indicated. The dye content was measured by butanol extraction, and expressed as per cent of control cells. Each curve is the average of three independent experiments. Bar represents SE; $DiOC_2(5)$ (▲); $DiOC_2(5)$ + CCCP (△); Rho 123 (●); Rho 123 + CCCP (○).

efficiency of DC3F cells treated with 1 μM Ditercalinium is observed at a uridine concentration of 20 μg/ml (data not shown). Figure 7 shows the cell survival as a function of Ditercalinium concentration in the presence of 20 μg/ml of uridine. These results indicate that uridine decreases Ditercalinium toxicity by about 10-fold ($CE_{50}$ = 0.42 μM compared to 4.4 μM). The same result is obtained in the presence of 20 μg/ml of cytidine. On the other hand, complementation with hypoxanthine, an exogenous precursor of purine nucleotide which does not interfere with the biosynthetic pathway of pyrimidine nucleotide has no effect on the toxicity of Ditercalinium. Reversal studies have also been performed on L1210 cells. The rescue by uridine and cytidine is better demonstrated when the cellular growth is measured in liquid medium during five to six generations than when the cells are seeded in agar (data not shown). This difference could be explained as an additional effect of agar whose exact composition is unknown.

*Ditercalinium cytotoxicity is increased by 2-deoxyglucose*

The above results show that mitochondrial $O_2$ consumption is significantly reduced 80 hr after a 24-hr treatment with Ditercalinium, suggesting an almost complete inactivation of mitochondrial function. Since previous results showed that Ditercalinium stimulates glycolysis [13], we studied the

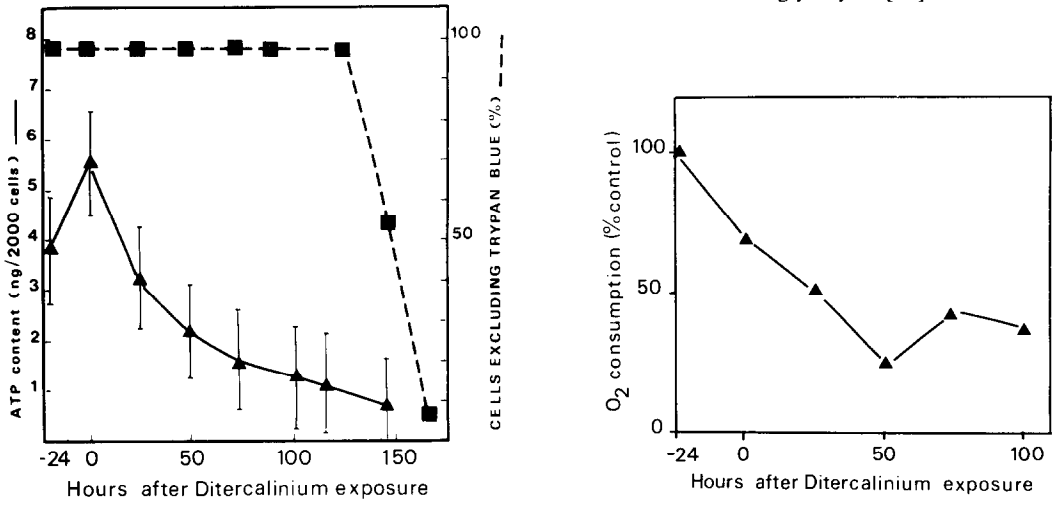


Fig. 5. Effect of Ditercalinium on ATP content (A) and oxygen consumption (B). L1210 cells ($10^5$ cells/ml) were treated with 0.2 μM Ditercalinium during 24 hr and grown in drug-free medium. (A): Intracellular ATP of L1210 cells (▲) was determined by bioluminescence. Each point was averaged from four separate experiments; bar represents SE. Cellular viability was measured by Trypan blue exclusion (■). (B): Oxygen consumption rate of treated cells was expressed as percentage of control. Results were the average of two independent experiments.

respiration-deficient by chloramphenicol and ethidium bromide were auxotrophic for pyrimidines. We examined the effect of various concentrations of uridine (1–40 μg/ml) on the plating efficiency of control and Ditercalinium-treated DC3F cells. Uridine alone has no consequence on the growth of control cells. The maximum enhancement of the cloning

toxicity of Ditercalinium in the presence of an inhibitor of glucose phosphorylation, 2-deoxyglucose. Ditercalinium and 2-deoxyglucose were not applied simultaneously to the cells but sequentially in order to prevent side effects of 2-deoxyglucose on the uptake of Ditercalinium by the cells. Cells were first incubated for 4 hr with Ditercalinium concentrations

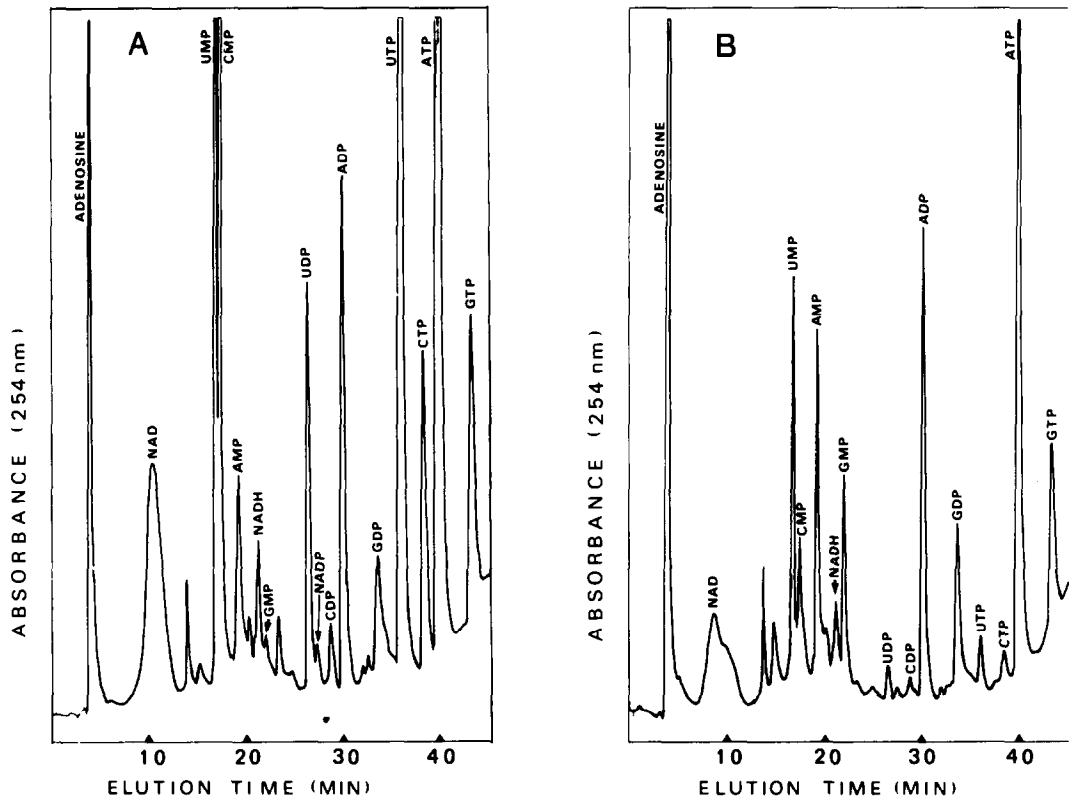


Fig. 6. HPLC separation of nucleotides obtained with control and Ditercalinium-treated cells. DC3F cells were incubated with 1.6 μM Ditercalinium for 4.5 hr and plated in drug-free MEM. Nucleotides were extracted at days 1, 2 and 3 after the treatment, and quantitated by HPLC. (A): control cells. (B): Ditercalinium-treated cells grown during 71 hr after drug removal.

Table 2. XTP content of Ditercalinium-treated DC3F cells

| Time (hr) | ATP (%) | GTP (%) | CTP (%) | UTP (%) |
|---|---|---|---|---|
| 0 | 100 | 100 | 100 | 100 |
| 27 | 103 | 92 | 87 | 99 |
| 50 | 44 | 35 | 13 | 11 |
| 71 | 35 | 62 | 5 | 3 |

DC3F cells were treated for 4.5 hr with 1.6 μM Ditercalinium and plated in drug-free MEM. Nucleotides were extracted at days 1, 2 and 3 after treatment, and quantitated by HPLC. For control cells, the 5′-triphosphate nucleotide concentrations in pmol/10 million cells were: ATP, 15,800; GTP, 5000; UTP, 17,600; CTP, 7200. XTP content of treated cells at each time was expressed in per cent of the control. Experiments were done in duplicate.

ranging from 0.1 μM to 2 μM, and then seeded in agar containing 0.1 mM or 0.3 mM deoxyglucose. As shown in Fig. 8, the toxicity of the drug was enhanced by a factor of 6.5 in the presence of 0.3 mM deoxyglucose ($CE_{37}$ of Ditercalinium is 0.52 μM compared to 0.08 μM in the presence of deoxyglucose). When Ditercalinium-treated cells were grown in the presence of 0.3 mM deoxyglucose, the typical abortive colonies caused by Ditercalinium were not observed. These results indicate that Ditercalinium cytotoxicity is moderately increased by an inhibitor of glycolysis and suggest that inhibition of ATP synthesis is not the only consequence of mitochondrial DNA loss.

*Antileukemic activity* in vivo *of Ditercalinium in presence of 2-deoxyglucose*

As an increase of the antitumor activity *in vivo* of Rho 123 in presence of 2-deoxyglucose has been demonstrated [25, 26], the effect of deoxyglucose was tested on the antitumoral activity of Ditercalinium in mice. DBA/2 mice were grafted with L1210 cells ($10^5$ cells i.p. on day 0) and treated with 10 mg/kg of Ditercalinium the next day (i.p. day 1). A daily injection of deoxyglucose (500 mg/kg i.p.) was made during 10 days (day 1 to 11). As shown in Table 3. high doses of 2-deoxyglucose are not toxic on leukemic mice. Ditercalinium alone significantly prolong survival of leukemic mice (T/C = 158%), but there is no effect of deoxyglucose on the survival time of Ditercalinium-treated mice (T/C = 148%).

## DISCUSSION

The cytotoxicity of Ditercalinium on cultured cells is unique in that it induces a delayed cell death five to six generations after drug treatment [10, 11]. Previous studies suggested that mitochondria might represent the main target of Ditercalinium. Cell variants deficient in mitochondrial respiration are much

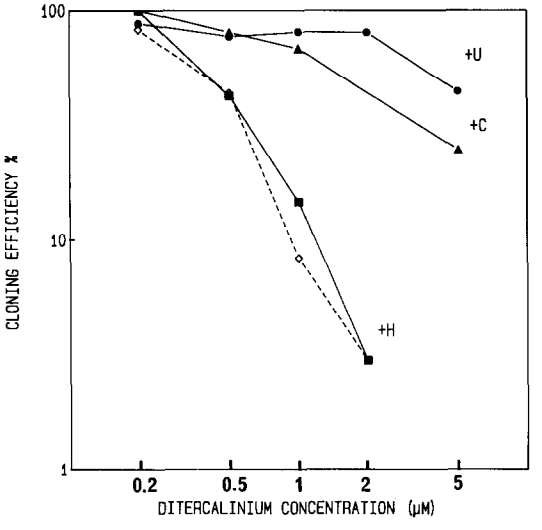


Fig. 7. Effect of nucleosides on the plating efficiency of cells treated for 3 hr with Ditercalinium. DC3F cells ($10^5$ cells) were incubated with various concentrations of Ditercalinium either alone (◇), with 20 μg/ml uridine (●), with 20 μg/ml cytidine (▲) or with 14 μg/ml hypoxanthine (■). After 3 hr, cells were collected and plated in medium without or with the respective nucleoside at the indicated concentration. The cloning efficiency of Ditercalinium-treated cells was determined and the results are presented as percentage of control. The cloning efficiency of control cultures was about 70%. Experiments were carried in triplicate.

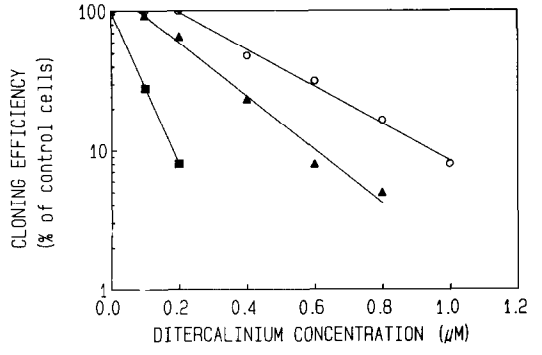


Fig. 8. Effect of 2-deoxyglucose on Ditercalinium cytotoxicity. L1210 cells ($10^5$/ml) were incubated for 4 hr with various concentrations of Ditercalinium without deoxyglucose. Cells were then seeded in Ditercalinium-free soft Noble agar but containing 0.1 mM (▲), 0.3 mM (■) or without (○) deoxyglucose. Absolute colony-forming abilities were respectively 50% for control cells, 50% and 45% in presence of 0.1 mM and 0.3 mM deoxyglucose. The results are percentage of cloning efficiency in the absence of Ditercalinium. Abortive colonies resulting from the treatment with Ditercalinium disappeared in deoxyglucose-containing agar. These results are the average of three independent experiments.

more resistant to Ditercalinium than the parental cell line. In addition, Ditercalinium causes the selective degradation of mitochondrial DNA [13]. To determine whether mitochondrial DNA degradation was

Table 3. The antitumor activity of Ditercalinium is not potentiated by 2-deoxyglucose

| Treatment | Median survival time (days) | T/C (%) |
|---|---|---|
| Control | 10.1 (20) | 100 |
| Ditercalinium (10 mg/kg, day 1) | 16 (10) | 158 |
| 2-Deoxyglucose (0.5 g/kg, days 1–11) | 10.3 (10) | 100 |
| Ditercalinium (10 mg/kg, day 1) plus 2-deoxyglucose (0.5 g/kg, days 1–11) | 15 (10) | 148 |

Survival of mice bearing L1210 leukemia treated with Ditercalinium, 2-deoxyglucose or both. L1210 cells ($10^5$ cells) were injected i.p. in DBA/2 mice. Drug treatments were started 24 hr later and injections were made i.p. The number of mice used in each group is indicated in parentheses.

the main source of mitochondrial injury and cytotoxicity, or whether Ditercalinium acts directly on the respiratory chain or other cellular components, we studied mitochondrial and cellular function during the time which separates the treatment from cell death. The results presented in this paper indicate that mitochondrial and cellular functions are only altered after the loss of mitochondrial DNA and, therefore, the cytotoxic effects of Ditercalinium are a consequence of its action at the level of mitochondrial DNA.

Cells treated with Ditercalinium show morphological changes at the level of mitochondria, as observed using electron microscopy. Alterations of mitochondrial ultrastructure were previously described for various agents acting on different mitochondrial components such as MGBG [27, 28], chloramphenicol [29, 30], ethidium bromide [30], lonidamine [31], gossypol [32] and novobiocin [33]. For all these drugs, the mitochondrial modifications are reversible. In contrast, the mitochondrial alterations induced by Ditercalinium are irreversible. The physiological activity of mitochondrial double membranes in cells treated with Ditercalinium is not evident, even though these membranes are observed in electron microscopy of cells subjected to prolonged exposure to the drug.

Ditercalinium has two positive charges located on the pyridinium nitrogens, and is therefore expected to be concentrated in mitochondria as a result of the mitochondrial electric membrane potential. Cellular localization of the drug was determined by comparison of its distribution with $DiOC_2(5)$, a well characterized mitochondrial fluorescent probe. By this approach, Dequalinium which is a positively charged lipophilic compound was localized in the mitochondria of epithelial cells [34], where the mitochondrial membrane potential is more negative than in L1210 cells [19, 20]. Similar localization of $DiOC_2(5)$ and Ditercalinium suggests preferential mitochondrial localization of Ditercalinium in L1210 cells. In spite of an increase of Ditercalinium quantum yield on DNA binding, only a slight fluorescence

enhancement is detected in the nucleus of living cells. In contrast, Ditercalinium fluorescence in dead cells, is almost exclusively observed in the nucleus. In a study related to the metabolism and the liver toxicity of Ditercalinium [35], the drug was found mainly localized into the mitochondria of cultured hepatocytes. These intracellular distributions of Ditercalinium in living and dead cells are similar to the distributions observed in DC3F cells treated with ellipticine derivatives [36].

Comparison of the cellular uptake of Ditercalinium before and after CCCP treatment at a dose which causes a complete collapse of membrane potential (Fig. 3B) shows that only 25 to 30% of this uptake is dependent on membrane potential. This fraction may correspond to the mitochondrial compartmentalization of Ditercalinium and indicates that a large fraction of the drug is retained by non-energized membranes. CCCP treatment protects the cells from Ditercalinium toxicity by a factor of three. This protection is most probably limited as a result of a large intracellular pool of Ditercalinium inside the cytosol.

All the measurements done in this work indicate that there is no direct effect of Ditercalinium on the mitochondrial respiratory chain. There is no immediate drop of the ATP pool, $O_2$ consumption and mitochondrial electric membrane potential as observed after treatment with oligomycin, an inhibitor of the mitochondrial F1-F0 ATPase [37]. All the metabolic alterations resulting from the mitochondrial damage are delayed and appear once the drug has been removed. These alterations most likely result from the early degradation of mitochondrial DNA. If the mitochondrial DNA degradation is the only effect of Ditercalinium, one expects to see an irreversible and progressive inactivation of the respiratory chain. The rate of inactivation should correspond to the turnover of the proteins coded by mitochondrial DNA. It was previously observed [13] that the cytochrome *c* oxidase activity of which three subunits are coded by mouse mitochondrial DNA decreases exponentially after Ditercalinium treatment with a half-life of 24 hr. The ATP pool and $O_2$ consumption also decrease at the same rate. Inactivation of mitochondria can be compensated by stimulation of glycolysis to maintain ATP levels. A stimulation of glycolysis was previously observed after Ditercalinium treatment [13]. In some cases, mammalian cells were a defect in aerobic respiration have been obtained, indicating that glycolysis can completely replace respiration for ATP production [38–41]. When the glycolytic pathway is inhibited by 2-deoxyglucose in the presence of Ditercalinium, the toxicity of Ditercalinium is enhanced and the abortive colonies indicative of delayed cytotoxicity are no longer observed. With no energy available, cell death occurs without delay. Therefore, this suggests that the cytotoxicity of Ditercalinium is not caused only by delayed suppression of respiration.

The effect of 2-deoxyglucose on Ditercalinium antitumor activity on L1210 leukemia could not be demonstrated as described with Rhodamine 123 on bladder carcinoma and Ehrlich ascite tumors [25] and on adenocarcinoma cells [26]. *In vitro*, 2-deoxyglucose was continuously in agar during 14 days for cloning experiments. *In vivo*, deoxyglucose cannot probably be maintained at a level high enough to be efficient on the antitumor activity of Ditercalinium.

Chicken embryo fibroblasts treated during a long time with ethidium bromide or chloramphenicol became devoid of mitochondrial DNA and became auxotrophic for uridine [24]. This auxotrophy for pyrimidines appears to result from a deficiency in dihydro-orotate dehydrogenase activity. This enzyme catalyses the conversion of dihydro-orotate to orotate, a key step in the synthesis of UMP [42]. Dihydro-orotate dehydrogenase is a lipoprotein which is located in the inner mitochondrial membrane and requires a functional mitochondrial electron transport chain for activity [43]. Thus, the indirect inactivation of the mitochondrial enzyme dihydro-orotate dehydrogenase which requires a functional mitochondria electron transport chain for activity was suggested to be the critical event for the cytotoxicity of ethidium bromide and chloramphenicol. When we analysed the 5′-ribonucleotide pools of Ditercalinium-treated DC3F cells, we observed a selective fall of the uridine and cytidine 5′-ribonucleotide pools. Furthermore, a significant decrease of Ditercalinium toxicity was observed when cells are incubated with either uridine or cytidine. The absence of reversal of toxicity with hypoxanthine demonstrates that the pyrimidine pathway is selectively inhibited as a consequence of Ditercalinium effect.

The effect of Ditercalinium on pyrimidine pools is observed 50 hr after drug removal and a direct effect of Ditercalinium on dihydro-orotate dehydrogenase can be discarded. We have shown in this study that Ditercalinium has no direct effect on oxygen consumption. For these reasons, Ditercalinium has a mechanism different from orotate analogs [44], dichloroallyl lawsone, lapachol [45], DUP785 [46], 1,1,1-trifluoro-3-thenoylacetone [47] or from inhibitors of the electron transfer chain [48]. Ditercalinium leading to the loss of mitochondrial DNA has an indirect effect on the enzyme as it has been demonstrated for ethidium bromide and chloramphenicol [24]. The major difference between these agents and Ditercalinium is the time required to observe the loss of the mitochondrial DNA. A long-time incubation with ethidium bromide is necessary while a 24-hr contact at a low dose with Ditercalinium is sufficient. The pyrimidine starvation resulting from indirect inhibition of dihydro-orotate dehydrogenase could explain, in part, the antitumor activity of this drug as it has been described for antimetabolites used in cancer chemotherapy such as 5-fluorouracil, azapyrimidine nucleosides [49–51].

Cells derive their energy from either mitochondrial oxidative phosphorylation and/or glycolysis. Many tumor cells possess an abnormal energy metabolism in comparison to most normal mammalian cells exhibiting an increased utilization of glycolysis for ATP production [52, 53]. Johnson *et al.* [18] have observed that many carcinoma cells exhibit a high mitochondrial membrane potential compared to normal cells. This is responsible for an enhanced concentration of cationic dyes in these tumor cells. Such differences could provide a strategy for the design of

antitumor drugs having enhanced therapeutic indexes.

To maintain growth while respiration is crippled, tumor cells could possibly derive their energy needs from glycolysis alone. However, these cells must maintain an energized mitochondrial membrane to support dihydro-orotate dehydrogenase activity and prevent pyrimidine starvation. In the absence of respiration, they could only maintain such an energized membrane through the generation of an ion gradient and therefore a much larger electric potential. For this reason, these characteristics might render tumor cells more sensitive to cationic antimitochondrial agents such as Ditercalinium.

*Acknowledgements*—These studies were supported by CNRS, INSERM, Association pour la Recherche sur le Cancer (ARC, Villejuif, France) and Université P. et M. Curie (Paris VI). The authors are very grateful to Drs M. Lebret, C. Auclair and C. Gosse for fruitful discussions. We wish to thank G. Bretou from Service d'Experimentation Animale, Institut Gustave Roussy and C. Buffenoir in our laboratory for their technical assistance. We thank Dr T. O'Connor for his critical reading of the manuscript.

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