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INHIBITION OF MELANOGENESIS IN HUMAN MELANOMA CELLS BY NOVEL ANALOGUES OF THE PARTIAL HISTAMINE (H2) AGONIST NORDIMAPRIT

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Abstract—A series of analogues of the weak histamine (H2) agonist *S*-[2-(*N,N*-dimethylamine)ethyl] isothiourea (nordimaprit) was produced to investigate the possibility that bulky substituents on the tertiary amine of nordimaprit would enhance potency for depigmentation and killing of melanoma cells. Cell survival studies showed that neither an increase in lipophilicity nor an increase in size of these groups produced selective toxicity, with only the *N,N*-diisobutyl derivative being more effective than the *N,N*-diisopropyl derivative in killing the constitutively pigmented human melanoma cell line MM418. The most hydrophobic analogue, the *N,N*-dibenzyl derivative, was also the most toxic to all cell lines tested. The association of toxicity with lipophilicity was confirmed by the piperidine derivative having greater toxicity than the less hydrophobic morpholine analogue. The ability to decrease tyrosinase activity was lost when lipophilicity and size of the N-terminal groups were increased, but these analogues produced marked depigmentation, even greater than that found with either nordimaprit or the diisopropyl derivative. Surprisingly, an increase in tyrosinase activity was achieved, the most potent agent being the *N*-ethyl-*N*-anilino analogue which caused complete depigmentation (0.6% of control) and elevated tyrosinase activity (148%) in MM418 cells after 1 month of treatment. This indicates that nordimaprit and its derivatives possess two different mechanisms of depigmentation, the first being tyrosinase dependent and the second being tyrosinase independent. The latter pathway is yet to be elucidated but appears to require a high degree of hydrophobicity.

Melanin biosynthesis is a unique and widely studied aspect of melanoma differentiation, in particular the regulation of the rate limiting enzyme tyrosinase [1, 2]. Previously, histaminergic control of tyrosinase activity was demonstrated by an increase in activity when melanoma cells were treated with the histamine H2 antagonists, cimetidine and ranitidine [2, 3], and a decrease in activity following treatment with the H2-specific agonist dimaprit [2].

When human melanoma cells were treated in culture with the weak histamine (H2) 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), there was a significant enhancement of potency for tyrosinase inhibition and cell killing as the carbon chain of the tertiary amine increased. Only the former effect was mediated by the H2 receptor [4]. This suggested that both cell toxicity and inhibition of tyrosinase activity were directly related to hydrophobicity. It has also been claimed that there is enhanced affinity to all three histamine receptors when lipophilic groups are suitably located on the agonist or antagonist [5]. A new series of more hydrophobic and larger analogues of nordimaprit has therefore been produced to define more closely the structural requirements for biological activity, in particular in the hope of achieving a more selective killing of melanotic cells. A wide variety of hydrophobic groups were chosen including the diisobutyl, dibenzyl and ethylaniline derivatives, and analogues where the tertiary amine was fixed into a ring structure to restrict the access of ligands to the distal side of the tertiary amino group. A theophylline derivative was included because theophylline, a phosphodiesterase inhibitor, is a strong stimulating agent for melanogenesis in a number of human and mouse melanoma cell lines [1, 6].

These new derivatives lacked both the ability to destroy selectively melanotic cells and to inhibit tyrosinase. However, they were able to produce complete depigmentation of a heavily melanized cell line at non-toxic levels, with a paradoxical increase in tyrosinase activity.

MATERIALS AND METHODS

Synthesis of diisobutylaminoethanol. All equipment was cooled to below 10°. Diisobutylamine (35 mL, 0.3996 mol) was dissolved in 30 mL of ethanol in one flask. To the other flask was added ethylene oxide (8.8 g, 0.1998 mol). The temperature of the latter was raised to 10° and ethylene oxide was slowly added into the flask containing the amine over 2 hr with the aid of dry nitrogen. The reaction was allowed to warm to room temperature and then refluxed for 2 hr. The solvent was then removed *in vacuo* and the product purified by column

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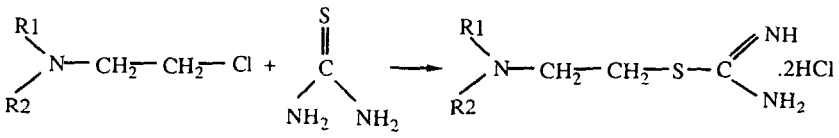
‡ Abbreviations: DENOR, *S*-[2-(*N,N*-diethylamino)-ethyl]isothiourea; DINOR, *S*-[2-(*N,N*-diisopropylamino)-ethyl]isothiourea; PBS, phosphate-buffered saline, pH 7.2.

chromatography to yield a clear yellow oil. Yield 36.18%, $\eta = 1.434$. ^{1}H NMR: (dimethyl sulphoxide) 0.82 (12H, d, $J = 6.25$ Hz), 1.64 (2H, multiplet), 2.1 (4H, d, $J = 3.93$ Hz), 2.42 (2H, t, $J = 4.29$ Hz), 3.42 (2H, t, $J = 2.5$ Hz) ppm.

Synthesis of 2-chloroethyl diisobutylamine HCl. 2-Diisobutylamino ethanol (2 g, 0.0115 mol) was dissolved in chloroform (5 mL) and cooled in an ice bath. Thionyl chloride (1.5 mL, 0.01725 mol) in chloroform (10 mL) was added dropwise over 1 hr. The reaction was allowed to warm to room temperature, refluxed for 3 hr and the solvent removed by distillation *in vacuo*. The resulting oil was dissolved in ethyl acetate and the final product allowed to crystallize overnight at 0°. Yield 77.8% m.p. 110°, ^{1}H NMR: 1.03 (12H, d of d, $J = 3$, 3.5 Hz), 2.18 (2H, multiplet), 3.13 (4H, d of d, $J = 3.6$, 4 Hz), 3.64 (2H, t, $J = 6.5$ Hz), 3.96 (2H, t, $J = 6.5$ Hz) ppm.

Synthesis of compounds I–VI. Compounds I–VI were synthesized following the general procedure of Hino *et al.* [7]. The respective ethylchlorides (2-chloroethyldibenzylamine, 2-chloroethylethylaniline, 2-chloroethylpiperidine, 2-chloroethylmorpholine and 2-chloroethyltheophylline) purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) were refluxed with thiourea under the appropriate conditions (Table 1). The structures (Fig. 1) were confirmed by ^{1}H NMR (Bruker AMX500, Germany) and i.r. spectroscopy (Perkin-Elmer 599, Norwalk, U.S.A.), and the relative molecular mass was determined by fast atom bombardment mass spectrometry (Kratos M525 RFA).

All compounds were soluble in water and the solutions were filter sterilized before use. L-Dopa was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).



Thiourea

Compound	R1	R2	MWt
DINOR	$CH(CH_3)_2$	$CH(CH_3)_2$	276.25
I	C_4H_9	C_4H_9	304.33
II	$CH_2—C_6H_5$	$CH_2—C_6H_5$	372.35
III	C_6H_5	C_2H_5	259.8

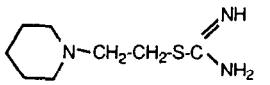
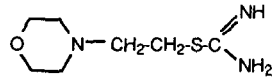
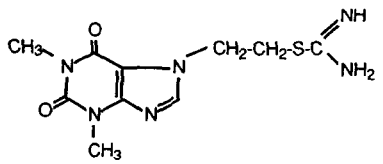
IV		260.23
V		261.20
VI		318.79

Fig. 1. Synthesis and structures of isothiouronium compounds.

Cell culture. The origin and properties of the human melanoma cell lines MM96L, MM96E, MM418 and A2058, the human cervical carcinoma line HeLa, and the human virus-transformed keratinocyte line KJD-1/SV40 have been described previously [8–13]. 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 nicotinamide, 3 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid and either 5% or 10% (v/v) foetal calf serum. The monoclonal antibody (MoAb) B8G3 was used as the hybridoma supernatant. Routine assays for mycoplasma by Hoechst dye 33258 were negative [14].

Cell survival. Cell survival was determined by a modified colony assay [15], in which cells were plated into a 96-well microtitre plate (Nunc, Denmark) at 1×10^3–2×10^3/cells/well and allowed to attach overnight. The cells were continuously exposed 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, pH 7.2 (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.

Tyrosinase assay. Tyrosinase (dopa oxidase) activity was determined as follows. Cells were plated into 10 cm dishes at 5×10^5–1×10^6 cells/10 mL, allowed to attach overnight and then continuously treated with drugs 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. Triplicate 15 μL aliquots of the supernatant were added to 150 μL of a mixture of 7.6 mM dopa and 50 mM phosphate, pH 6.8, in the wells 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 [2].

PAGE-western blotting. For immunoblotting, approximately 2×10^7 cells were sonicated in 500 μL of lysis buffer (10 mM dithiothreitol, 20% glycerol, 1% sodium dodecyl sulphate, 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 6–10% polyacrylamide gradient gel and electrophoresed in 25 mM Tris containing 192 mM glycine and 0.1% sodium dodecyl sulphate, pH 8.3. Relative molecular mass standards were purchased from Sigma.

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. Vis-

Table 1. Synthesis and properties of nordimaprit analogues

Compound*	Refluxing solvent	Reflux time (hr)	Recrystallizing solvent	Yield (%)	m.p. (°C)	^{1}H NMR (ppm)
I	Ethanol	20	Ethanol	61	213–215	(D_2O) 1.06 (12H, d, J = 8.4 Hz), 2.14 (2H, multiplet), 3.09 (4H, d, J = 6.3 Hz), 3.56 (4H, d of t, 4.2, 6.9 Hz)
II	Ethanol	30	Ethyl acetate: ethanol	100	183–185	(D_2O) 3.32 (2H, t, J = 8.4 Hz), 3.47 (2H, t, J = 8.4 Hz), 4.44 (4H, s), 7.54 (10H, multiplet, Ar)
III	Ethanol	8	Ethyl acetate: ethanol	59	121–123	(D_2O) 1.11 (3H, t, J = 7.1 Hz), 3.35 (2H, t, J = 6.8 Hz), 3.4 (2H, q, J = 5.9 Hz), 3.66 (2H, t, J = 5.5 Hz), 6.89 (1H, t, J = 7 Hz), 6.69 (2H, d, J = 8.4 Hz), 7.37 (2H, t, J = 8 Hz)
IV	Methanol	24	Ethanol	81	208–210	(D_2O) 1.64 (2H, s), 1.91 (4H, s), 3.29 (4H, s), 3.47 (2H, t, J = 7.6 Hz), 3.55 (2H, t, J = 7.3 Hz)
V	Methanol	15	Methanol	48	225–226	(D_2O) 3.41 (4H, s), 3.57 (4H, multiplet), 3.99 (4H, s)
VI	Methanol	8	Methanol	96	250–252	(D_2O 3.38 (3H, s), 3.55 (3H, s), 3.67 (2H, t, J = 6.1 Hz), 4.67 (2H, t, J = 6.1 Hz), 8.06 (1H, s)

* Structures shown in Fig. 1.

Table 2. Toxicity of DINOR and analogues I–VI in human cell lines

Cell type	D_{37} (μM)*						
	DINOR	I	II	III	IV	V	VI
Melanoma							
MM96E	65	40	8	48	56	>100	40
MM96L	125	29	16	76	61	86	36
MM418	14	10	34	100	79	>100	90
A2058	75	73	13	100	92	>100	>100
Non-melanoma							
HeLa	78	35	18	97	40	>100	46
KJD	80	>100	28	81	54	>100	57

* Dose required to reduce survival to 37%; interpolated from the combined response curve of two to four experiments.

ualization of protein bands was achieved by incubation with alkaline phosphatase-linked anti-mouse immunoglobulin G antibody (Silenus, Melbourne, Australia) diluted 1 in 1000 in PBS containing 0.02% Tween 20, followed by NBT–BCIP substrate [0.75 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 0.75 mM nitroblue tetrazolium (NBT), 5 mM $MgCl_2$ and 0.1 M Tris, pH 9.5] [16, 17]. Band intensities were measured by a Scanning Computer Densitometer and analysed 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 the protein standard.

Melanin content. Cells were harvested by trypsin-EDTA, washed in PBS, lysed with water, pelleted, then dissolved in Soluene-350 (Packard, Groningen, The Netherlands). The absorbance of the solution was read on a Cary 4E UV-Visible Spectrophotometer (Varian, Melbourne, Australia) at 405 nm against standards of dopa-melanin dissolved in Soluene-350. HeLa cells were used as a non-pigmented control cell line. Results were expressed as μg of melanin/mg protein [18].

RESULTS

The structures and physical properties of the nordimaprit analogues are shown in Fig. 1 and Table 1, respectively.

Cell survival

Investigation of the toxicity of these analogues in melanoma and non-melanoma cell lines (Table 2) showed that compared with DINOR the more hydrophobic diisobutyl analogue (I) showed increased killing with a degree of selectivity for the pigmented MM418 cells (Table 2, Fig. 2). The dibenzyl compound (II), the most hydrophobic analogue synthesized, was extremely toxic to all cells tested with a D_{37} of 8 μM for the MM96E cell line and 18 μM for HeLa; MM418 was more resistant to

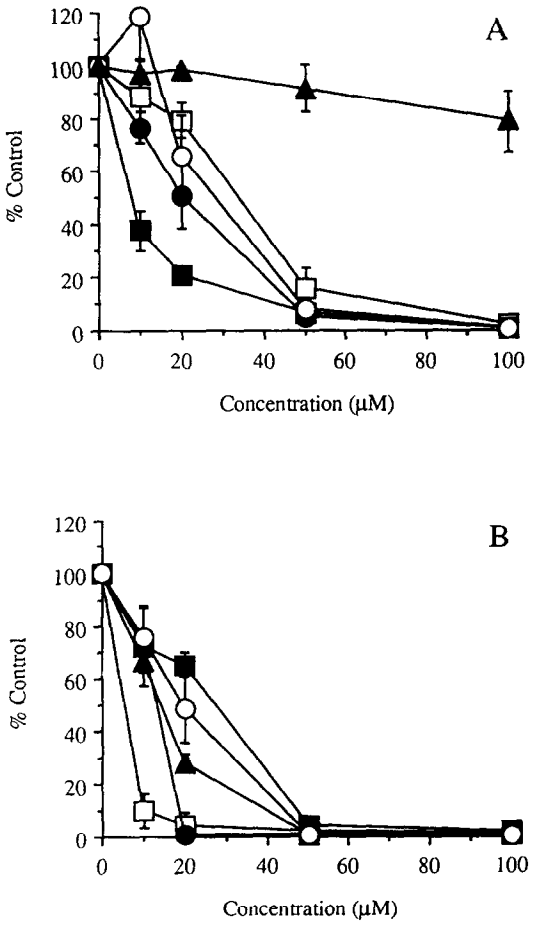


Fig. 2. Dose–response of cell survival for compound I (A) and compound II (B) in human malignant melanoma cell lines MM96E (□), MM418 (■), MM96L (●) and the non-melanoma cell line HeLa (○), and KJD-1/SV40 (▲). Points represent the means and SE of three experiments.

Table 3. Effect of nordimaprit analogues on tyrosinase activity in MM96E and MM418 cells treated for 4 days

Drug	Treatment Dose (μM)*	MM96E	MM418
DINOR	100	3†	52
	50	11	96
	20	59	112
	10	73	131
I	50	100	82
	20	100	98
	10	69	74
II	10	76	140
	5	100	80
III	100	124	110
	50	108	136
	20	103	110
	10	106	107
IV	50	79	97
	20	79	96
	10	84	96
V	100	73	70
	50	94	99
	20	95	105
	10	97	100
VI	100	63	NT‡
	50	75	90
	20	87	100
	10	100	NT

* Maximum dose applied was limited by drug toxicity.

† Enzyme rate was calculated as the change in A490/min/mg protein, and was determined from the mean of triplicates, after treatment with drugs simultaneously for 4 days.

‡ Not tested.

(II) than the other melanoma cell lines. The association of toxicity with lipophilicity was more rigorously demonstrated by the respective toxicities of the piperidine (IV) and morpholine (V) derivatives, where the less lipophilic but structurally identical morpholine derivative was markedly less toxic than the piperidine analogue in all cell lines tested, all but MM96L cells having a D_{37} greater than 100 μM. Compounds II–VI did not show selective toxicity to the pigmented MM418 cell line.

Table 5. Comparison of melanin content and tyrosinase activity of MM418 cells following long-term treatment (>1 month)

Treatment	Melanin content (% control)*	Tyrosinase activity (% control)
DINOR (20 μM)	13	65 ± 5
I (20 μM)	8 ± 5	97 ± 3
II (10 μM)	5 ± 4	148 ± 3
III (50 μM)	0.6 ± 0	232 ± 22
IV (20 μM)	6 ± 4	198 ± 21
V (50 μM)	61 ± 1	248 ± 8
VI (50 μM)	51 ± 5	111 ± 19

* Mean and SE (N = 3).

Tyrosinase activity

None of these agents directly inhibited tyrosinase activity when added to an assay mixture (results not shown). The following studies refer to cells cultured in the presence of drugs for various periods. Increasing the size or hydrophobicity of the tertiary amine in DINOR caused loss of inhibition of tyrosinase, at the doses tested on both MM96E and MM418 cell lines (Table 3). The two aromatic analogues (compounds II and III) induced an increase in activity in MM418 cells after 4 days of continuous treatment. Subsequent comparisons were made using cells treated for 4 days (Table 4) or after 4 weeks when depigmentation had stabilized (Table 5). The highest tolerated concentration of drug was used in each case, the actual level being somewhat higher than the D_{37} values, presumably because of reduced toxicity at the higher cell densities used for tyrosinase assays. After long term treatment (>1 month) (Table 5) of the constitutively pigmented cell line MM418 at non-toxic doses, under which conditions pre-existing melanin became diluted by cell proliferation, there was an increase in tyrosinase activity to approximately double that of control, particularly with compounds II–V. This contrasted with that of DINOR where a decrease in activity (65%) occurred.

Melanin content

The increase in tyrosinase activity induced by

Table 4. Comparison of melanin content and tyrosinase activity of MM418 cells following short-term treatment (4 days)

Treatment	Melanin content (% control)*	Tyrosine activity (% control)
Nordimaprit (50 μM)	72.31 ± 12	61 ± 7
DINOR (20 μM)	55 ± 11	68 ± 0
I (20 μM)	68 ± 12	98 ± 3
II (10 μM)	42 ± 12	140 ± 21
III (50 μM)	49 ± 13	136 ± 5
IV (20 μM)	49 ± 15	96 ± 5
V (50 μM)	75 ± 12	99 ± 1
VI (50 μM)	78 ± 10	90 ± 5

* Mean and SE (N = 3).

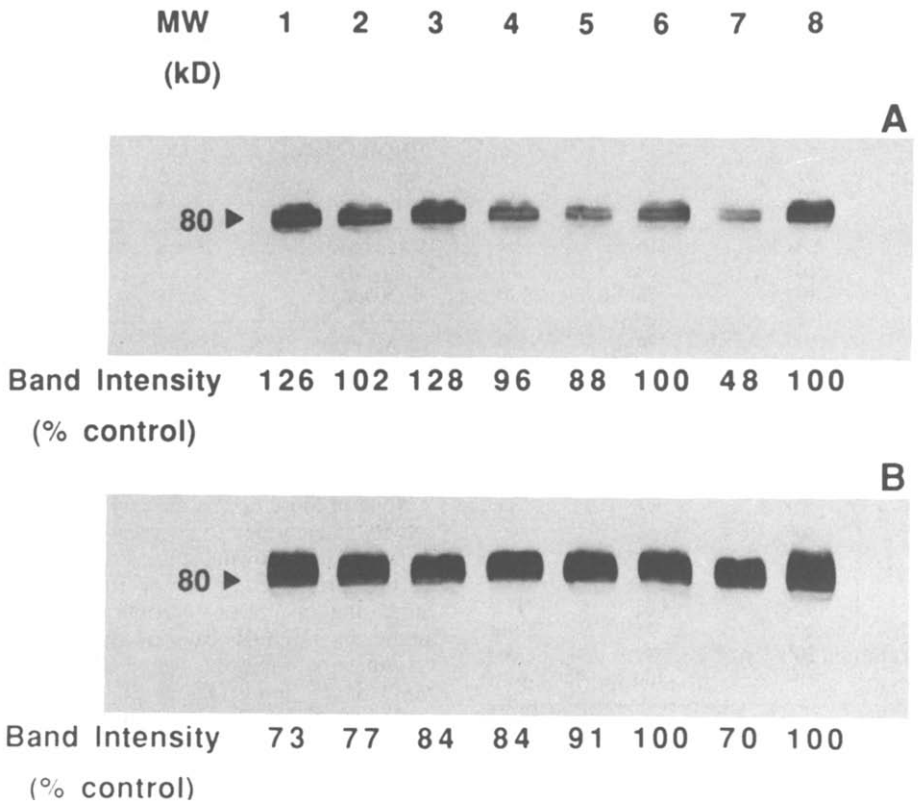


Fig. 3. Expression of B8G3 antigen in MM96E (A) and MM418 (B) detected by western blotting following 4-day treatment of cells with drugs. Lane 1, 50 μM VI; lane 2, 50 μM V; lane 3, 20 μM IV; lane 4, 20 μM III; lane 5, 10 μM II; lane 6, 20 μM I; lane 7, 20 μM DINOR; lane 8, control (untreated cells).

drugs II–V was not reflected in the amount of melanin in both short-term (4 days) and long-term treated (>1 month) MM418 cells (Tables 4 and 5). After 4 days continual treatment, the most effective depigmenting agents were compounds II, III and IV, where all cells had a melanin content of less than 50% of untreated cells but an enzyme activity of 140%, 136% and 96%, respectively. The tyrosinase inhibitors, nordimaprit and DINOR, produced a decrease in melanin content to 72% and 55%, respectively. When comparing melanin content in long-term treated cells, there was almost complete loss of melanin and subsequent depigmentation in all but those cells treated with compounds V and VI which only gave a decrease to 61% and 51%, respectively.

Expression of b-*locus protein*

Immunoblotting of lysates from drug-treated cells using B8G3, a monoclonal antibody which recognizes the melanosomal *b*-locus protein, showed that expression of the antigen in treated MM96E cells was suppressed by DINOR, the compound which caused a decrease in tyrosinase activity (Fig. 3). There was no significant change in expression in cells treated with compounds II and III where an increase in tyrosinase activity and depigmentation was seen in treated MM418 cells.

DISCUSSION

The diethyl and the diisopropyl (DINOR) derivatives of nordimaprit were more toxic than nordimaprit, particularly to the heavily melanized MM418 cell line, and were stronger inhibitors of tyrosinase activity in MM96E cells [4]. In the present study, the addition of one extra carbon to form the diisobutyl analogue (compound I) was found to increase toxicity, with MM418 cells remaining the most susceptible to killing. However, any further increases in hydrophobicity of these groups caused a non-specific increase in toxicity and the ability to inhibit tyrosinase was lost. The toxicity of these compounds may be mediated through the mechanism by which nordimaprit and dimaprit inhibit lymphocyte proliferation [19]. It was proposed that the non-ionized moiety crosses into the lysosomes where it becomes protonated and results in an increase in pH and interference of lysosomal function which is necessary to stimulate cell growth. Nordimaprit was found to be more potent than dimaprit and histamine, and inhibition of lymphocyte proliferation was not

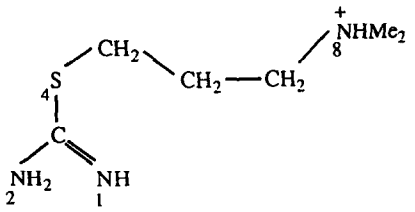


Fig. 4. Monocation of dimaprit.

blocked by the histamine H2 antagonists, cimetidine and metiamide [19, 20]. This may also explain the hydrophobic dibenzyl analogue (compound II) being more toxic than the ethylaniline derivative (III) as anilines have lower pK_a values compared to benzylamines.

Increasing hydrophobicity and also more importantly the size of the groups on this amine also destroy the ability to inhibit tyrosinase activity. DINOR and nordimaprit are thought to act through the H2 receptor [4]. Increasing the size of the groups on this amine most likely hinders the binding capacity of these compounds to the receptor and hence loss of activity. Empirical atom–atom potential calculations for dimaprit suggest that its agonist action is related to the characteristic $N_2 \cdots N_8$ and $S_4 \cdots N_8$ distances (Fig. 4) being similar to histamine in the gauche conformation [21]. A piperidine analogue of dimaprit showed negligible H2 activity when tested *in vitro* on the rat gastric acid secretion and guinea-pig atrium. This was attributed to the molecule being unable to fold the functional groups into positions that resemble those of histamine, presumably due to the steric interactions from Van der Waal's forces [22, 23]. Therefore, the best structure–activity relationship appears to be achieved by the diisopropyl analogue, with any further increases causing loss of flexibility and conformation necessary for H2 receptor binding.

Although there was no loss of tyrosinase activity, these sterically hindered compounds, particularly compounds I–IV, were able to depigment the MM418 cell line. The reason for this inhibition of pigmentation is not clear as few agents are able to cause depigmentation without affecting tyrosinase activity [18]. One such agent is the serotonin uptake antagonist, DU 24565 (6-nitroquipazine). It presumably facilitates this decrease in melanogenesis by depressing intracellular dopa levels, either by efflux from the cell or through some effect on dopa transport and metabolism [18]. This would prevent dopa from undergoing polymerization to melanin [24]. Some 2-aryl-1,3-thiazolidines inhibited melanogenesis via masked sulphydryl compounds acting distal to tyrosinase by sequestering the dopaquinone required in the pigmentation pathway [25]. Here, this is not possible as diverting dopaquinone would prevent the formation of dopachrome which is measured in the assay used. However, if the present drugs are metabolized to free sulphydryl compounds within the cell, putative quinones occurring further down the pigmentation pathway may be affected by a similar mechanism. Alternatively, the drugs could be sequestered by and disrupt subcellular organelles, particularly the melanosomes in which melanin is synthesized and packaged for transport into keratinocytes. Imokawa [6] found that aberrant internal structures of melanosomes, caused by specific inhibition of core carbohydrate synthesis with tunicamycin and glucosamine, resulted in a marked loss of melanogenesis with no substantial change in tyrosinase activity [6]. On the other hand, after short-term treatment (4 days) there was no change in expression of the *b*-locus protein (B8G3) antigen which is thought to play some structural or enzymatic role in melanosomes [4]. The loss of melanin coincided with a paradoxical increase in tyrosinase activity. Possibly, this increase is due to a feedback response of the cell to depigmentation leading to increased activity of tyrosinase or, more simply, less inactivation of tyrosinase by copolymerization with melanin.

These new nordimaprit analogues have revealed an alternative but as yet undefined means for controlling pigmentation in human melanoma cells. Elucidation of the target molecules may provide further understanding of the complex process of pigmentation.

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