Pergamon

Biochemical Pharmacology, Vol. 48, No. 1, pp. 121–130, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0110-7

REDUCTION OF DNA SYNTHESIS, PIGMENT SYNTHESIS, PIGMENTATION GENE mRNA AND RESISTANCE TO UVB IN HUMAN MELANOMA CELLS TREATED WITH ANALOGUES OF A HISTAMINE (H2) AGONIST

GREGORY A. FECHNER, JOANNA MICHEL,* RICK A. STURM,† JEFF J. JACOBS and PETER G. PARSONS*‡

Pharmacy Department, The University of Queensland, 4072; *Queensland Cancer Fund Research Unit, Queensland Institute of Medical Research, Herston, Queensland 4029; and the †Centre for Molecular Biology and Biotechnology, The University of Queensland, 4072, Australia

(*Received* 29 *November* 1993; *accepted* 24 *February* 1994)

Abstract—Two groups of *S*-[2-(*N*,*N*-dialkylamino)ethyl]isothiourea derivates which depigmented melanoma cells either with inhibition of tyrosinase (group 1, R = methyl, isopropyl) or without inhibition of tyrosinase (group 2, R = benzyl, phenyl) were studied. Treatment of human melanoma cells with non-lethal doses of group 1 drugs led to a reduction in the levels of mRNA for the pigmentation genes tyrosinase, tyrosinase-related protein-1 and Pmel 17. The group 1 drug *S*-[2-(*N*,*N*-diisopropylamino)ethyl[isothiourea] (DINOR) (R = isopropyl) produced only moderate inhibition of DNA, RNA and protein synthesis in three cell lines during the first 24 hr of treatment, and there was no correlation between the extent of inhibition and long-term toxicity. A group 2 drug (R = benzyl) rapidly inhibited DNA synthesis in an amelanotic melanoma cell line (MM96E) sensitive to killing by the drug; association of the latter with inhibition of RNA or protein synthesis was less clear. MM96E cells were also sensitive to killing by reactive oxygen species. In pigmented melanoma cells (MM418), incorporation of [^{125}I]thiouracil, a false precursor of melanin, increased during the first 24 hr of treatment with DINOR whereas a group 2 drug (R = phenyl) inhibited incorporation of [^{125}I]thiouracil. Cells depigmented by treatment with drugs from either group suffered the same amount of DNA damage as pigmented cells after UVB irradiation, as judged by inhibition of DNA synthesis, but did not recover as well as pigmented cells, whether or not drug was present during recovery. These results suggested that (1) group 1 agents down-regulated message for several pigmentation genes, possibly at the transcriptional level; (2) the toxicity of group 2 drugs was related to reactive oxygen species; and (3) melanin protected cells from UVB by enhancing cellular recovery.

Key words: melanoma; pigmentation; ultraviolet light; histamine agonists; gene expression

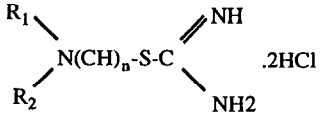
The increase in pigmentation seen after UV exposure, known as the delayed tanning response, is stimulated by UVB (290–315 nm) and to a lesser extent UVC. It commences 48–72 hr after irradiation and reaches a maximum at 7 days [1–3]. It is thought that the UV-filtering properties of melanins protect proliferating cells situated on the basal membrane of the epidermis and even the dermis itself [4]. UVB induced pigmentation is considerably more absorbing of UVB than the constitutive epidermal melanin pigment [3]. Kitano and Hu [5] found that pigmented B16 melanoma cells were more resistant to UVB cytotoxicity than non-pigmented B16 cells. Westerhof *et al.* [6] demonstrated that in a subject with vitiligo, the pigmented skin required twice the 310 nm UV energy to elicit erythema compared to vitiliginous skin.

However, it has also been shown that melanization does not provide complete protection of the epidermis from some of the deleterious effects of UVB radiation. Vermeer *et al.* [1] found that epidermal Langerhans cells were altered when heavily pigmented skin of black individuals were irradiated with low doses of UVB. Melanocytes from black and white subjects suffered similar toxicity when exposed to simulated sunlight where the highest photon energy was in the UVB range [7]. It is also known that chronic exposure to UV radiation results in mitogenic stimulus and proliferation of melanocytes [2, 8]. Therefore, the response of melanocytes and melanoma cells to UV radiation and in particular UVB is varied and complex.

There are currently a wide range of compounds which produce depigmentation *in vitro* and *in vivo*, either by inhibiting tyrosinase activity or by disrupting the pigmentation pathway post-tyrosinase [9]. Melanogenesis inhibitors can be divided into three groups [10, 11], comprising direct inhibitors of tyrosinase, compounds that indirectly cause loss of tyrosinase activity by cytotoxicity or inhibition of enzyme synthesis or processing and molecules that act independently of tyrosinase.

Two groups of *S*-[2-(*N*,*N*-dialkylamino)] isothiourea derivatives (Fig. 1) based on the H_2 agonist dimaprit reversibly depigment melanoma cells on long-term, non-toxic treatment in culture, either with inhibition of tyrosinase (group 1, R = methyl, isopropyl) or without inhibition of tyrosinase (group 2, R = benzyl, phenyl) [12]. Selective toxicity was found for certain cell lines at higher doses. To further

‡ Corresponding author.



R1	R2	n	
Group 1			
CH_3	CH_3	2	Nordimaprit
$CH(CH_3)_2$	$CH(CH_3)_2$	2	S,2-(N,N-diisopropylamino)ethylisothiourea (DINOR)
Group 2			
CH_2-C_6H_5	CH_2-C_6H_5	2	S,2-(N,N-dibenzylamino)ethylisothiourea (DBNOR)
C_6H_5	C_2H_5	2	S,2-(N-ethyl,N-phenylamino)ethylisothiourea (ANNOR)

Fig. 1. Stucture of nordimaprit and its analogues.

Table 1. Melanin content of MM418 cells following long-term treatment (>1 month)

Treatment	Melanin content (% control)
Nordimaprit (20 μM)	2
DINOR (20 μM)	6.5
ANNOR (50 μM)	1

elucidate the mechanism of action of these drugs their effects on macromolecular synthesis and mRNA levels of pigmentation genes were studied, and showed that depigmented cells were more susceptible to killing by UVB.

MATERIALS AND METHODS

Cell culture. The origin and properties of the human melanoma cell lines MM96E and MM418 and the human cervical carcinoma line HeLa have been described previously [13–15]. The human melanoma cell line MM96E is a slightly pigmented, near-diploid-subline of MM96, which was derived from a melanoma metastasis. The MM418 cell line is constitutively pigmented, partially aneuploid and was derived from a heavily pigmented primary melanoma [16]. Depigmented cell lines of the pigmented MM418 cell were established by continual treatment (drugs were added twice a week when cells were passaged) for >1 month with either 20 μM nordimaprit,* 20 μM DINOR or 50 μM ANNOR (the ethylaniline derivative of nordimaprit). Melanin contents [12] for each cell line are shown in Table 1 and, unless indicated, the cells were continually treated with drugs. 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, 100 IU/mL penicillin, 0.17 mM streptomycin, 3 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid and either 5% or 10 (v/v) foetal calf serum. Routine assays for mycoplasma by Hoechst dye 33258 were negative [17].

Macromolecular synthesis. DNA, RNA and protein syntheses were determined using the general procedure of Parsons *et al.* [18]. Cells (MM96E, MM418 and HeLa) were seeded into 96-well plates (Nunc, Denmark) at 5×10^4 cells/well and allowed to attach overnight. The cells were continuously exposed to the nordimaprit derivatives, DINOR [19] and DBNOR [12] for varying times from 1 to 24 hr. The cells were then labelled with [^{3}H-methyl]-thymidine (10 μCi/mL, 5 Ci/mmol, Amersham, U.K.) for 45 min. Cells were washed with 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).

RNA synthesis was determined as above except that after continuous treatment with drugs, the cells were labelled with [5-^{3}H]uridine (20 μCi/mL, 5 Ci/mmol, Amersham, U.K.) for 1 hr. Protein synthesis was measured using the same procedure as above. The cells were labelled using [^{35}S]methionine (10 μCi/mL, 500 mCi/mmol, Amersham, U.K.). Cells were then washed, harvested and counted as above.

Cell survival. Cell survival was determined by a modified colony assay [20], in which cells were plated into a 96-well microtitre plate (Nunc, Denmark) at 5×10^3–1×10^4 cells/well and allowed to attach overnight. The cells were continuously exposed to drugs at the doses used for macromolecular synthesis at varying times from 1 to 24 hr. After 24 hr the

* Abbreviations: ANNOR, *S*-[2-(*N*-ethyl,*N*-phenylamino)ethyl]isothiourea; DBNOR, S-[2-(*N*,*N*-dibenzylamino)ethyl]isothiourea; DINOR, *S*-[2-(*N*,*N*-diisopropylamino)ethyl[isothiourea; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; nordimaprit, *S*-[2-(*N*,*N*-dimethylamino)ethyl[isothiourea]; TRP-1, tyrosinase related protein-1.

drugs were removed and replaced with fresh media. The cells were allowed to recover for 4 days and then labelled with [^{3}H-methyl]thymidine (2 μCi/mL; 5 Ci/mmol) for 4 hr. Cells were then washed, harvested and counted as above.

Incorporation of [^{125}I]2-*thiouracil.* Melanin synthesis was determined by measuring the incorporation of [^{125}I]2-thiouracil. 2-Thiouracil was labelled with ^{125}I using the method of Coderre and Halle [21]. Thiouracil (60 μg/mL in a 100 mM sodium phosphate buffer, pH 7) was added to a glass pill tube coated with 100 μg of IodoGen (Pierce, Rockford, IL, U.S.A.) and to this was added 5 μL of carrier-free Na^{125}I (0.5 mCi, Amersham, U.K.). After 5 min, 50 μL of KI solution (120 μg/mL) was added to complete iodination and the solution stirred for 30 min. This was transferred to a microfuge tube and 50 μL of 20 mM DTT was added to reduce any disulphide bonds. The solution was then microfuged and the supernatant analysed by thin layer chromatography for unbound ^{125}I ions. Unbound ^{125}I was removed by purifying down a small basic ion exchange column in the OH^- form.

To determine the effect that nordimaprit, DINOR and ANNOR had on melanin turnover, MM418 cells were plated out in a 96-well microtitre plate (Nunc, Denmark) at 5×10^4 cells/well and allowed to attach overnight. Cells were then treated for varying times up to 24 hr at non-toxic doses. In the final hour of treatment, [^{125}I]2-thiouracil was added to each well. The medium was removed and the cells washed and detached by incubating with 0.02% trypsin and 0.1 mM EDTA in PBS for 30 min at 37°. Cells were then harvested and added to scintillation tubes for counting in a gamma counter (Auto-Gamma 5000 series, Packard, Downers Grove, IL, U.S.A.).

Northern blotting. Cells (MM418 and MM96E) were plated at 2×10^6 cells/20 mL and allowed to attach overnight. They were then treated continuously for 4 days with either 50 μM nordimaprit or DINOR (20 μM DINOR for long-term treated, >1 month, MM418 cells). Cells were then washed with sterile PBS, harvested and RNA analysed on northern blots as previously described [22]. The tyrosinase, TRP-1 and pMel 17 probes were used as previously [22]. A GAPDH probe was prepared from pHGAP [23], and β-actin as an EcoRI-BamHI insert of pHFβA-3'UT [24]. Each of the probes were radiolabelled by random priming [25] and used at a concentration of 10^5–10^6 cpm/mL in the hybridization after heating for 5–10 min at 100°. Band intensities were analysed by Imagequant software (Molecular Dynamics, CA, U.S.A.).

Cell survival following UVB irradiation. Pigmented MM418 cells and depigmented MM418 cell lines were suspended in PBS and added to 5 mL Petri

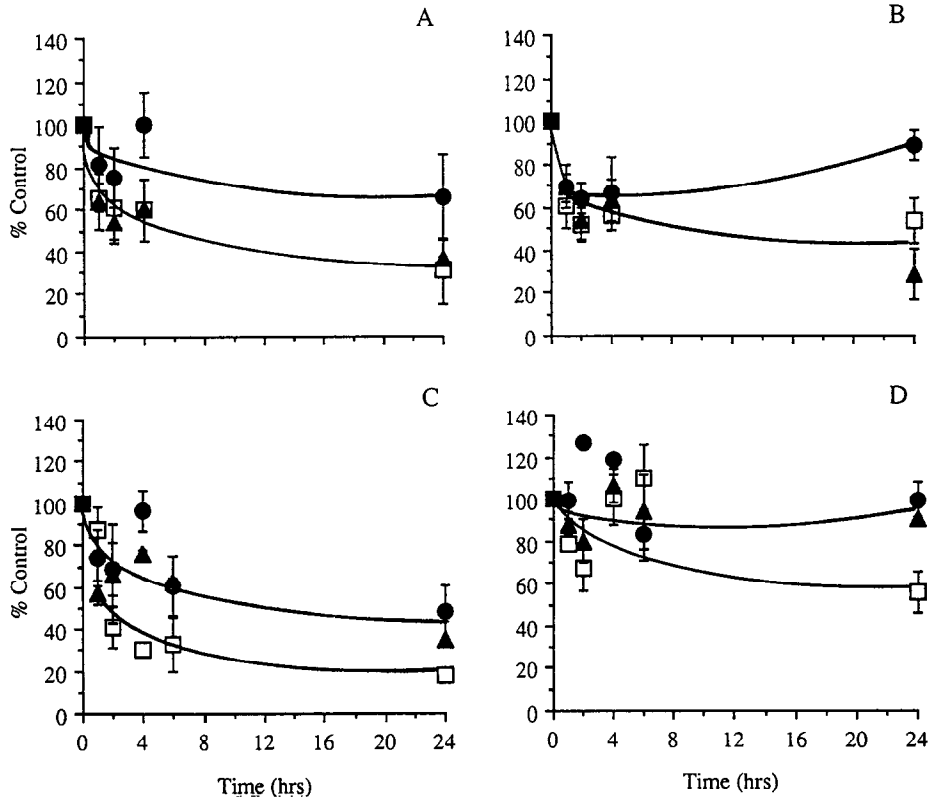


Fig. 2. Temporal effect of 50 μM DINOR on macromolecular synthesis in HeLa (□), MM96E (▲) and MM418 (●) cells. A, DNA synthesis: B, RNA synthesis; C, protein synthesis; D, cell survival. Points represent the means and SEs of three experiments.

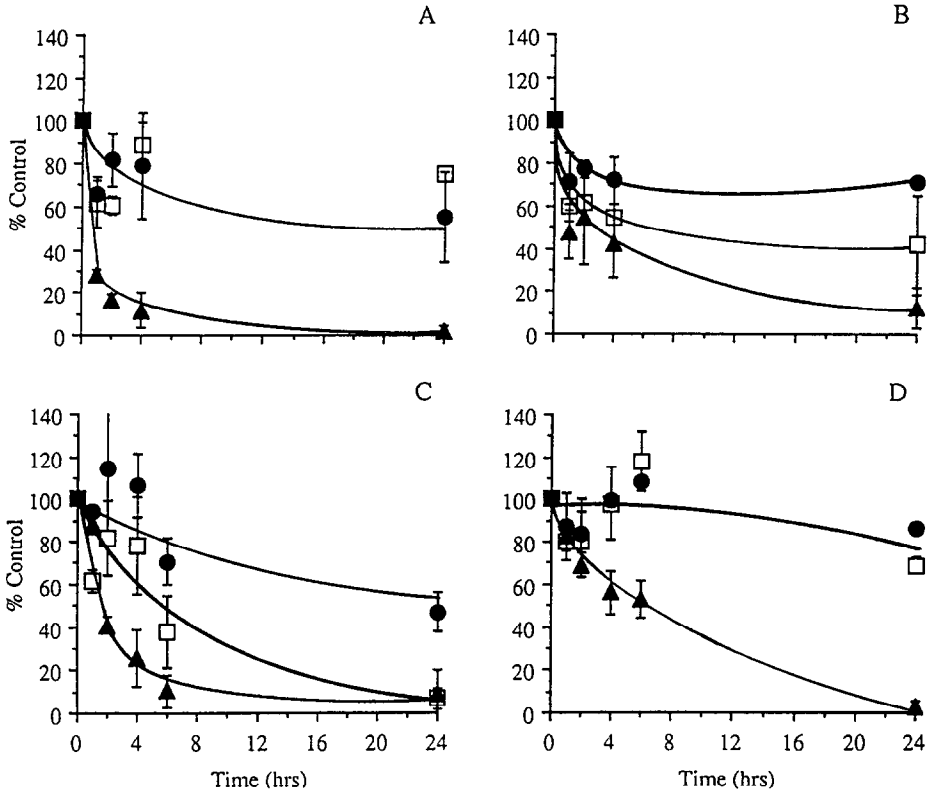


Fig. 3. Temporal effect of 50 μM DBNOR on macromolecular synthesis in HeLa (□), MM96E (▲) and MM418 (●) cells. A, DNA synthesis: B, RNA synthesis; C, protein synthesis; D, cell survival, determined by DNA synthesis 4 days after drug treatment. Points represent the means and SEs of three experiments.

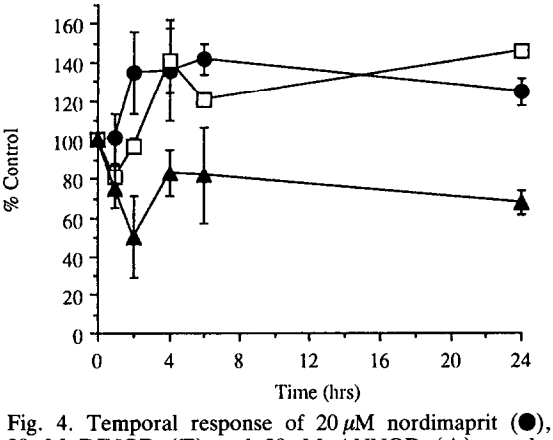


Fig. 4. Temporal response of 20 μM nordimaprit (●), 20 μM DINOR (□) and 20 μM ANNOR (▲) on the synthesis of new melanin in MM418 cells, determined by incorporation of [^{125}I]thiouracil for the last hour of treatment. Points represent the means and SDs of triplicates.

dishes at 7.5×10^5 cells/5 mL. Each dish was irradiated with UVB radiation (2 J m^{-2} s^{-1}) for 0.25, 0.5, 1 and 2 min. Flux was measured with an IL-1700 radiometer. After each treatment a sample was removed. The cells from each sample were resuspended in fresh culture medium and seeded into a 96-well microtitre plate (Nunc, Denmark) to measure DNA synthesis after 1 day (initially, 5×10^4 cells/well), 2 days (2×10^4 cells/well), 3 days (5×10^3 cells/well) or 4 days (2×10^3 cells/well), with and without drugs, as follows. After the appropriate recovery time, the cells were labelled with [^{3}H-methyl]thymidine (2 μCi/mL, 5 Ci/mmol, Amersham, U.K.) for 4 hr. The cells were then washed with 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} (UV fluorescence which gave 37% survival) was interpolated from the dose–response curves.

Tyrosinase assay. Tyrosinase (dopa oxidase) activity for cells treated with UVB radiation was determined as follows. MM96E cells were seeded into 10 mL Petri dishes at 1×10^6 cells/10 mL, allowed to attach overnight and treated continuously with 20 μM nordimaprit for 4 days. The media was then replaced with 10 mL PBS and the cells irradiated for 0.25, 0.5, 1 and 2 min with UVB radiation (2 J m^{-2} s^{-1}). The PBS was removed and fresh culture medium without 20 μM nordimaprit added and cells allowed to recover for 0, 4, 24, 48 and 72 hr. The cells were then washed with PBS and

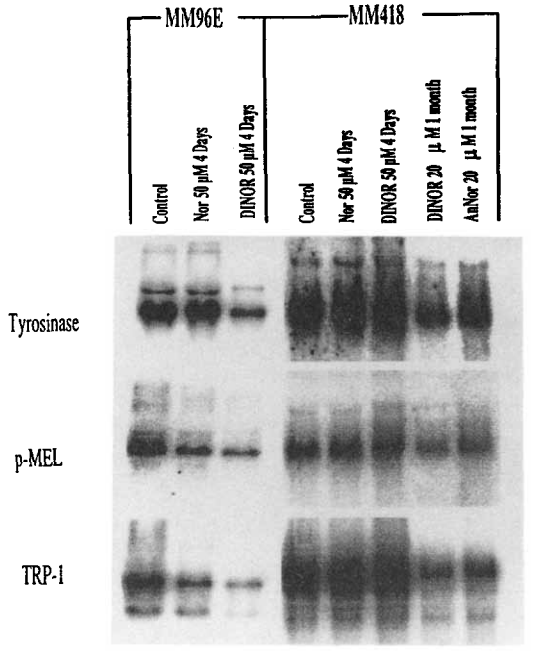


Fig. 5. Northern blot of RNA from MM96E and MM418 treated for 4 days with 50 μM nordimaprit or DINOR, and from long-term treated MM418 cells (20 μM DINOR). Quantitative comparisons after normalization against β-actin are given in Table 2.

pelleted. Tyrosinase (dopa oxidase) 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 [16].

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.

RESULTS

DNA, RNA and protein synthesis

A temporal study of DNA, RNA and protein synthesis in HeLa, MM96E and MM418 cells was carried out using the nordimaprit analogues DINOR (Fig. 2) and DBNOR (Fig. 3).

DNA synthesis was measured by the incorporation of [^{3}H]thymidine after treatment with drugs for various times up to 24 hr (Figs 2A and 3A). The melanoma cell line MM418, previously found to be the most susceptible to the toxicity of these compounds when the treatment was continuous [19], displayed the least inhibition of DNA synthesis. Treatment with DBNOR rapidly inhibited DNA synthesis in MM96E (Fig. 3A), but not in MM418 or HeLa cells. Changes in RNA synthesis, measured by [^{3}H]uridine incorporation (Figs 2B and 3B), broadly followed those found for DNA synthesis. HeLa cells showed an rapid decrease during the first 2 hr and then a steady decline over 24 hr. Once again the MM418 cells were resistant to inhibition of RNA synthesis, after an initial decrease. Protein synthesis (Figs 2C and 3C) decreased in all cell lines following treatment with both drugs, with DBNOR being the more effective agent.

To determine whether there was a relationship between toxicity and inhibition of DNA, RNA and protein synthesis, a cell survival study was carried out using the same doses and treatment times as above. Cells were labelled with [^{3}H]thymidine 4 days (2–3 cell doubling times) after drug treatment, when DNA synthesis would reflect recovery and clonogenic cell survival. HeLa was somewhat more susceptible to DINOR than the two melanoma cell lines (Fig.

Table 2. mRNA of pigmentation genes TRP-1, tyrosinase and Pmel 17 in melanoma cells, normalized to β-actin

Treatment	Gene				
	β-Actin	TRP-1	Tyrosinase	Pmel 17	GAPDH
4 days					
MM96E control	100	100	100	100	100
Nordimaprit (50 μM)	100	24	79	32	48
DINOR (50 μM)	100	23	61	40	42
MM418 control	100	100	100	100	100
Nordimaprit (50 μM)	100	84	115	79	89
DINOR (50 μM)	100	142	158	123	124
Long-term*					
DINOR (20 μM)	100	29	67	38	53

* MM418 cells treated for >1 month.

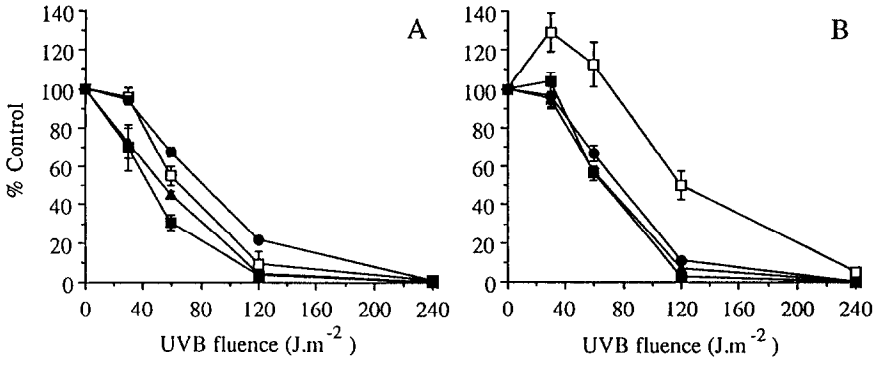


Fig. 6. Inhibition of DNA synthesis in MM418 cells by UVB radiation. The cells were pigmented controls (□) or were depigmented by previous treatment for 1 month with 20 μM nordimaprit (▲), 20 μM DINOR (■) or 50 μM ANNOR (●). No drugs were present during the post-irradiation period. A, 24 hr after irradiation; B, 72 hr after irradiation. Points represent the means and SEs of three experiments.

2D). DBNOR had little effect on HeLa and MM418 cells but even a 4–6 hr treatment inhibited the proliferation of MM96E and few cells survived a 24 hr treatment (Fig. 3D).

Melanin synthesis

[^{125}I]Thiouracil, a false precursor of melanin, could be used to follow the synthesis and degradation of melanin [26]. In this study, only the pigmented MM418 cells could be used because amelanotic melanoma cell lines did not incorporate thiouracil to any significant extent [27]. The cells were treated for various times up to 24 hr with non-toxic doses of nordimaprit (20 μM), DINOR (20 μM) and ANNOR (20 μM). Nordimaprit and DINOR were chosen because they were the two best inhibitors of tyrosinase activity, while ANNOR produced the greatest depigmentation of MM418 cells without inhibiting tyrosinase. The results were expressed as a percentage of the untreated control cells, which gave an incorporation of about 2000 cpm per well (Fig. 4). Both nordimaprit and DINOR produced an increase in [^{125}I]thiouracil incorporation of up to 140% of controls and were able to sustain this level for 24 hr. The ethylaniline derivative produced a 50% decrease in thiouracil incorporation after 3 hr of treatment; incorporation then increased, with partial recovery by 4 hr and a subsequent gradual decline.

Detection of mRNA by northern blotting

The effect of the tyrosinase inhibitors, nordimaprit and DINOR on the mRNA levels of the pigmentation genes, tyrosinase, TRP-1 and Pmel 17, was determined by northern blotting of RNA from treated cells. Message for β-actin, an abundant structural protein, was used as an internal control to correct for differences in the amount of RNA loaded. The results (Fig. 5, Table 2) showed that MM96E cells treated for 4 days with nordimaprit (50 μM) or DINOR (50 μM) displayed decreased message of all three pigment genes examined, with TRP-1 (24% and 23% of the control, respectively) and Pmel 17 (32% and 40% of the control, respectively) having the most prominent decreases. Message for the GAPDH gene was also lowered (Table 2).

Treatment of MM418 cells with nordimaprit or DINOR (50 μM) for 4 days resulted in relatively small changes in mRNA levels. However, MM418 cells grown over the long term (>1 month) in the presence of DINOR (20 μM) exhibited a decrease in mRNA of all three genes compared to control cells, with TRP-1 (29%) and Pmel 17 (30%) being the most affected. GAPDH was also decreased in these cells.

DNA synthesis/cell survival of UVB treated cells

Twenty-four hours after UVB irradiation, the pigmented MM418 cells showed similar dose–response curves for inhibition of DNA synthesis as chemically-depigmented cultures (Fig. 6A). By the second day, the pigmented MM418 control cells displayed slightly enhanced resistance, and after 3 and 4 days, survival of the control cells was clearly greater than that of the non-pigmented cells (Fig. 6B). UV-induced enhancement in the growth of pigmented cells (130% of the unirradiated control) was found 3 days after 30 J m^{-2} of UVB (Fig. 6B). The recovery of pigmented genes from UVB was compared with depigmented cells by plotting the D_{37} values against time after irradiation (Fig. 7A). DNA synthesis and cell survival following UVB were not affected by the presence of drugs during the post-irradiation period (Fig. 7B).

Tyrosinase activity in UVB-treated cells

MM96E cells were chosen for study rather than the pigmented MM418 cells because it had been found previously [19] that tyrosinase activity in the latter responded slowly to these drugs. In cells not treated with drugs, tyrosinase activity decreased following irradiation, recovered at 48 hr and subsequently declined (Fig. 8). In addition, MM96E cells were treated for 4 days with 20 μM nordimaprit to inhibit tyrosinase activity, then irradiated with

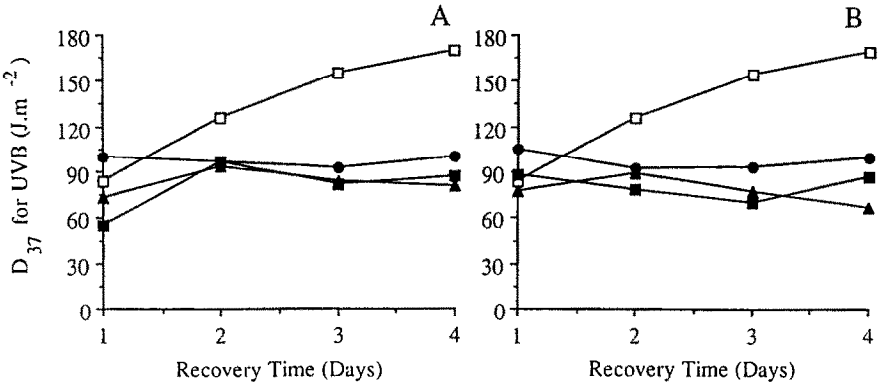


Fig. 7. D_{37} values for UVB inhibition of DNA synthesis in MM418 cells (□) and MM418 cells previously depigmented by treatment with 20 μM nordimaprit (▲), 20 μM DINOR (■) or 50 μM ANNOR (●). A, no drugs present post-irradiation; B, drugs were present at the same level as pre-irradiation.

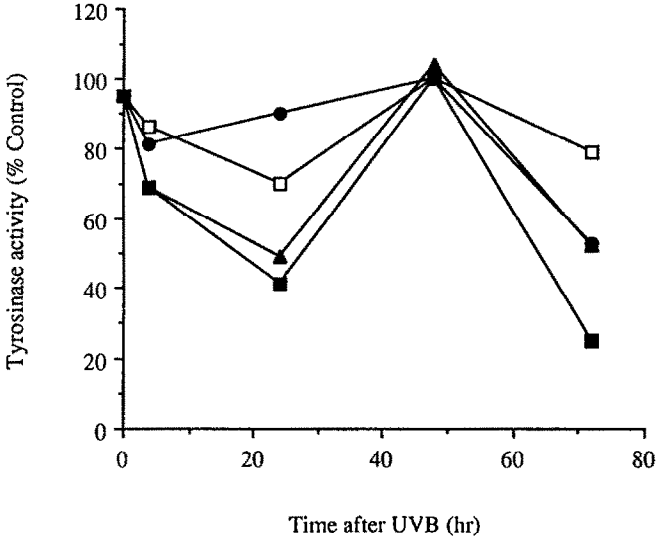


Fig. 8. Tyrosinase activity in MM96E cells following irradiation with 30 (□), 60 (●), 120 (▲) and 240 (■) J m^{-2} UVB. Control activity was 0.35 A_{490}/min/mg protein. Points represent the means of triplicates; SDs < 10%.

UVB and cultured without drug. The tyrosinase activity of treated cells was expressed as the percentage of activity in non-treated control cells irradiated with the same dose of UVB (Fig. 9). After release from treatment with nordimaprit, the initial tyrosinase activity (50% of the control) increased over 24–72 hr to reach approximately the control level. Tyrosinase activity in cells given 120 J m^{-2} UVB increased to 150% of the control at 48 hr.

DISCUSSION

It was decided to investigate the temporal changes in DNA synthesis along with RNA and protein synthesis in an attempt to determine the mechanism of toxicity of these isothiuronium compounds, particularly DINOR which in previous studies was found to be selectively toxic to the melanoma cell line MM418 over a 5 day period of continuous exposure, and DBNOR which depigmented these cells [12, 19]. When cells were treated for only 24 hr, cell survival, as judged by loss of DNA replication 4 days later, showed MM418 and MM96E cells to be more resistant than HeLa. In the latter, toxicity appeared to correlate with early inhibition of DNA synthesis; an appreciable dose-dependent block in the G_2 + M phase was also found (results not shown). Protein synthesis was also inhibited over a similar time period in HeLa, suggesting that DINOR has multiple effects in cells. MM96E and MM418 cells displayed inhibition of DNA synthesis at the early time points but this was not reflected in decreased

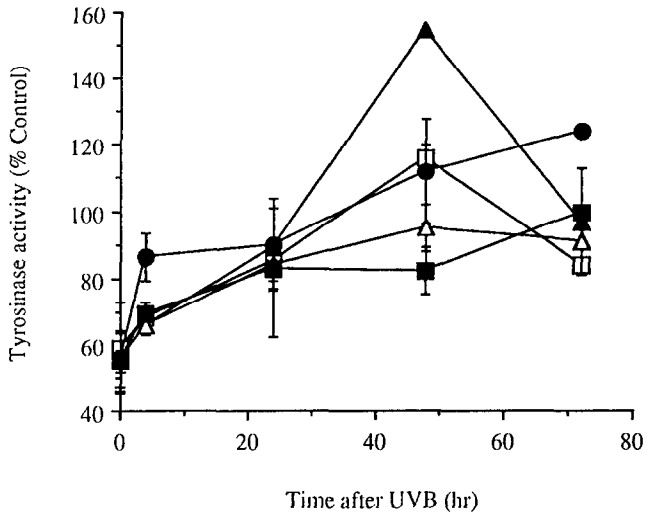


Fig. 9. The effects of UVB radiation on the recovery of tyrosinase activity in MM96E pre-treated with 20 μM nordimaprit for 4 days. Cells were irradiated with 0 (□), 30 (●), 60 (▲) and 240 (■) J m^{-2} UVB. Activity expressed as a percentage of untreated cells irradiated with the same dose of UVB. Points represent the means and SEs of three experiments.

survival, possibly because DINOR was detoxified by metabolism or, in the case of MM418, bound to melanin.

Short treatment times with DBNOR greatly increased the sensitivity of MM96E cells compared with MM418 and HeLa, and was associated with rapid inhibition of DNA synthesis. Compared with HeLa and MM418, MM96E cells are sensitive to killing by oxygen radicals generated from autoxidation of catechols, which also rapidly inhibit DNA synthesis [28]. DBNOR may therefore inhibit one or more of the cellular enzymes that detoxify oxygen species, such as catalase, superoxide dismutase or enzymes of glutathione metabolism. This could also be relevant to the depigmentation mechanism.

Concerning pigment synthesis, MM418 cells treated with nordimaprit or DINOR showed somewhat increased incorporation of [^{125}I]thiouracil over the first 24 hr, a period in which tyrosinase was not markedly inhibited in these cells [19]. This may have been due to an excess of dopaquinone being available because of drug-induced depletion of the competing endogenous nucleophiles, glutathione and cysteine. By contrast, ANNOR decreased the melanin content but not tyrosinase activity, and produced an immediate decrease in the incorporation of thiouracil. This suggested that the target was a post-tyrosinase step closely coupled to pigment synthesis, possibly a radical protection function as proposed for the catalase activity of TRP-1 [29].

Northern blot analysis of the levels of tyrosinase mRNA of nordimaprit-and DINOR-treated cells showed marginal inhibition of tyrosinase mRNA expression in MM96E cells after 4 days of treatment, insufficient to account for the loss of enzyme activity seen previously. Tyrosinase mRNA levels in MM418 cells increased slightly over the same period, which was not unexpected because tyrosinase activity was also found to increase [19]; long-term treatment was needed to decrease the mRNA level, along with tyrosinase activity and melanin content. This suggested that consecutive transcriptional changes were necessary for depigmentation of MM418 cells.

mRNA of two other pigmentation genes, particularly of TRP-1 and GAPDH (an abundant sulphydryl enzyme), was depleted more than tyrosinase mRNA. How these genes interact with each other in the overall regulation of pigmentation is unknown, and the present studies did not distinguish between decreased transcription or decreased stability of the mRNA. Transcription of some genes is sensitive to thiols, including glutathione, providing a link to the action of nordimaprit and DINOR if these drugs are metabolized to sulphydryl compounds. Long-term DINOR-treated MM418 cells also showed disruption of the structure of melanosomes and loss of TRP-1 protein [19]. Thus, depigmentation by such drugs may result from transcriptional down-regulation of TRP-1, possibly in concert with other genes.

The UVB experiments with pigmented and depigmented cells from the same cell line extended the results reported previously [30], where MM418 cells were found to be resistant to the cytotoxicity of UVB irradiation compared to the amelanotic cell line MM253cl. Enhanced survival was not considered to be due to shielding by melanin because there was no resistance to UVC [31]. In the present study identical levels of inhibition of DNA synthesis in pigmented and depigmented cells at early times (1–2 days) after UVB irradiation indicated that they suffered the same amount of initial damage to DNA. The subsequent enhanced recovery of the pigmented controls pointed to a delayed melanin-mediated protective (or "healing") response. This was a

previously unrecognized property of melanotic cells. Besides its ability to absorb UV and visible light, melanin is thought to act as a free-radical scavenger in the skin, trapping the free radicals produced during UVB irradiation [4], but such properties do not account for the present results where protection was not seen until cells completed one doubling time. Early inhibition of DNA synthesis, independent of template damage, is a protective mechanism to facilitate repair of damaged DNA [32], and may be enhanced in pigmented cells by mechanisms not yet understood. The effect is unlikely to be drug-induced *per se* because MM418 cells depigmented with both types of drug were equally susceptible to UVB.

Following 30 J m^{-2} of UVB radiation, proliferation of pigmented MM418 cells was increased compared with controls. Proliferation of melanocytes and melanoma cells was influenced by certain growth factors released following UV irradiation [33–35], including leukotriene C_4 [36], transforming growth factor TGFα [37] and basic fibroblast growth factor [38]. The fact that these stimuli were more effective on pigmented melanoma cells may be related to their more differentiated phenotype.

In human melanocytes UVB irradiation enhanced pigmentation [39] but at high doses decreased tyrosinase activity by 10% and melanin content by 40% [40]. In the MM96E melanoma cell line tyrosinase activity was decreased by UVB except at 48 hr after irradiation, when activity temporarily recovered. The reason for this is not known, but the time is similar to that for maximum UV-induced pigmentation in the skin. The overshoot of tyrosinase activity in cells pre-treated with drug following irradiation with 30 and 60 J m^{-2} may have arisen from delayed recovery of naturally occurring inhibitors found in amelanotic melanoma cells and skin [11, 41].

Acknowledgements—This work was supported by an Australian Postgraduate Research Award (GAF) and the National Health and Medical Research Council, Canberra.

REFERENCES

1. Vermeer M, Schmieder GJ, Yoshikawa T, van der Berg J, Metzman MS, Taylor JR and Streilein JW, Effects of ultraviolet B light on cutaneous immune responses of humans with deeply pigmented skin. *J Invest Dermatol* **97**: 729–734, 1991.
2. Ortonne JP, The effects of ultraviolet exposure on skin melanin pigmentation. *J Intern Med Res* **18**: 8C–17C, 1990.
3. Kollias N, Sayre RM, Zeise L and Chedekel MR, Photoprotection by melanin. *J Photochem Photobiol B: Biol* **9**: 135–160, 1991.
4. Schallreuter KU and Wood JM, Free radical reduction in the human epidermis. *Free Rad Biol Med* **6**: 519–532, 1989.
5. Kitano Y and Hu F, The effect of ultraviolet light on mammalian pigment cells *in vitro*. *J Invest Dermatol* **52**: 25–31, 1969.
6. Westerhof W, Bebelaar D, Cormane RH, Langelaar J and Middelkamp HJ, The protective role of epidermal melanin in a patient with porphyria variegata and vitiligo. *Acta Dermato-Venereal (Stockholm)* **61**: 23–28, 1987.
7. Yohn JJ, Lyons MB and Norris DA, Cultured human melanocytes from black and white donors have different sunlight and ultraviolet A radiation sensitivities. *J Invest Dermatol* **99**: 454–459, 1992.
8. Elder DE, Human melanocytic neoplasma and their etiologic relationship with sunlight. *J Invest Dermatol* **92**: 297S–303S, 1989.
9. Imokawa G, Analysis of initial melanogenesis including tyrosinase transfer and melanosome differentiation through interrupted melanization by glutathione. *J Invest Dermatol* **93**: 100–107, 1989.
10. Mishima Y, Hatta S, Ohyama Y and Inazu M, Induction of melanogenesis suppression: Cellular pharmacology and mode of differential action. *Pigment Cell Res* **1**: 367–374, 1988.
11. Ando S, Ando O, Suemoto Y and Mishima Y, Tyrosinase gene transcription and its control by melanogenic inhibitors. *J Invest Dermatol* **100**: 150S–155S, 1993.
12. Fechner GA, Jacobs JJ and Parsons PG, Inhibition of melanogenesis in human melanoma cells by novel analogues of the partial histamine (H2) agonist nordimaprit. *Biochem Pharmacol* **46**: 47–54, 1993.
13. Gey GC, Coffman WD and Kubicek MT, Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res* **12**: 264–265, 1952.
14. Maynard K and Parsons PG, Cross-sensitivity of methylating agents, hydroxyurea, and methotrexate in human tumor cells of the Mer^-phenotype. *Cancer Res* **46**: 5009–5013, 1986.
15. Pope JH, Morrison L, Moss DJ, Parsons PG and Mary R, Human malignant melanoma cell lines. *Pathology* **11**: 191–195, 1979.
16. McEwan MT and Parsons PG, Regulation of tyrosinase expression and activity in human melanoma cells via histamine receptors. *J Invest Dermatol* **97**: 868–873, 1991.
17. Chen TR, *In situ* detection of mycoplasma contamination in cell culture by fluorescent hoescht 33258 stain. *Exp Cell Res* **104**: 255–262, 1977.
18. Parsons PG, Favier D, McEwan M, Takahashi H, Jimbow K and Ito S, Action of cysteaminylphenols on human melanoma cells *in vivo* and *in vitro*: 4-*S*-cysteaminylphenol binds protein disulphide isomerase. *Melanoma Res* **1**: 97–104, 1991.
19. Fechner GA, Parsons PG, McEwan MT, Jacobs JJ and Takahashi H, Antiproliferative and depigmenting effects of the histamine (H2) agonist dimaprit and its derivatives on human melanoma cells. *Biochem Pharmacol* **43**: 2083–2090, 1992.
20. Parsons PG, Carter FB, Morrison L and Mary R, Mechanism of melphalan resistance developed in vitro in human melanoma cells. *Cancer Res* **41**: 1525–1534, 1981.
21. Coderre JA and Halle DA, Direct electrophilic iodination of 2-thiouracil using Iodo-Gen™. *Int J Radiat Appl Instrum* **4A**: 795–763, 1989.
22. Wong SSC, Sturm RA, Michel J, Zhang X-M, Danoy PAC, McGregor K, Jacobs JJ, Kaushall A, Dong Y, Dunn IS and Parsons PG, Transcriptional regulation of differentiation, selective toxicity and ATGCAAAT binding of bisbenzimidazole derivatives in human melanoma cells. *Biochem Pharmacol* **47**: 827–837, 1994.
23. Tso JY, Sun XH, Kao TH, Reece KS and Wu R, Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res* **13**: 2485–2502, 1985.
24. Ponte E, Gunning P, Blau H and Kedes L, Human actin genes are single copy for α-skeletal and α-cardiac actin but multicopy for β- and γ-cytoskeletal genes: 3′

untranslated regions are isotype specific but are conserved in evolution. *Mol Cell Biol* **13**: 1783–1791, 1983.
25. Feinberg AP and Vogelstein B, A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13, 1983.
26. Broxterman HJ, van Langevelde A, Bakker CM, Boer H, Journee-de Korver JG, Kaspersen FM, Kakebeeke-Kemme HM and Pauwels EKJ, Incorporation of [^{125}I]-5-iodo-2-thiouracil in cultured hamster, rabbit melanoma cells. *Cancer Res* **43**: 1316–1320, 1983.
27. Parsons PG and Allen BJ, Accumulation of chlorpromazine and thiouracil by human melanoma cells in culture. *Aust J Exp Biol Med Sci* **64**: 517–526, 1986.
28. Parsons PG, Modification of dopa toxicity in human tumour cells. *Biochem Pharmacol* **34**: 1801–1807, 1985.
29. Halaban R and Moellmann G, Murine and human b locus pigmentation genes encode a glycoprotein (gp 75) with catalase activity. *Proc Natl Acad Sci USA* **87**: 4809–4813, 1990.
30. Musk P and Parsons PG, Resistance of pigmented human cells to killing by sunlight and oxygen radicals. *Photochem Photobiol* **46**: 489–494, 1987.
31. Anderson RR and Parrish JA, Optical properties of human skin. In: *The Science of Photomedicine* (Eds. Regan JD and Parrish JA), pp. 147–194. Plenum Press, New York, 1982.
32. Glazer PM, Greggio NA, Metherall JE and Summers WC, UV-induced DNA-binding proteins in human cells.*Proc Natl Acad Sci* **86**: 1163–1167, 1989.
33. Romerdahl CA, Donawho C, Fidler IJ and Kripke ML, Effect of ultraviolet-B radiation on the *in vivo* growth of murine melanoma cells. *Cancer Res* **48**: 4007–4010, 1988.
34. Diffey BL, Solar ultraviolet radiation effects on biological systems. *Phys Med Biol* **36**: 299–328, 1991.
35. Chenevix-Trench G, Cullinan M, Ellem KAO and Hayward NK, UV induction of transforming growth factor alpha in melanoma cell lines is a posttranslational event. *J Cell Physiol* **152**: 328–336, 1992.
36. Morelli JG and Norris DA, Influence of inflammatory mediators and cytokines on human melanocyte function. *J Invest Dermatol* **100**: 191s–195s, 1993.
37. Ellem KAO, Cullinan M, Baumann KC and Dunstan A, UVR induction of TGFα: a possible autocrine mechanism for promotion of epidermal carcinogenesis. *Carcinogenesis* **9**: 797–801, 1988.
38. Yaar M and Gilchrest BA, Human melanocyte growth and differentiation: a decade of new data. *J Invest Dermatol* **97**: 611–617, 1991.
39. Friedman PS, Gilchrest BA, Ultraviolet radiation induces pigment production by cultured human melanocytes. *J Cell Physiol* **133**: 88–94, 1987.
40. Onodera H, Horikoshi T, Eguchi H and Fukuzawa K, Effect of UV-irradiation on DNA damage and replication of cultured human melanocytes. *J Invest Dermatol* **94**: 562, 1990.
41. Flesch P and Rothman S, Role of sulphydryl compounds in pigmentation. *Science* **108**: 505–506, 1948.