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ANTIPROLIFERATIVE AND DEPIGMENTING EFFECTS OF THE HISTAMINE (H2) AGONIST DIMAPRIT AND ITS DERIVATIVES ON HUMAN MELANOMA CELLS

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Abstract—Human melanoma cells were treated in culture with the histamine (H2) agonist *S*-(3-(*N*-*N*-dimethylamino)propyl)isothiourea (dimaprit), a partial agonist, *S*-(2-(*N*,*N*-dimethylamino)ethyl)-isothiourea (nordimaprit), and two analogues of nordimaprit, *S*-(2-(*N*,*N*-diethylamino)ethyl)isothiourea (DENOR) and *S*-(2-(*N*,*N*-diisopropylamino)ethyl)isothiourea (DINOR), to investigate the effects on toxicity and tyrosinase activity. Cell survival studies showed highest toxicity in the constitutively pigmented human melanoma cell line MM418, DINOR being the most effective agent. Toxicity was not blocked by the H2 antagonist cimetidine. Dimaprit and its derivatives decreased tyrosinase activity in the amelanotic human melanoma cell line MM96E and inhibited expression of a melanosomal antigen. Loss of tyrosinase activity could be prevented by cimetidine and ranitidine, an H2 antagonist. Although the tyrosinase activity in MM418 cells was much more resistant to inhibition by these agents compared with that in MM96E cells, prolonged growth in the presence of non-toxic levels of DINOR caused a decrease in tyrosinase activity and subsequent depigmentation. Ultrastructural examination of the depigmented cells showed a decrease in the number of melanized melanosomes and the appearance of premelanosomes. These results indicate that bulky substituents on the tertiary amine group in nordimaprit significantly enhance potency for depigmentation and cell killing but only the former effect is mediated by the H2 receptor.

Metastatic melanoma, a tumour embryonically related to the neural crest, resists treatment with standard cytotoxic agents [1]. However, the presence of a unique metabolic pathway for the production of the pigment melanin provides a possible means of designing a specific chemotherapeutic agent [1, 2]. Attempts to utilize the toxicity of 3,4-dihydroxyphenylalanine (dopa‖) and it derivatives suffer from lack of specificity, as dopa and tyrosine are widely distributed [2, 3]. Thus, work has centred on the bi- (and possibly tri-) functional enzyme tyrosinase which is the rate-limiting enzyme critical in the formation of melanin [2, 4–6]. Its regulation is an important control point in the pigmentation response [6].

Recently it has been shown that the histamine (H2) agonist *S*-(3-(*N*,*N*-dimethylamino)propyl)-isothiourea (dimaprit) and the partial agonist *S*-(2-(*N*,*N*-dimethylamino)ethyl)isothiourea (nordimaprit) cause a decrease in tyrosinase activity and subsequent pigmentation in human melanoma cells, the most dramatic effect being the complete depigmentation of a constitutively melanizing cell line by nordimaprit [7]. Histaminergic control of pigmentation was supported by the decreased expression of two tyrosinase antigens, 5C12 and 2B7, following treatment with nordimaprit and an increase in tyrosinase activity by the H2 antagonist, cimetidine. Treatment of murine melanoma cells with the H2 antagonists, cimetidine and ranitidine, stimulated tyrosinase activity and inhibited cellular proliferation of human melanoma cells [8]. Previously, histamine has been demonstrated to cause an H1-mediated increase in proliferation of human melanoma cells [9] and an increase in intracellular cAMP which was effectively blocked by the H2 antagonists cimetidine and ranitidine [10]. It has also been reported that H2 antagonists alone or in combination with other agents can induce remission of melanoma and certain other tumours in humans and animals [8].

Following UV irradiation, there are increases in PGE2, PGD2 and histamine levels in the skin [11]. This is understandable because UV irradiation causes a plethora of immunological and pharmacological effects in the skin, including a local inflammatory process [12, 13]. It has been suggested that prolonged release of a high concentration of histamine from mast cells may induce hyperpigmentation in lesions such as urticaria pigmentosa and systemic mastocytosis and may therefore be an important mediator of cellular response to sunburn injury and subsequent proliferation and pigmentation [11]. This is particularly important as exposure to sunlight, particularly the UV component, has been implicated

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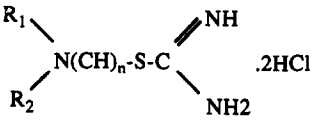
‖ Abbreviations: dimaprit, *S*-(3-*N*,*N*-dimethylamino)-propyl)isothiourea; nordimaprit, *S*-(2-(*N*,*N*-dimethylamino)ethyl)isothiourea; DENOR, *S*-2-(*N*,*N*-diethylamino)-ethylisothiourea; DINOR, *S*-2-(*N*,*N*-diisopropylamino)-ethylisothiourea; dopa, 3,4-dihydroxyphenylalanine; PBS, phosphate-buffered saline, pH 7.2.



R1	R2	n	
CH_3	CH_3	3	Dimaprit
CH_3	CH_3	2	Nordimaprit
C_2H_5	C_2H_5	2	2-(Diethylamino)ethylisothiourea (DENOR)
$CH(CH_3)_2$	$CH(CH_3)_2$	2	2-(Diisopropylamino)ethylisothiourea (DINOR)

Fig. 1. Structure of dimaprit and its derivatives.

as a contributing factor in the etiology of melanoma [13]. This investigation examined the cytotoxicity and effects on tyrosinase activity of dimaprit, nordimaprit and two homologues of nordimaprit, in human melanoma and non-melanoma cell lines. Tyrosinase activity was found to be regulated through the H2 receptor but toxicity was not.

MATERIALS AND METHODS

Drugs. Dimaprit, nordimaprit, *S*-(2-(*N*,*N*-diethylamino)ethyl)isothiourea (DENOR) and *S*-(2-(*N*,*N*-diisopropylamino)ethyl)isothiourea (DINOR) were synthesized by refluxing the appropriate chloroalkyl amine with thiourea in ethanol as described previously [14–16] to give the resulting isothiourea as a dihydrochloride. The structures of the compounds (see Fig. 1) were confirmed by ^{13}C NMR, ^{1}H NMR and infrared spectroscopy. All compounds were readily soluble in water and the solutions were filter sterilized before use. Other drugs were purchased from the following companies: L-dopa (Sigma Chemical Co., St Louis, MO, U.S.A.), cimetidine (Tagamet®) (Smith, Kline & French Philadelphia, PA, U.S.A.) and ranitidine (Zantac®) (Glaxo).

Cell culture. The origin and properties of the human melanoma cell lines MM96L, MM96E, MM418 and MM253c1, the human cervical carcinoma line HeLa, the human ovarian tumour lines JAM and CI 80-13S and the human virus-transformed keratinocyte line KJD-1/SV40 have been described previously [17–22]. Cells were cultured in 5% CO_2/air at 37° in Roswell Park Memorial Institute medium 1640 (Flow Laboratories, Sydney, Australia) containing 1 mM pyruvate, 0.2 mM nicotamide, 100 IU/mL penicillin, 0.17 mM streptomycin, 3 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid and either 5 or 10% (v/v) fetal calf serum. The monoclonal antibody B8G3 was used as the hybridoma supernatant. Routine assays for *Mycoplasma* by Hoechst dye assays were negative [23].

Cell survival. Cell survival was determined by two methods. In a modified colony assay [24], cells were plated onto a 96-well microtitre plate (Nunc, Denmark) at 1×10^3–2×10^3 cells/well and allowed to attach overnight. The cells were exposed continuously to drugs for 5 days and then labelled with [^{3}H-*methyl*]thymidine (2 μCi/mL, 5 Ci/mmol, Amersham, U.K.) for 4 hr. Cells were washed with phosphate-buffered saline (PBS), detached with 0.02% trypsin and 0.1 mM EDTA in PBS, harvested onto glass fibre sheets and counted in a Betaplate counter (LKB, Finland). The D_{37} (dose required to achieve 37% survival) was calculated from the dose–response curves.

For the colony counting method, 1×10^3 cells were added to a 5-mL dish and allowed to attach overnight. On the following day, 100 cells were counted at random to calculate colony multiplicity [25]. Cells were then exposed continuously to drug until colonies (≥50 cells) were formed (7–10 days). The dishes were washed with PBS, fixed with methanol, washed again with PBS and stained with Giemsa. The colonies were then counted and corrected for initial multiplicity and the surviving fractions calculated.

Tyrosinase assay. Tyrosinase (dopa oxidase) activity was determined as follows. Cells were plated into 10-mL petri dishes at 5×10^5–1×10^6 cells/10 mL, allowed to attach overnight and then treated continuously with drug for 4 days. The cells were washed, scraped into 10 mL of ice-cold PBS and pelleted. Tyrosinase activities were obtained by sonicating cells in 200 μL of lysis buffer (50 mM sodium phosphate, pH 6.8, containing 1% Triton X-100) per 1×10^7 cells, followed by centrifugation in a microfuge for 30 min at 4° to remove melanin and debris. A 15-μL aliquot of the supernatant was added to 150 μL of a mixture of 7.6 mM dopa and 50 mM phosphate, pH 6.8, in the well of a microtitre plate. The increase in absorbance at 490 nm was read at 2-min intervals in an ELISA reader (Model EC310, Bio-Tek Instruments). Rates were calculated as the change in absorbance/min/mg protein, based on the initial rate [7].

PAGE-western blotting. For immunoblotting, approximately 2×10^7 cells were sonicated in 500 μL

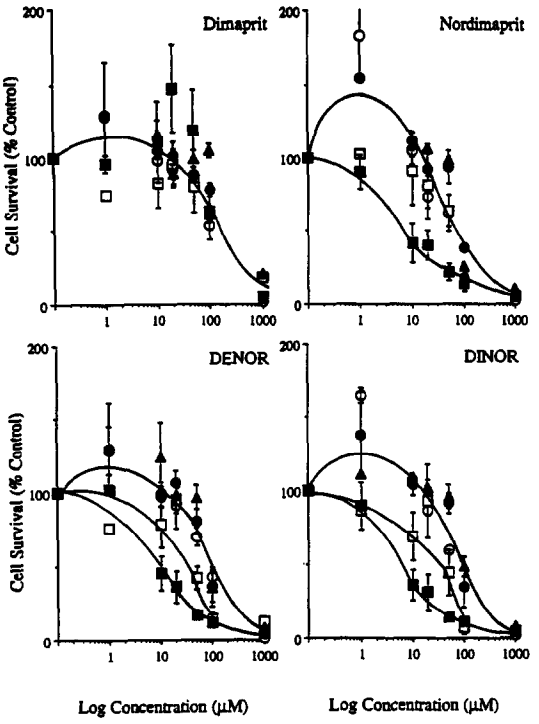


Fig. 2. Dose–responses of cell survival for dimaprit, nordimaprit and derivatives of nordimaprit (DENOR and DINOR) in human malignant melanoma cell lines MM96L (●), MM96E (□) and MM418 (■) and non-melanoma cell lines CI 80–13S (▲) and HeLa (○). Points represent the means and SE of 2–4 experiments.

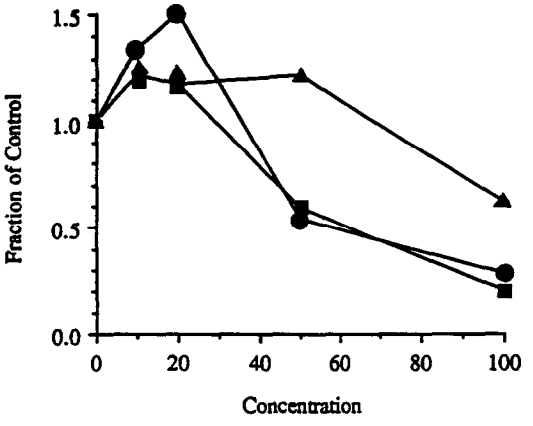


Fig. 3. Dose–response of cell survival for dimaprit-treated MM96E (●), MM418 (■) and HeLa (▲) cells as measured by inhibition of colony formation. Points represent the means of duplicates.

of lysis buffer (100 mM dithiothreitol, 20% glycerol, 10% sodium dodecyl sulfate, 10 mM Tris, pH 7.4, and 2 mM phenylmethylsulphonyl fluoride) for 2 min. Cell lysates were then immersed in boiling water for 2 min. After centrifugation for 10 min, 10-μL samples of supernatants adjusted to 1 mg of protein/mL were applied to a 6–15% polyacrylamide gradient gel and electrophoresed in 25 mM Tris containing 192 mM glycine and 0.1% sodium dodecyl sulphate, pH 8.3. Molecular weight standards were purchased from Sigma Chemical Co.

Immunoblotting was performed by the transfer of proteins onto nitrocellulose in transfer buffer (20% methanol in 10 mM Na_2CO_3, pH 9.9) for 3 hr at 30 V. The nitrocellulose was then blocked and incubated with the hybridoma supernatant. Visualization of protein bands was achieved by incubation with alkaline phosphatase-linked anti-mouse IgG antibody (Silenus, Melbourne, Australia) diluted 1 and 1000 in PBS containing 0.02% Tween 20, followed by nitroblue tetrazodium – 5-bromo-4-chloro-3-indolyl phosphate 5-bromo-4-chloro-3-indolyl phosphate substrate 0.75 mM, 0.75 mM nitroblue tetrazolium, 5 mM $MgCl_2$ and 0.1 M Tris, pH 9.5) [26, 27]. Band intensities were measured by a Scanning Computer Densitometer and analysed

Table 1. Toxicity of dimaprit and its derivatives in human cell lines

Cell type	D_{37} (μM)			
	Dimaprit	Nordimaprit	DENOR	DINOR
Melanoma				
MM96L	400	165	130	125
MM96E	310	80	70	65
MM418	310	30	24	14
MM253c1	300	70	60	60
Non-melanoma				
JAM	450	90	130	88
HeLa	240	80	110	72
CI 80-13S	700	120	130	120
KJD	>1000	90	80	80

* Dose required to reduce survival to 37%; interpolated from the combined response curve of 2–4 experiments.

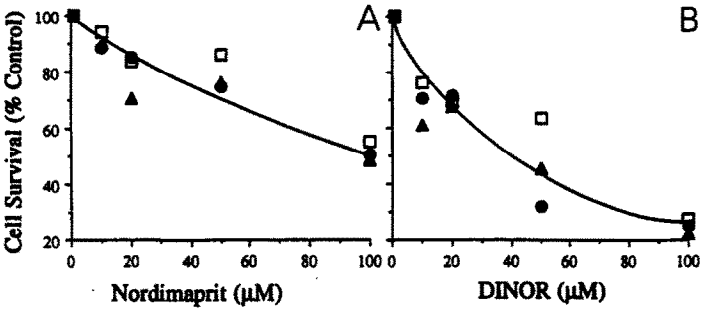


Fig. 4. Effect of the histamine (H2) antagonist cimetidine on survival of nordimaprit- and DINOR-treated cells. A: Nordimaprit alone (▲), cimetidine (100 μM) and nordimaprit (□), cimetidine (30 μM) and nordimaprit (□), cimetidine (30 μM) and nordimaprit (●). B: DINOR alone (▲), cimetidine (100 μM) and DINOR (□), cimetidine (30 μM) and DINOR (●). Points represent the means of duplicates.

by Imagequant software (Molecular Dynamics, CA, U.S.A.).

The amount of protein in each sample was determined by incubating 10 μL of supernatant from cell lysates (in duplicate) with 100 μL of BCA working reagent (Pierce Chemical Co., U.S.A.) for 30 min at 37°. Absorbance was read on an ELISA scanner at 540 nm. Bovine serum albumin was used as protein standard.

Electron microscopy. MM418 cells (treated and untreated) were harvested by scraping into 10 mL PBS, pH 7.2, pelleted and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3. The pellet was washed and post-fixed with 1% OsO_4 prior to embedding in epoxy resin. Sections were examined with a JOEL 12 EX transmission microscope.

RESULTS

Cell survival

The dimaprit dose–responses for cell survival were similar for all cell lines tested (Fig. 2 and Table 1), except that the KJD line was highly resistant. Several cell lines including the melanized cell line MM418 had enhanced growth at low doses of dimaprit. This was partly due to enhancement of cell clonogenicity because some enhancement of colony formation was found when cell survival was tested under conditions which allowed the number of colonies to be counted (Fig. 3). Nordimaprit and its homologues were selectively toxic to the melanized line MM418, and to a lesser extent to MM96E and MM253c1 (Fig. 2 and Table 1). It was also clear that increasing the alkyl chain length of the tertiary amine group of nordimaprit caused an increase in toxicity, with DINOR giving a D_{37} value as low as 14 μM for MM418 cells. This toxicity was not blocked by the H2 antagonist, cimetidine (Fig. 4).

Tyrosinase activity

Tyrosinase (dopa oxidase) activity was decreased in MM96E cells by all drugs during a 4-day treatment period (Fig. 5A). The most potent inhibitor was DINOR, giving approximately 50% inhibition at the relatively non-toxic dose of 20 μM. Dimaprit was found to be the least effective drug, with only 50% inhibition at 100 μM.

When MM418 cells (Fig. 5B) were treated with the same dose range there was an increase in activity at low doses. Only DINOR was able to inhibit activity effectively and this inhibition occurred at doses which had been found previously to be relatively toxic. However, MM418 cells adapted to growth during prolonged treatment with these compounds leading to a decrease in tyrosinase activity and subsequent establishment of a depigmented line (results not shown).

It was therefore decided to use MM96E for further analysis due to high tyrosinase activity and low melanin production. In a study of the temporal response of tyrosinase activity in MM96E cells, DINOR again proved the most effective agent with a 40% decrease in activity after only 6 hr of treatment and little or no activity after 7 days (Fig. 6). Recovery of tyrosinase activity following continual treatment of MM96E cells for 4 days with dimaprit, nordimaprit or DINOR is shown in Fig. 7. A gradual recovery of enzyme activity was observed, with complete recovery 7 days after removal of drug.

To determine whether inhibition of tyrosinase activity was mediated through the histamine H2 receptor, MM96E cells were incubated with the H2 antagonists ranitidine and cimetidine, alone and in combination with one of the previous drugs. As seen in Table 2, ranitidine or cimetidine alone increased tyrosinase activity and antagonized the inhibition of tyrosinase by dimaprit and its derivatives.

Immunoblotting

Immunoblotting of lysates from drug-treated cells using B8G3, a monoclonal antibody that gives identical bands to TA99 [28] in human melanoma cell lines, showed that expression of the antigen was suppressed by dimaprit, nordimaprit and DINOR following treatment of MM96E and MM418 cells for 4 days (Fig. 8). The most dramatic effect was

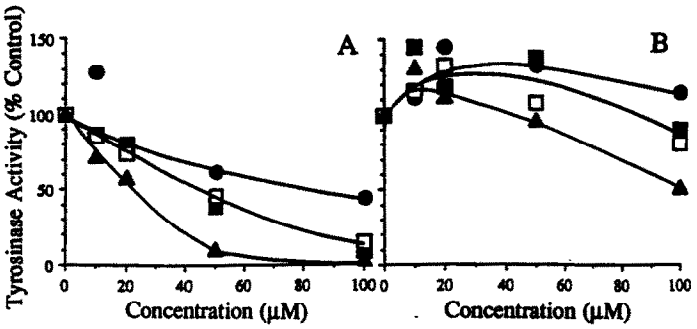


Fig. 5. Dose–response curves showing inhibition of tyrosinase activity in MM96E (control enzyme rate = 0.00125 A_{490}/min/mg protein) (A) and MM418 (control enzyme rate = 0.00115 A_{490}/min/mg protein) (B) following treatment with dimaprit (●), nordimaprit (■), DENOR (□) or DINOR (▲) for 4 days. Points represent the means of triplicates.

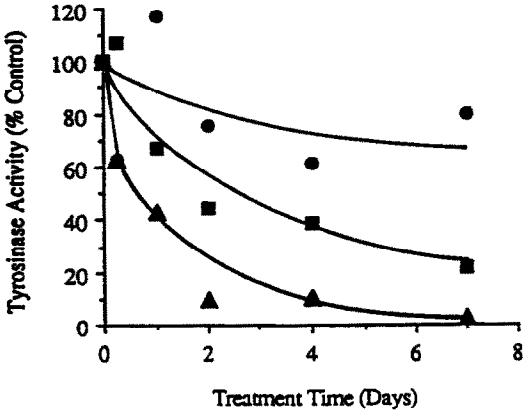


Fig. 6. Temporal response for inhibition of tyrosinase activity in MM96E cells following treatment with 50 μM dimaprit (●), 50 μM nordimaprit (■) or 50 μM DINOR (▲). Points represent the means of triplicates.

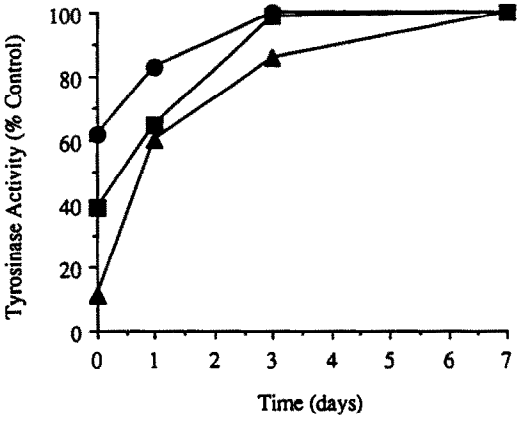


Fig. 7. Recovery of tyrosinase activity in MM96E cells following removal of 50 μM dimaprit (●), 50 μM nordimaprit (■) and 50 μM DINOR (▲) after treatment for 4 days. Points represent the means of triplicates.

Table 2. Effect of the H2 antagonists ranitidine and cimetidine on tyrosinase activity* in treated MM96E cells

	Ranitidine			Cimetidine	
Treament	0 μM	30 μM	100 μM	30 μM	100 μM
Control	100	154	164	105	122
Nordimaprit (50 μM)	39	107	128	56	50
DINOR (50 μM)	11	45	62	94	72
Dimaprit (50 μM)	62	86	81	72	—

* Enzyme rate was calculated as the change in A_{490}/min/mg protein, and was determined from the mean of triplicates, after treatment with drugs simultaneously for 4 days.

observed after treatment of MM96E cells with DINOR. This produced a decrease in band intensity (immunoreactivity) to only 15% of the control.

Electron microscope

Electron microscopy of treated MM418 cells grown in the presence of DINOR for a prolonged period displayed distinct changes in subcellular organelles. Low power examination of untreated cells (Fig. 9a) showed melanosomes as electron dense structures in varying stages of melanization and in relatively large numbers. Treated cells (Fig. 9b) contained

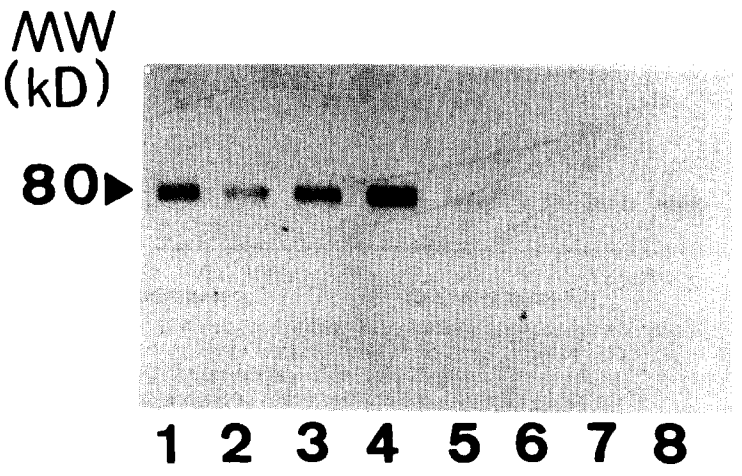


Fig. 8. Expression of B8G3 antigen in MM418 (lanes 1–4) and MM96E (lanes 5–8) detected by western blotting following 4-day treatment of cells with drugs. Lane 4, MM418 control; lane 8, MM96E control; lanes 1 and 5, 50 μM dimaprit; lanes 2 and 6, 50 μM DINOR; lanes 3 and 7, 50 μM nordimaprit.

fewer well-melamized melanosomes and no new melanization appeared to be taking place.

When studied under high power, untreated cells (Fig. 9c) were found to contain melanosomes with melanization occurring at different stages on filamentous fragments typical of stage I and II melanosomes. The subcellular components of the cells appeared to be well developed, particularly the golgi apparatus, mitochondria and endoplasmic reticulum. In treated cells (Fig. 9d) the inner filaments of melanosomes were poorly developed (horseshoe appearance) and lacked pigment. The golgi apparatus and endoplasmic reticulum were poorly developed and less evident.

DISCUSSION

This investigation provides further insight into the regulation of tyrosinase activity by histamine (H2) agonists in human melanoma cells [7]. Since altering the isothiourea group of dimaprit causes loss of histaminergic activity [14], it was decided to investigate the effect of modifying the tertiary amine of nordimaprit, the more potent inhibitor of tyrosinase activity. Increasing the size of the alkyl chain on this end of the molecule increased toxicity and produced more effective inhibitors of tyrosinase activity, suggesting a common dependence on hydrophobicity for these mechanisms. However, the finding that toxicity was not modulated by H2 receptor antagonists indicates that the dimaprit analogues can enter the cell and reach relevant targets independently of histamine receptors.

Toxicity studies revealed that the heavily melanized line MM418 was the cell line most susceptible to killing, particularly by DINOR, yet its tyrosinase activity was less sensitive to inhibition by short term treatment. MM418 is very sensitive to killing by DL-buthionine (*S,R*)-sulfoxime, presumably due to glutathione depletion and to the loss of protection from endogenous radicals produced by melanin synthesis [29]. DINOR or its metabolites might therefore act similarly by compromising the antioxidant defences of the cell. The difference between the tyrosinase response of MM418 and MM96E cells suggests that coordinate regulation of tyrosinase activity and melanogenesis occurs in cells undergoing constitutive pigment synthesis.

It is of interest that nordimaprit and its analogues DENOR and DINOR are more potent inhibitors of tyrosinase activity than dimaprit, the more effective H2 agonist. These compounds may be metabolized into a more active histaminergic form by the melanoma cells or they may bind to the H2 receptor with greater efficiency than dimaprit but are unable to evoke a normal histaminergic response. In due course it will be of interest to further enhance potency by modification of the nordimaprit structure so that relevant target molecules can be identified by affinity chromatography.

One of the functional effects of these compounds on pigment organelles was identified by ultrastructural examination of treated MM418 cells. The filamentous structures in melanosomes on which melanin deposition occurs were absent or poorly developed in treated cells, where premelanosomes (stage I melanosomes) predominated. Taken in conjunction with the decrease in B8G3 premelanosomal antigen expression, these results suggests that the drugs down regulate expression of other melanosomal proteins as well as of tyrosinase itself. This is significant because the B8G3 antigen appears to be identical with the *b* locus protein which may play a structural [30] or enzymatic role [4, 31] in melanosomes. Nordimaprit analogues might therefore be useful for selective destruction of melanotic cells and for analysing the coordinate regulation of expression of the proteins involved in pigment synthesis.

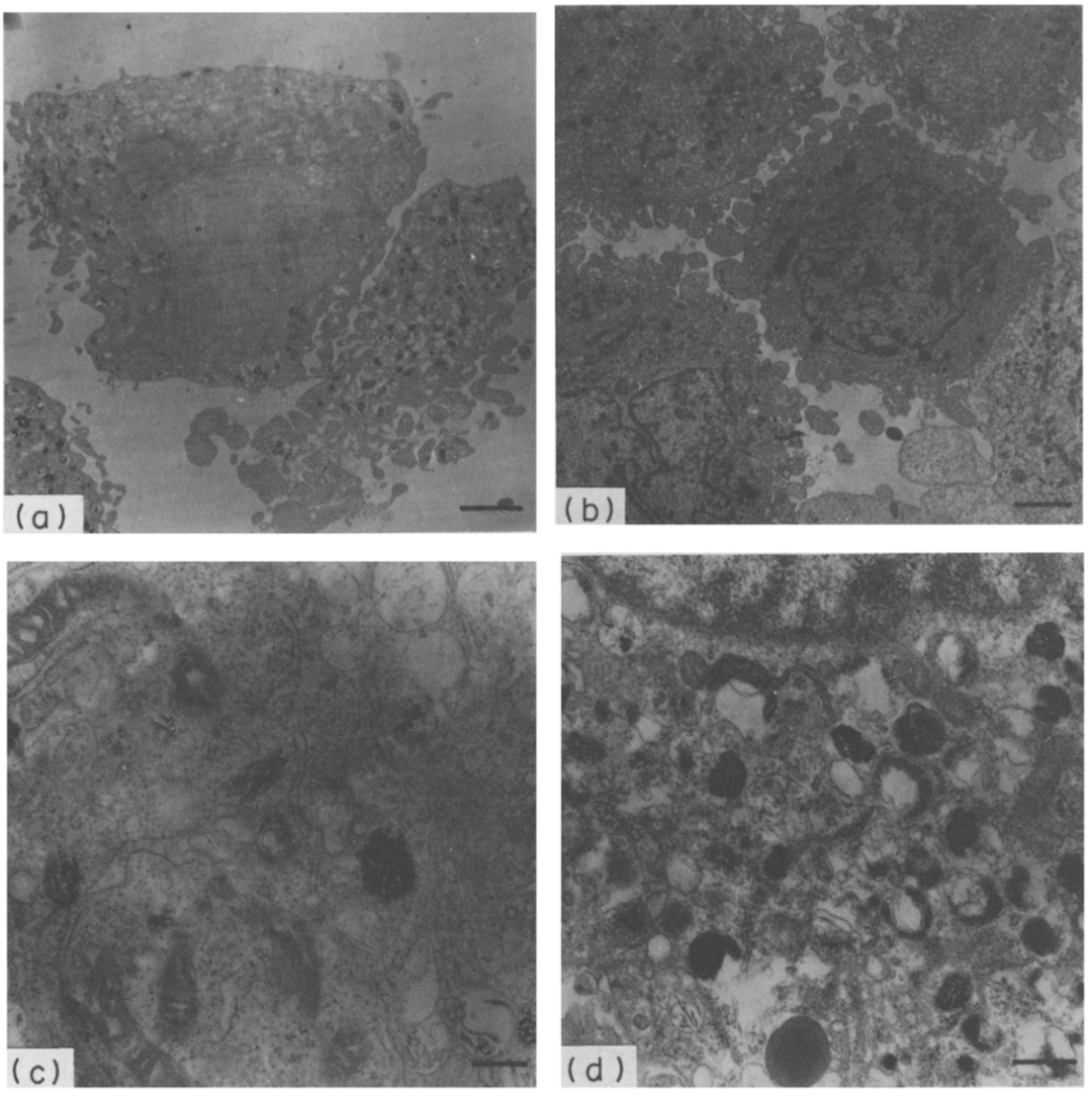


Fig. 9. Ultrastructural study of MM418 cells after long term DINOR treatment (6 months). Untreated MM418 cells (a) showing highly melanized melanosomes in large number. Bar; 2 μM. Following treatment with 50 μM DINOR (b), the number of fully melanized melanosomes was reduced. Bar; 2.5 μm. At higher magnification, untreated MM418 cells (c) showed melanosomes at various stages with melanization occurring on inner filamentous structures. Bar; 250 nm. Cells treated with 50 μM DINOR (d) had decreased melanization, with the inner filaments of melanosomes being poorly developed and frequently of horseshoe appearance. Bar 400 nm.

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