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Effect of Dequalinium on K1735-M2 Melanoma Cell Growth, Directional Migration and Invasion *in vitro*

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Cationic lipophilic compounds have an antiproliferative effect on certain tumour systems *in vitro* and *in vivo*. We have investigated whether the cationic lipophilic compound dequalinium affects not only proliferation but also motility and invasion of the highly metastatic and highly invasive melanoma cell line K1735-M2. Proliferation was assessed in monolayer cultures and in multicellular spheroids, motility was estimated in the assay of directional migration, and invasiveness was tested through confrontation cultures of tumour multicellular spheroids with embryonic chick heart tissue evaluated by computerized image analysis. 2 μmol/l dequalinium impaired melanoma cell proliferation, reduced directional migration and significantly blocked invasion *in vitro*. On the ultrastructural level, dequalinium caused obvious changes in mitochondria of both melanoma and embryonic chick heart cells. The mechanisms of the antiproliferative, antimigrating and antiinvasive effects remain to be determined. Inhibition of protein kinase C, calmodulin antagonism, DNA intercalation and/or direct effects on mitochondrial functions may be considered.
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INTRODUCTION

TUMOUR CELL PROLIFERATION, proteolytic tissue degradation and the active movement of cancer cells into and through host tissue barriers are considered as crucial tumour cell properties during the metastatic cascade [1–3]. Various attempts have been made in the past to develop antimetastatic strategies, which affect one or more of these cellular features [for a review see 4]. Dequalinium, with two positive charges, belongs to this group of agents. It selectively accumulates in mitochondria of carcinoma cells and blocks energy production [5]. Besides an inhibitory effect on mitochondrial functions, dequalinium acts as a powerful DNA intercalator [6], as an antagonist of calmodulin [7] and as a protein kinase C inhibitor [8]. Dequalinium prolonged survival of mice with intraperitoneally implanted mouse bladder carcinoma MB49 and inhibited the growth of subcutaneously implanted human colon carcinoma CX-1 in nude mice and recurrent rat colon carcinoma W163 in rats [5]. Furthermore, it impaired growth and led to regression of chemically-induced mammary tumours [9] and blocked proliferation of the C_6 astrocytoma cell line *in vitro* [10]. These studies, however, have largely been focused on the antiproliferative effect of the compound. Using the highly invasive, highly metastatic murine melanoma cell line K1735-M2 we investigate whether dequalinium is able not only to exert an antiproliferative, but also an antimigrating and antiinvasive effect *in vitro*.

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MATERIALS AND METHODS

Cells and culture conditions

A highly metastatic murine melanoma cell clone, K1735-M2, was derived from pulmonary metastases produced by the K1735 parental melanoma cell line growing at a subcutaneous site [11]. The cells were kindly provided by Dr I.J. Fidler, (M. D. Anderson Cancer Center, Houston, Texas). The cells were maintained as monolayers in Dulbecco's modified Eagle's Medium (Flow) supplemented with 10% fetal calf serum (Sera-Lab) and antibiotics (Flow), hereafter called complete culture medium.

Growth rates

Melanoma cells were seeded at a density of 1×10^5 cells/ml in 5 ml complete culture medium in 25 ml tissue culture flasks (Costar). Cultures were trypsinised every 24 h over a period of 6 days and the cell number per flask was calculated with a Bürker haemocytometer (Assistent, Sondheim/Rhön, Germany).

Melanoma multicellular spheroids

Tumour cells were trypsinised from subconfluent monolayers and adjusted to approximately 1×10^6 cells/ml in complete culture medium. For reaggregation the cell suspension was seeded into Petri dishes with a nonadherent surface (Falcon) and incubated at 37°C in a humidified CO_2 incubator for 24 h. Thereafter the irregularly-shaped cell clusters were transferred to 100-ml spinner flasks (Bellco) and cultured for about 5 days on magnetic stirrers (type EOA-W, IKA-Werk, Staufen, Germany) at approximately 120 rpm regulated by a control unit (type ES5, IKA-Werk). Multicellular spheroids with a diameter of 200 μm were selected under a stereomicroscope (Wild) equipped with an ocular grid.

Determination of directional migration

Individual spheroidal aggregates of melanoma cells, 200 μm in diameter, were explanted in each well of 24-well plates (Costar) and cultivated with or without 2 μmol/l dequalinium up to 120 h. One set of experiments was performed with melanoma multicellular spheroids, which had been incubated with 2 μmol/l dequalinium for 72 h prior to the assay of directional migration. In order to examine the reversibility of the dequalinium-effect the compound was removed from the culture medium after 120 h and measurement was continued for another 120 h. Photographs were taken every 24 h under an inverted microscope (Zeiss) at an objective magnification factor of 2.5. On black and white prints, the area occupied by the cells radially migrating from the multicellular spheroid was measured interactively by tracing on the digitiser board of the Videoplan image analysis system (Kontron). For each time and each experiment at least 10 spheroids were measured and the average increase of the mean radius was calculated as μm/day by linear regression analysis.

Precultured chick heart fragments

Heart ventricles from 9-day-old chick embryos were aseptically dissected into small fragments with microscissors and maintained in spinner culture for 4–6 days. The resulting rounded fragments were selected for 400 μm in diameter and used as a stroma analogue in the *in vitro* invasion assay.

In vitro *invasion assay*

The method described by Mareel *et al.* [12] was slightly modified. Individual melanoma multicellular spheroids were placed in close contact with rounded fragments of embryonic chick heart tissue on top of a semi-solid agar medium. After an incubation period of 60–90 min in a humidified CO_2 incubator at 37°C the confrontation pairs were transferred to each well of 24-well plates (Costar), the bottom of which had been covered with semi-solid agar medium. The confrontation cultures were cultivated in complete culture medium or in complete culture medium plus 2 μmol/l dequalinium for 48 or 120 h. One series of experiments was performed with melanoma multicellular spheroids, which had been incubated with 2 μmol/l dequalinium for 72 h in spinner culture prior to the invasion assay and treatment was continued during confrontation culture.

For light microscopical observation confrontation cultures were fixed in Bouin–Hollande's solution, embedded in tissue-freezing medium (Reichert-Jung, Vienna, Austria) and snap frozen in liquid nitrogen.

Immunohistochemistry

3 μm cryostat sections from confrontation cultures were mounted with a polyclonal rabbit anti chick heart antiserum (kindly provided by Dr M.M. Mareel and Dr G. De Bruyne, University Hospital, Gent, Belgium) diluted 1:50 in Tris–buffer pH 7,4/ normal swine serum 1:1 for 1 h. After careful rinsing in phosphate-buffered saline (PBS), a peroxidase-conjugated swine anti rabbit antiserum (Dako) was applied for 30 min. Staining was achieved by aminoethyl-carbazole and hydrogen peroxide.

Image analysis of melanoma cell invasion in vitro

Each confrontation culture was serially sectioned and the median section showing the tumour as well as the stromal component was selected for measurement as described previously [13].

In brief, the immunohistochemically-stained sections were viewed through an Axiomat bright field microscope (Zeiss) with a 10× objective, projected into a black and white Vidicon camera (Bosch), and fed into an IBAS image analysis system (Zeiss-Kontron). After various steps of image enhancement a binary image was discriminated of the total section and of the stromal component. After further binary image processing, the following parameters were derived by mathematical morphology:
STRAREA, area occupied by the stromal component, given in μm^2, TUMAREA, area occupied by the tumour component, given in μm^2, and INVASLOG = $-\log$ $((4 \times \pi \times \text{STRAREA})/\text{stromal contour})$, yielding values close to 0, when no invasion has taken place, and increases almost linearly, when invasion is taking place during the experiment. For each of the three parameters, the change over time was calculated by linear regression analysis.

Electron microscopy

Untreated (control) and incubated (2 μmol/l dequalinium for 48 and 120 h) K1735-M2 melanoma multicellular spheroids and chick heart fragments were fixed with 3% glutardialdehyde (Merck) in 0.06 mol/l phosphate buffer (pH 7.2) for 75 min at room temperature. Then the cells were rinsed several times in phosphate buffer (pH 7.2), post-fixed in 1% osmium tetroxide (Merck) for 2 h and again rinsed in phosphate buffer. After dehydration in ethanol, samples were embedded in Polarbed 812. Ultrathin sections were cut with a diamond knife on a Reichert ultramicrotome OmU2, stained with lead citrate (5 min) and uranyl acetate (15 min) and viewed with a Philips TEM CM 10.

Drugs

Dequalinium (Sigma) was dissolved in PBS at 2 mg/ml. After heating and exposure to ultrasonic waves, a stock solution of 100 μg/ml was prepared with culture medium. The filter-sterilised solution was further diluted to the final concentrations (1–3 μmol/l) with complete culture medium. 5-fluorouracil (Sigma) was dissolved in PBS, filter-sterilised and diluted to 5 μg/ml with complete culture medium.

RESULTS

Effect of dequalinium on melanoma cell proliferation

Growth of K1735-M2 melanoma cells was slightly inhibited by 1 μmol/l dequalinium, whereas 2 μmol/l of the compound showed a pronounced antiproliferative effect (Fig. 1). Cell viability was unimpaired at 2 μmol/l dequalinium as determined by trypan blue exclusion. Furthermore, after removal of the compound from the culture medium the cells started to divide, though the growth rates remained significantly decreased compared with untreated controls.

Effect of dequalinium on melanoma cell directional migration

Following the application of 2 μmol/l dequalinium K1735-M2 melanoma cell directional migration was reduced to mean (S.D.) 154 (9) μm/day, compared with 377 (22) μm/day in untreated controls ($P \leq 0.001$). Pretreatment of the melanoma multicellular spheroids for 72 h in spinner culture prior to the migration experiment further decreased the migration rates to 123 (6) μm/day ($P \leq 0.05$). Within 120 h after removal of the compound from the culture medium a migration rate of 220 (38) μm/day was determined, which indicates quick recovery of the cells in drug-free medium.

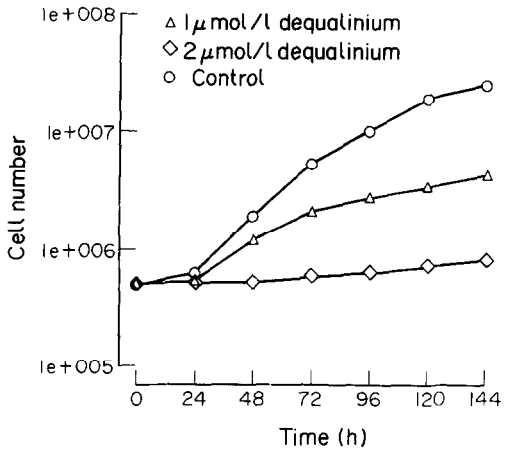

Fig. 1. Growth rates of K1735-M2 melanoma cells. (Ordinate: cell number per flask, le + 005 = 1 × 10^5).

Effect of dequalinium on melanoma cell invasion in vitro

2 μmol/l dequalinium significantly affected K1735-M2 melanoma cells in the *in vitro* invasion assay. In the control experiments the parameter TUMAREA, related to tumour cell proliferation, shows a significant increase over time, whereas the parameter remains almost constant in the dequalinium-experiment (Table 1). This result indicates that the growth inhibitory effect of the compound is also evident in multicellular spheroids. The antiinvasive activity is reflected by measuring parameter INVASLOG. This value increases in untreated controls indicating invasion, but shows nearly no change in the experiments with dequalinium, suggesting an antiinvasive effect.

Pretreatment of the melanoma multicellular spheroids with 2 μmol/l dequalinium for 72 h prior to the invasion experiment and continuous treatment during confrontation culture resulted in an even more pronounced inhibition of both tumour cell proliferation and invasion (Figs 2 and 3, Table 1).

The effect of a pure proliferation inhibitor, 5-fluorouracil, was tested for comparison. 5 μg/ml 5-fluorouracil significantly blocked melanoma cell proliferation, reflected by parameter TUMAREA. Melanoma cell invasion, however, was only moderately impaired (Fig. 3, Table 1).

Ultrastructure

Electron microscopical investigations showed that dequalinium caused obvious changes in mitochondria of both melanoma and embryonic chick heart cells. Compared with mitochondria of untreated controls, which usually had an elongated shape with a dense matrix and well-developed cristae (Figs 4a and 5a), changes were already visible after treatment with 2 μmol/l dequalinium for 48 h.

In melanoma cells swellings of the mitochondria with increased electron-translucent areas in the matrix and a decreased number of cristae were observed. Additionally, electron-opaque globular inclusions were frequently found in the matrix. Prolonged treatment (120 h) resulted in a further reduction of the cristae and matrix material (Fig. 4b). Granular electron-opaque inclusions of 0.1–0.2 μm in diameter were often present in form of globules of varying number in the matrix. Sometimes also irregularly-shaped inclusions occurred.

Mitochondria of embryonic chick heart cells also contained granular electron-opaque inclusions with a diameter up to 0.2 μm in a dense matrix already after an incubation period of 48 h. Furthermore, their cristae were often dilated but not reduced in number. In addition to those alterations also a slight reduction in the number of cristae and a loosened matrix material were sometimes found after treatment with 2 μmol/l dequalinium for 120 h (Fig. 5b).

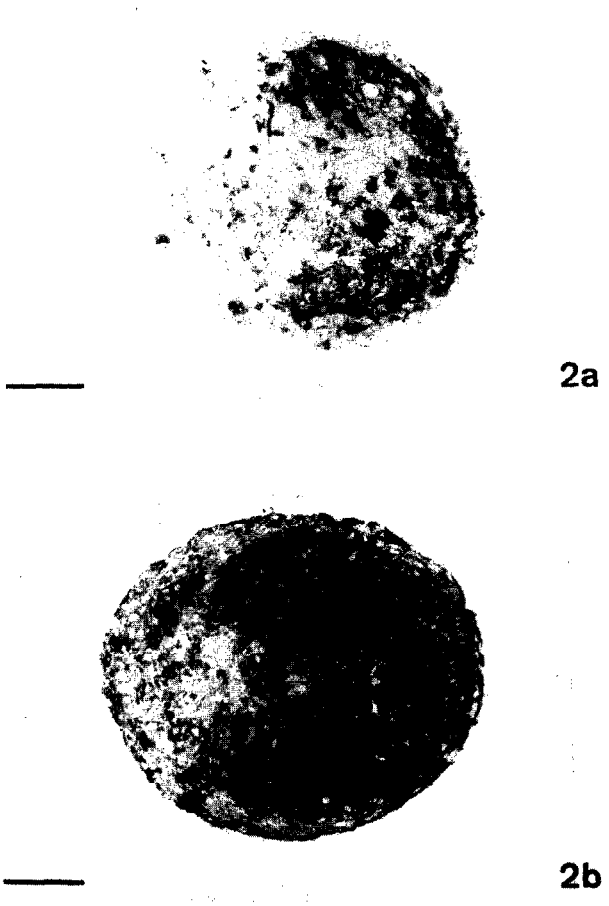

Fig. 2. Light micrographs of 3 μm cryostat sections from confrontations between K1735-M2 melanoma multicellular spheroids and rounded fragments of embryonic chick heart tissue. Cultures were fixed after 120 h and sections were stained with an antiserum against chick heart tissue. (a) untreated control, (b) melanoma cells were pretreated for 72 h with 2 μmol/l dequalinium prior to the invasion experiment and then continuously incubated with the compound during the confrontation culture. Scale bars = 100 μm.

Table 1. *Melanoma cell invasion* in vitro *defined by various measuring parameters, defined in Materials and Methods*

	K1735-M2, untreated control	K1735-M2 + 2 μmol/l dequalinium	K1735-M2 + 2 μmol/l dequalinium preincubated for 72 h	K1735-M2 + 5 μg/ml 5-fluorouracil
STRAREA	−5444.192	−3642.905	−527.324*	−7377.710
	(2703.223)	(4990.886)	(3273.222)	(2580.609)
TUMAREA	20907.365	2943.024*	78.106*	−20.372*
	(4858.049)	(4154.588)	(2161.919)	(1578.492)
INVASLOG	0.474	0.191	0.106*	0.260
	(0.122)	(0.263)	(0.183)	(0.132)

*Significant difference from K1735-M2, untreated control.

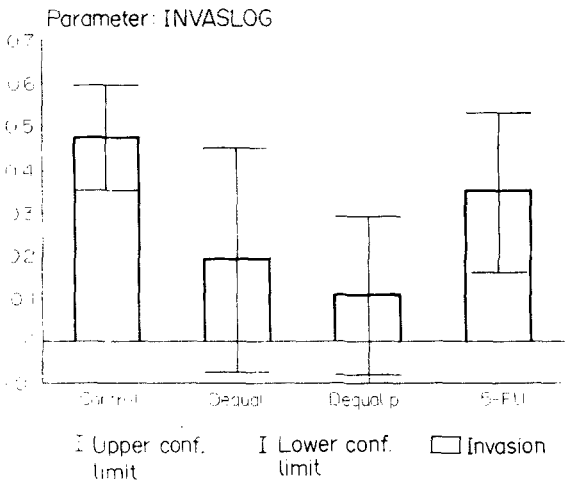

Fig. 3. Antiinvasive effect of dequalinium on melanoma cell invasion *in vitro*. Ordinate: Invasiveness defined by measuring parameter INVASLOG, abscissa: experiments. Control: K1735-M2, untreated control; Dequal.: K1735-M2 + 2 μmol/l dequalinium; Dequal.p.: K1735-M2 pretreated with 2 μmol/l dequalinium prior to the invasion experiment and continuous treatment during the confrontation culture; 5-FU: K1735-M2 + 5 μg/ml 5-fluorouracil. I: upper and lower confidential limit, respectively.

Besides those mitochondrial changes, all other cellular structures, including the cytoskeleton, remained unaffected on the ultrastructural level.

DISCUSSION

The antiproliferative effect of nontoxic concentrations of dequalinium on the K1735-M2 murine melanoma cell line, as demonstrated in our study, is in agreement with previous studies concerning other tumour systems including C_6 astrocytoma *in vitro* [10], subcutaneously implanted tumours in nude mice and rats [5] and chemically-induced mammary tumours [9].

To our knowledge, there are no previous data on lipophilic cations related to tumour cell motility. In the assay of directional migration, 2 μmol/l dequalinium demonstrated a pronounced antimigrating effect on K1735-M2 melanoma cells, indicating, that the antineoplastic action of lipophilic cations may in part be also mediated by impairment of cell motility. One has to admit, however, that an indirect influence of the antiproliferative effect on the assay of directional migration cannot be ruled out [14].

As far as invasion is concerned, we demonstrated a pronounced inhibitory effect of dequalinium in the *in vitro* invasion assay. Furthermore, impairment of invasion was significantly

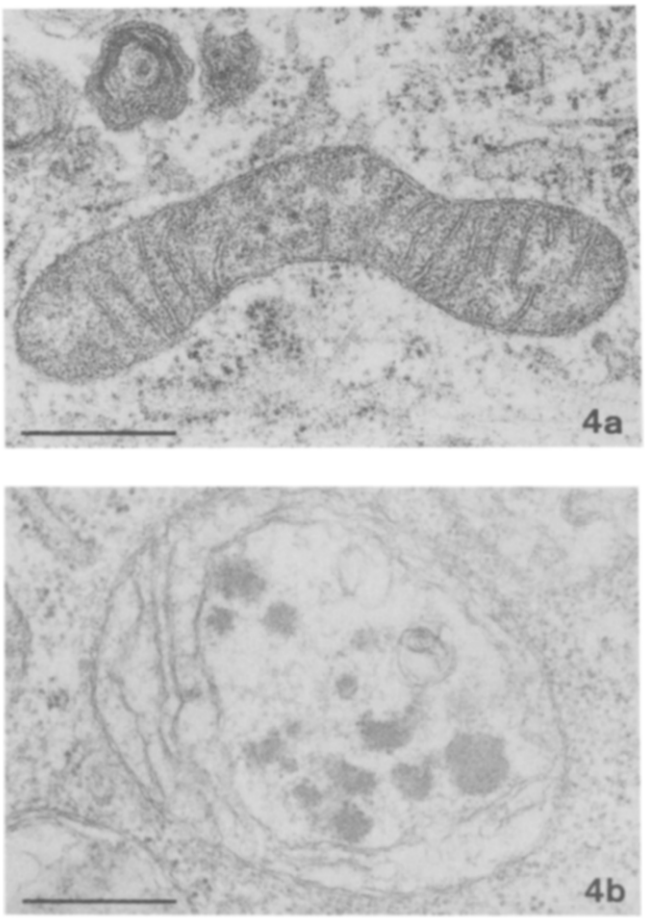

Fig. 4. Transmission electron micrographs of K1735-M2 melanoma cell mitochondria; (a) untreated control, (b) incubated with 2 μmol/l dequalinium for 120 h. Swollen mitochondrion with reduced and destructed cristae, electron-opaque inclusions and an electron-translucent matrix. Scale bars = 0.5 μm.

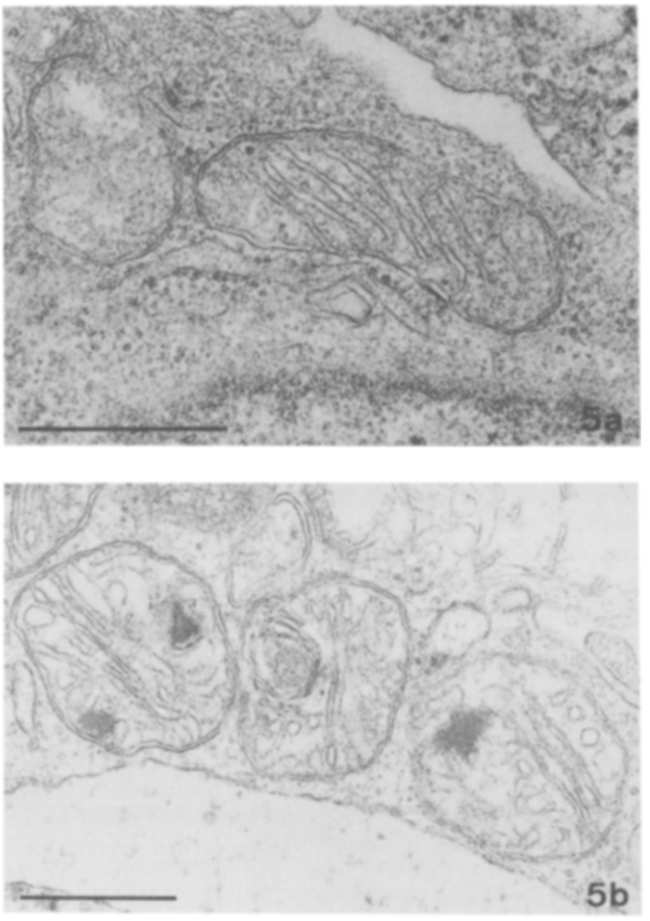

Fig. 5. Transmission electron micrographs of embryonic chick heart cell mitochondria; (a) untreated control, (b) incubated with 2 μmol/l dequalinium for 120 h. Some dilated cristae, loosened matrix material, and electron-opaque inclusions are present. Scale bars = 0.5 μm.

more pronounced in the dequalinium-experiments than in experiments using the pure antiproliferative agent 5-fluorouracil. Thus, mechanisms other than growth inhibition may contribute to the antiinvasive effect of dequalinium. Concerning the potential *in vivo* significance of this finding one has to take into account, that the embryonic chick heart model [12] provides a three-dimensional system with a living stroma and has already been demonstrated to mimic invasion *in vivo* to a great extent [15]. Furthermore, embryonic chick heart tissue closely resembles human tumour stroma with respect to proteoglycan composition and laminin, a glycoprotein exhibiting some implications for invasion has been demonstrated there [16]. Although tumour cells rarely invade heart, particularly melanoma proved to metastasise to the cardiac muscle [17].

The biochemical pathway, by which dequalinium exerts its effects in the K1735-M2 melanoma cells, remains to be determined. One potential target site may be protein kinase C, since dequalinium has been shown to be a potent protein kinase C inhibitor in certain *in vitro* systems [8]. Protein kinase C, in turn, is known to phosphorylate a large number of cytoskeleton-related proteins, e.g. microtubule-associated proteins [18], troponin I and T [19], heavy meromyosin [20], myosin light chain kinase [21] and vinculin [22], and may thus influence cell motility. Furthermore, dequalinium might act as a calmodulin antagonist [7] and/or as a DNA intercalating agent [6]. Our ultrastructural observations underline the potential role of the impairment of mitochondrial functions. In melanoma cells the compound caused a swelling of the mitochondria, exhibiting an electron-translucent matrix and altered cristae. In addition, electron-opaque inclusions occurred frequently. Also the mitochondria of normal host tissue were affected, but to a lesser degree. The electron-opaque inclusions observed in mitochondria of both melanoma and embryonic chick heart cells might represent either the accumulated compound itself or a reaction product of dequalinium with matrix components. Since we observed alterations in both cristae and matrix, impairment of main mitochondrial processes such as electron transport and oxidative phosphorylation can be expected. This assumption is supported by Anderson *et al.* [23], who found that quinolinium compounds interfere with the electron transport chain by inhibition of mitochondrial NADH oxidase, and by Weiss *et al.* [5], who reported impaired respiration and ATP production after dequalinium-induced mitochondrial changes.

Dequalinium has been shown to be 125-fold more toxic to human carcinoma cells than to normal epithelial cells [5]. The selective cytotoxicity of the compound has been suspected to arise from the higher membrane potential across membranes of mitochondria in carcinoma cells [24]. Accordingly, mitochondria of tumour cells are considered to accumulate lipophilic cations like dequalinium easier than mitochondria of normal cells. Nevertheless, a clear selective effect of dequalinium on tumour cells could not be confirmed in our study.

In conclusion, our data demonstrate that dequalinium does not only inhibit K1735-M2 melanoma cell growth, but also directional migration and invasion *in vitro*. Thus, other mechanisms than a pure antiproliferative effect may contribute to the antineoplastic action of dequalinium in certain experimental systems.

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