Histol Histopathol (1995) 10: 79-84

Histology and Histopathology

Effects of various antineoplastic agents on an established human melanoma cell line (G-361)

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Summary. An established human melanoma cell line was treated with several concentrations of three antineoplastic drugs: melphalan (0.016, 0.032, 0.16 µm), CCNU (1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea) (0.04, 0.21, 0.42 µM) and 4-OHA (4-hydroxyanisole) (4.01 x 10^{-4}, 1.20x10^{-3}, 2.4x10^{-3} µM), and the effects on cell growth and viability were compared.

24 hours after treatment, 4-OHA (ID_{50}=2.4X10^{-3} µM) was more cytotoxic than melphalan (ID_{50}=0.016 µM) and CCNU (ID_{50}=0.21 µM). However. after 96 hours exposure, the most effective drug was CCNU (growth rate = -1.277), which caused the death of the culture. This was followed by melphalan (growth rate = -1.024) and finally 4-OHA (growth rate= -0.69).

Similar ultrastructural cell injuries were observed after the use of the three drugs: the dilation of endoplasmic reticulum vesicles and the nuclear membrane; mitochondria swelling; and the existence of lamellar structures and cytoplasmic vacuoles.

Key words: Melphalan, CCNU, 4-Hydroxyanisole, Melanoma cells

Introduction

Melanoma is a neoplasia of low incidence, directly responsible for 67% of deaths by skin cancer. Although surgery is the only curative therapy for melanoma in its initial stages, complementary treatments of chemotherapy, radiotherapy and hyperthermia are frequently employed in order to stop metastatic growth. Since the level of response of metastatic melanoma to different treatments is very low, their use may be of marginal benefits, as documented by a variety of studies (Morgan et al., 1981; Cardenes, 1989; Vicente et al., 1990).

«In vitro» assay systems for screening potential anticancer agents have been widely used since the inception of clinical cancer chemotherapy. Tissue culture of melanocytes provides an obvious tool for investigating and monitoring the effects of these agents; and the relationship between *«in vitro»* drug sensitivity exhibited by cultures of human tumours and their «in vivo» counterparts argues for their continued use in drug evaluation programmes (Wilson, 1987).

Alkylating agents are one of the most effective groups of antineoplastic agents. Their cytotoxic effect seems to be due to their ability to react with cell macromolecules to induce multiple kinds of molecular lesions (Hansson et al., 1987). Melphalan and nitrosoureas, are two of the most widely used alkylating agents both in *«in vitro»* and *«in vivo»*. In addition, recent years have seen a growth in interest in certain depigmenting agents, such as 4-OHA. Its effect is thought to be due to the toxic products that are formed when the phenol is converted by the enzyme tyrosinase into a melanin-like product (Riley, 1969). This melanocytotoxic activity appears to be of potential therapeutic use in the treatment of melanotic melanoma.

In this report, we studied the effect of three antineoplastic drugs: melphalan; CCNU; and 4-OHA on the growth of a human melanoma cell line (G-361), and the ultrastructural changes originated by these agents.

Materials and methods

An established human melanoma cell line was used in this study. The G-361 cells (Flow Laboratories) were routinely propagated as monolayer cultures in McCOY'S 5A supplemented with 15% heat inactivated foetal calf serum, penicilin (100U/ml) and streptomycin (100 µg/ml) and incubated in a humidified atmosphere of 95% air and 5% carbon dioxide at 37 °C.

The drugs for *«in vitro»* study were melphalan, CCNU and 4-OHA.

Cells (250,000) were plated in 3.5 cm diameter tissue culture Petri dishes. After 24 hours the cells were incubated with various concentrations of melphalan (0.016, 0.032 and 0.16 µM), CCNU (0.04, 0.21 and 0.42 µM) and 4-OHA (4.01x 10^{-4}, 1.20x10^{-3} and 2.4x10^{-3}

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μM) for 4 days.

Duplicate cultures were established for each of the concentrations. The medium was replaced with fresh culture medium containing the desired concentrations of each drug at 48-hour intervals.

Cells were harvested separately from dishes every day and counted with a hemacytometer. Cell viability was evaluated by trypan blue dye exclusion.

The results were expresed as the surviving fraction of treated cultures with respect to the control culture. Doubling time was calculated from the curves.

The growth rate of treated cells compared with that of control cells was reckoned by a minimum cuadratic rectified fit of the exponential $y=\exp(a_0+a_1x)$.

The effectiveness of the various treatments was compared by a contrast of equality of the growth rates (a_1 parameter) of cultures treated with ID_{50} at 24 hours for each agent (dose required to reduce survival to 50%).

For the ultrastructural study, cells were harvested by trypsinization and centrifuged (240g, 10 minutes). Pellets obtained by centrifugation, were fixed in glutaraldehyde, contrasted with uranyl acetate and postfixed in osmium tetroxide. After dehydration, the samples were embedded in epon. Semithin sections were stained with toluidine blue for examination by light microscopy, selecting those areas of most interest. The ultrathin sections were then contrasted with uranyl acetate and lead citrate. For the ultrastructural photographic study, we used a Zeis EM 10C electron microscope.

Results

The growth curves of the control cultures showed a 24 hour lag phase, followed by an exponential phase of growth. Confluence was at 120 hours post-seeding. Doubling time was approximately 60 hours and cell viability (viable cell/total cells x 100) was kept between 85 and 90% throughout the experiment.

After exposure to the three agents, it was noted that all of them had a cytotoxic effect within 24 hours which was more marked at higher concentrations. ID_{50} calculated at 24 hours were approximately 0.016 μM for melphalan, 0.21 μM for CCNU and 2.4×10^{-3} μM for 4-OHA. Inhibition of culture growth increased with length of exposure to the agents, as is shown in Table 1.

In CCNU-treated cultures, death of the culture (99.9% inhibition) occurred at almost 72 hours, whereas in those treated with 4-OHA and melphalan, death of the culture had still not been observed by the end of experiments. The inhibiton of the culture growth at 96 hours was 93.2% for 4-OHA and 82.86% for melphalan

Table 1. Growth inhibition of ID_{50} (μM) 24 hour-treated cultures (%control).

DRUG	ID_{50}	EXPOSURE TIME (hours)			
		24	48	72	96
Melphalan	0.016	57.5	67.17	76.31	82.86
CCNU	0.21	55.2	83.3	99.9	100
4-OHA	2.4×10^{-3}	42.2	70.7	91.2	93.2

Table 2. Growth rate and rate of decrease in viability of ID_{50} 24 hour-treated cultures.

DRUG	ID_{50} (μM)	GROWTH RATE	VIABILITY
Melphalan	0.016	-0.400	0.074
CCNU	0.21	-1.277	0.948
4-OHA	2.4×10^{-3}	-0.69	0.356

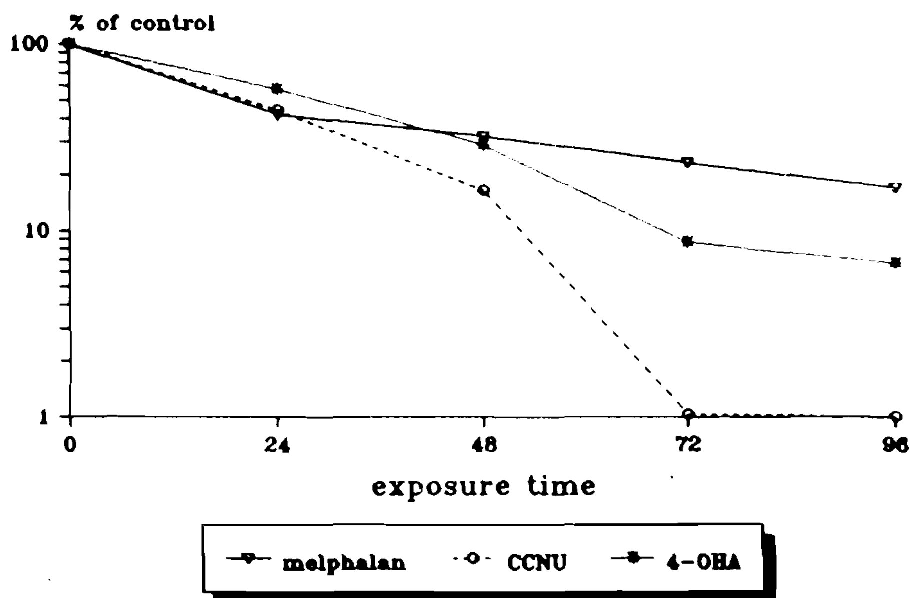

Fig. 1. Survival of ID_{50} (24 hour-treated cultures) (% control). Melphalan: 0.0165 μM; CCNU: 0.21 μM and 4-OHA: 2.4×10^{-3} μM.

(Fig. 1). The growth rates of the surviving fractions of treated cultures with their respective ID_{50} and variations in cell viability calculated by statistical analysis are shown in Table 2.

The inhibitory effect of the three agents was clear, since the growth rates of the cultures were negative in all of them. The cultures which decreased more quickly were those treated with CCNU, which showed significant differences to those treated with melphalan ($p < 0.001$) and 4-OHA ($p < 0.001$).

Cell viability diminished with time for the three agents used, which shows that there was cell death. The cultures treated with CCNU, which showed the greatest decrease in viability, showed significant differences to those treated with 4-OHA ($p < 0.001$) and melphalan ($p < 0.001$). The cultures treated with melphalan showed very little variation of viability. This seems to indicate that the agent inhibits culture growth, although cellular death is

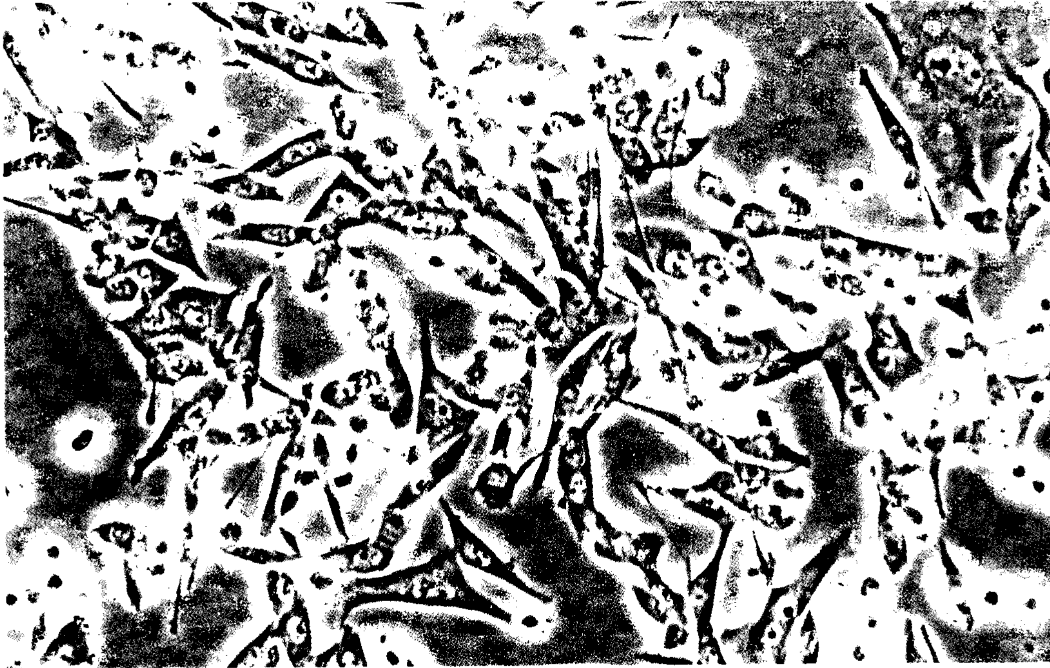

Fig. 2. Control culture at 48 hours. Melanocytes show an elongated, strellate appearance, and round nuclei with one or two nucleoli. x 200

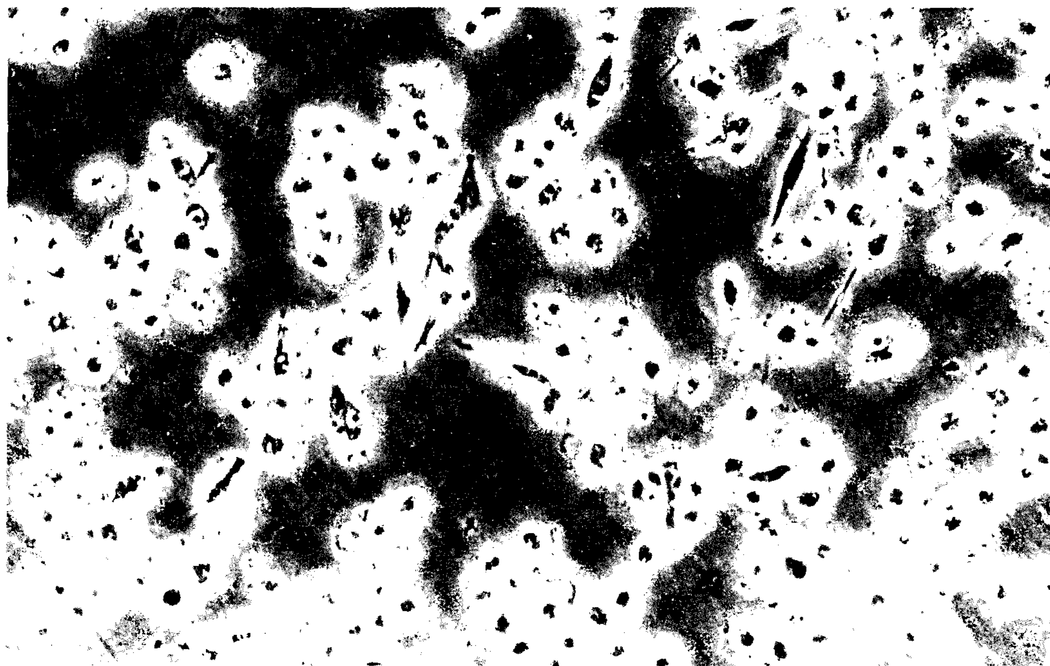

Fig. 3. 2.4×10^{-3} µM 4-OHA culture at 48 hours. Melanocytes exhibit loss of the cytoplasmic processes and membrane bending. x 200

lower than in those with CCNU ($p< 0.001$) and 4-OHA ($p< 0.02$).

Through a light microscope, *«in vitro»* cells had an elongated or stellate appearance, generally with 3 cytoplasmic processes. Most had round nuclei with several (2 or 3) nucleoli, though the presence of cells with 2 and even 3 nuclei was also noticed (Fig. 2). After exposure to the different agents, cells exhibited similar morphological changes with all the applied treatments. When exposure time to the agents increased, disappearance of the cytoplasmic processes and bending of the cell surface were noted. The cells acquired a round morphology, with an increased refringency, indicating the loss of adherence to substrate (Fig. 3).

Ultrastructurally, cells showed the typical organellae of protein-secreting cells and the distinctive organella: melanosomes. Nuclei were large, with regular edges and irregularly dispersed. The cytoplasm showed abundant mitochondria, microtubuli, microfilaments, free ribosomes or polyribosomes, endoplasmic reticulum and Golgi apparatus. Melanosomes were of varied appearance and in different stages of maturation (Fig. 4). After exposure to the agents, the alterations noted differed very little from those of the control cultures. The most noteworthy observations were the presence of irregularities at the cell and nuclear edges, organelle swelling, dilation of endoplasmic reticulum vesicles and nuclear membrane, mitochondria swelling, and the existence of lamellar structures and lipidic vacuoles. Melanosomes showed no changes except for their tendency to gather at cell periphery (Fig. 5).

Discussion

In recent years, cultures of human tumours have been increasingly used in the study of tumoral biology at a cellular level, as well as in pharmacological assays. The use of *«in vitro»* assay system for the screening of potential anticancer agents has been encouraged by the relationship between drug sensitivity exhibited by primary cultures of human tumours and their *«in vivo»* counterparts (Wilson, 1987). In the treatment of cancer, alkylating agents still represent an important group of antineoplastic drugs. In this group, melphalan and nitrosureas are the most widely used drugs, in both *«in vitro»* and *«in vivo»* assays. In recent years a de-

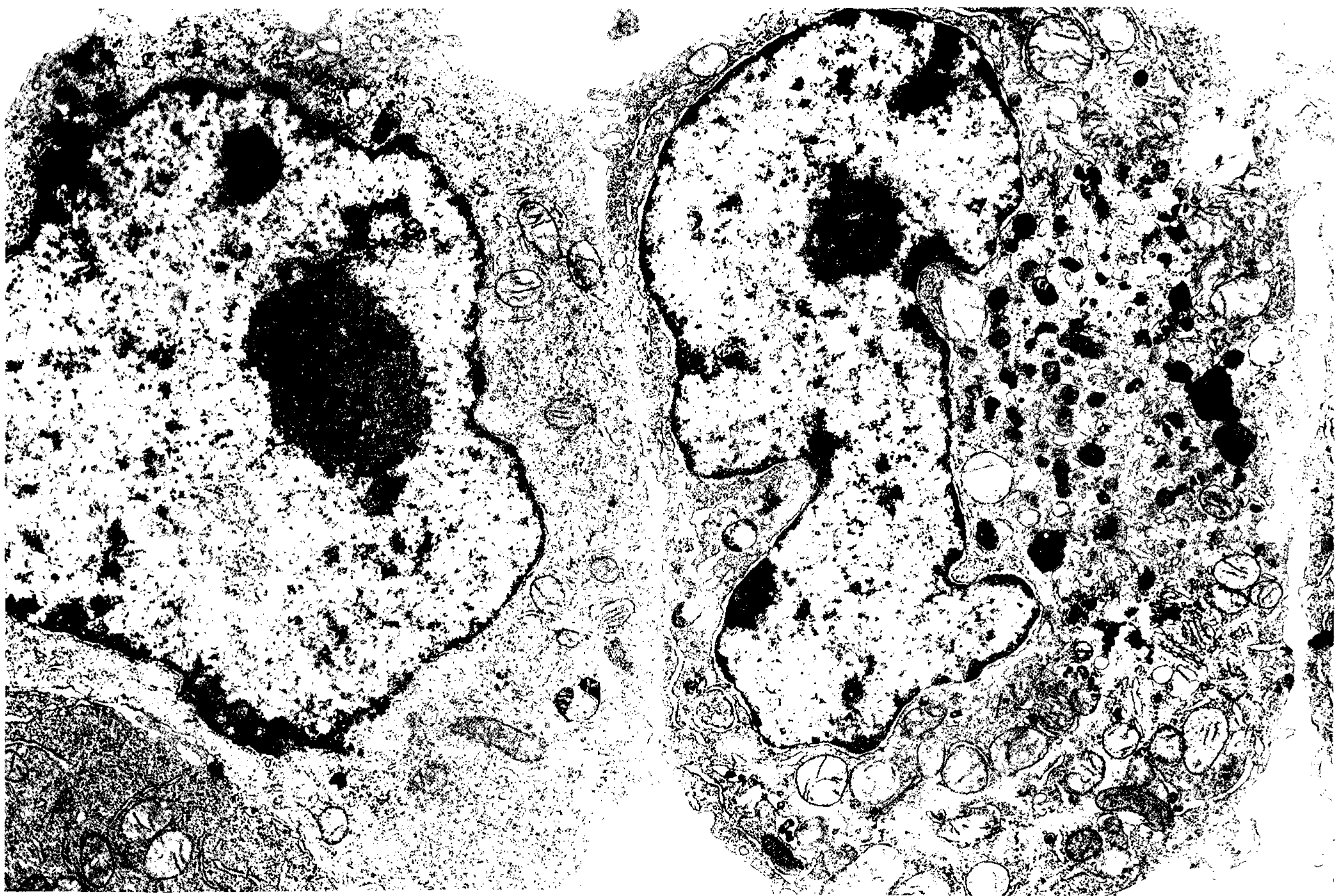

Fig. 4. Melanocytes from control cultures at 48 hours. The nuclei are large and abundant mitochondria, ribosomes, endoplasmic reticulum, Golgi apparatus and melanosomae are showed. x 5,000

pigmenting agent, 4-OHA, whose melanocytotoxic activity was first illustrated by Riley (1969) has been used in an experimental way.

In our study, the effects of these three agents (melphalan, CCNU and 4-OHA) on the G-361 human melanoma cell line were compared. 24 hours after their application, the most effective agent seemed to be 4-OHA, it had the lowest ID_{50} ($2.4x10^{-3}$ µM), followed by melphalan (0.016 µM) and lastly CCNU (0.21 µM). this greater efficacy of 4-OHA, was also noted in a similar study performed on B16-F10 murine melanoma and Harding-Passey with short exposure times (Garrido, 1991). Nevertheless, in our study, by increasing the exposure time to 96 hours, we observed that the cultures which decreased most rapidly were those treated with CCNU, while those treated with 4-OHA and melphalan decreased more slowly, as the growth rates of the surviving cell fractions show. This suggests a decrease in the inhibitory effect of 4-OHA with time.

Other studies carried out with human melanoma cells (NEL-MI) observed the inhibitory action of 4-OHA on the *«in vitro»* growth of that line at concentrations above 10 µM, a concentration of 1 mM being needed to cause the death of the culture (Kulkarni and Nathanson, 1989). In the same way, it was noted that in a 72-hour assay time, the inhibitory effect of the agent decreased with increasing cell density. According to these authors, the clinical implication of this observation might be pessimistic if patients with large tumors of higher body burden of tumor cells were less responsive to the tumor growth inhibitory properties of 4-OHA. On the other hand, in studies performed *«in vivo»* on B16 melanoma implanted in mice, a certain potential to suppress metastasis when the tumor spread is lower has been observed (Klanclertz and Chapman, 1986).

Although in our study, the decrease in the inhibitory effect of these agents with time is not related to an increase in cell density, we consider it worthwile to carry on studying the variations with time of the inhibitory properties of 4-OHA. Prolonged drug exposure using a

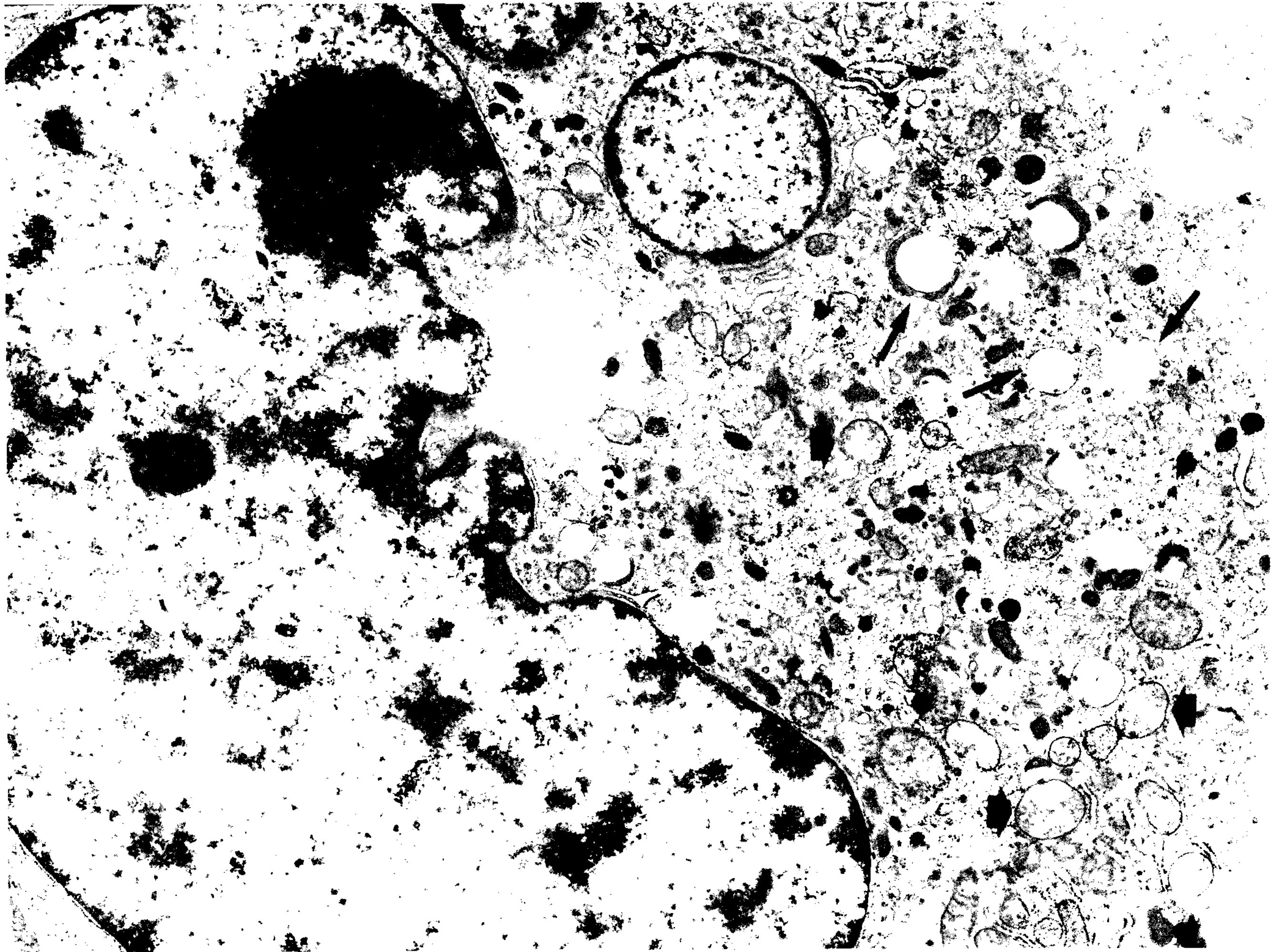

Fig. 5. Melanocyte from 0.42 µM CCNU culture at 24 hours. No important modifications can be observed except lobulate nucleus, mitochondria swelling (thick arrows) and cytoplasmic vacuoles (thin arrows). x 10,000

variety of cancer chemotherapeutic agents has been shown to result in gradually decreasing ID_{50} values, as exposure times increase (Wilson, 1987). The penetration rate of the drug may also be a limiting factor when short exposure times are used.

Finally, the question of length of drug exposure becomes one of practicality. Pharmacokinetic data show that maximum exposure to drug occurs in the first hour after intravenous injection (Wilson, 1987).

Our results show the cytotoxic effect of the three agents on this human melanoma cell line. Although in all cases, inhibiton on culture growth is evident, cellular death is more obvious in cultures treated with CCNU at 4-OHA. Cultures treated with melphalan show growth inhibition although cell viability only decreases slightly. This suggests that few cells die. We cannot be certain whether this is due to the growth of a resistant population or the implanted in mice, it was noted that, though the proliferation ability of treated tumours with melphalan decreased significantly, the mytotic index was higher in the treated groups, because non-affected cells showed a higher mytotic activity (Vicente et al., 1990).

With respect to the ultrastructural observations, previous studies reported several alterations due to the drug action, consisting of a peripheral spread of heterochromatin, swelling with dilation of the membrane system (nuclear membrane, Golgi and endoplasmic reticulum) degranulation of ribosomes and frequent vacuolization (Li and Laszlo, 1985; Li and Mak, 1985; Vicente et al., 1990). In this study we observed similar alterations corresponding to involutive phenomena, which are typical features of cell injury.

Acknowoledgements. We wish to thank A. Pina Gil for her valuable technical assistance. This work has been partially supported by a grant from the D.G.C.Y.T. (Nº PM88-0093). Ministerio de Educación y Ciencia, Spain.

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Accepted September 26, 1994