Regulation of Tyrosinase Expression and Activity in Human Melanoma Cells via Histamine Receptors

Max T. McEwan and Peter G. Parsons
Queensland Institute of Medical Research, Brisbane, Queensland, Australia

In cultured human melanoma cells, histamine H_1 (mepyramine) and H_2 receptor antagonists (cimetidine, ranitidine, impromidine) increased tyrosinase activity, whereas H_2 agonists (dimaprit, nordimaprit) decreased activity. Mixtures of agonist and antagonist either decreased or increased tyrosinase activity, depending on the relative concentrations of each drug. Nordimaprit, the most effective inhibitor, lowered tyrosinase activity significantly within 36 h and caused a slower loss of tyrosinase protein as judged by reactivity with two monoclonal antibodies. Prolonged treatment of a melanotic cell line with nordimaprit led to complete loss of pigment, with no loss of the 56-kDa melanosomal antigen 1C11. Cells remained amelanotic for 8 weeks after removal of the drug and, even after 26 weeks, melanin content and tyrosinase expression and activity had not fully recovered. Nordimaprit increased the rate of degradation of tyrosinase and of nordimaprit binding proteins. Whereas nordimaprit did not directly inhibit tyrosinase, lysates of treated cells contained an inhibitory activity that partitioned approximately equally across a 10-kDa ultrafiltration membrane. Overall, these results showed that melanogenesis can be controlled via histamine receptors, the mechanism for the H_2 agonist nordimaprit consisting of three components: induction of a tyrosinase inhibitor, increased degradation of tyrosinase, and long-term down-regulation of tyrosinase expression. *J Invest Dermatol 97:868–873, 1991*

Human skin tans on exposure to ultraviolet light (UV) due to the tyrosinase-catalyzed production in melanosomes of melanin, a pigment that absorbs UV and traps free radicals, including reactive oxygen species [1]. UV increases the number of dopa-positive cells in the epidermis by stimulating melanogenic activity and the rate of melanocyte proliferation; the rate of transfer of melanosomes to surrounding keratinocytes is also increased [2,3].

The cascade of cellular events that lead to these changes, particularly the onset of melanization, remains largely unexplained at the biochemical level. Candidates for mediators of UV stimulation of melanogenesis include cholecalciferol and prostaglandins. On sun exposure, cholecalciferol is synthesized in the epidermis from its precursor 7-dehydrocholesterol [4] but, as human melanocytes show decreased tyrosinase activity treatment with the active hormonal form 1,25-dihydroxy-vitamin D_3 [5], no clear sequence of stimulatory events has been elucidated using these agents.

The melanogenic effect of UVB (280–320 nm) is blocked in murine melanoma by indomethacin, suggesting that prostaglandins mediate this stimulation, but most prostaglandins inhibit melanocyte growth and only a few stimulate melanization through increased tyrosinase activity [6,7]. Stimulation of tyrosinase activity has been causally linked to the inhibition of cell growth by terminal differentiation involving melanin synthesis and the attendant toxicity of its by-products [5]. Again, the responses to eicosanoids do not appear to explain the in vivo phenomena.

Melanocytes originate in the embryonic neural crest and retain the typical dendritic appearance of neural cells. The initial tyrosinase reaction, conversion of tyrosine to dopa, allows cells to produce neurotransmitters that lie metabolically downstream from this reaction, via dopamine. We previously showed that human melanoma cells produce catecholamines and serotonin in culture [8]. Further, a serotonin uptake inhibitor prevented melanization without inhibiting tyrosinase activity, suggesting that a major differentiation pathway in melanoma cells was influenced by neurologic mechanisms [8]. We have now found that histamine agonists and antagonists control tyrosinase expression and melanization, a phenomenon demonstrated most potently as inhibition of melanization by H_2 receptor agonists.

MATERIALS AND METHODS

Chemicals Nordimaprit, dimaprit, N-methyl dimaprit, mepyramine, and impromidine were kindly donated by Smith, Kline and French Research Ltd., Hertfordshire, U.K. Cimetidine and ranitidine were obtained for intravenous use from a hospital pharmacy. All other drugs were purchased from Sigma, St. Louis, MO, U.S.A. All drugs were diluted or dissolved in culture medium and filter sterilized using a 0.22 μm sterile nitrocellulose filter (Millipore, MA, U.S.A.).

Cell Culture The MM96E human melanoma cell line (>100 passages) is a slightly pigmented, near-diploid subline of MM96 [8], derived from a melanoma metastasis, whereas the MM418 human cell line (>50 passages) is constitutively pigmented and partially aneuploid, and was derived from a heavily pigmented primary melanoma [9]. Routine assays for Mycoplasma using agar culture were negative. Cells were cultured as monolayers twice weekly in 15-cm plates using Roswell Park Memorial Institute medium 1640 supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 3 mM 4-(2-hydroxyethyl)-1-pipera-

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Reprint requests to: Dr. P. G. Parsons, Queensland Institute of Medical Research, Bramston Terrace, Herston, Brisbane, Qld., Australia 4006.

Abbreviations:
MoAb: monoclonal antibody
PBS: 50 mM phosphate, 0.1 M NaCl (pH 7.2)
UV: ultraviolet light

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zine ethanesulfonic acid. For treatments of longer than one passage, fresh drug was added with the new culture medium. Except for the cell-survival studies, all assays of drug-treated and control cells were carried out on the same number of cells.

Cell survival was determined by a clonogenic-type assay [9]. Cultures (1000 cells per microtiter well) were treated in duplicate with five drug levels and after incubation for 6 d were pulsed with ^{3}H-thymidine for 4 h, the cells detached with trypsin and lysed, and washed into glass fiber discs for liquid scintillation counting. The D_{37} (dose required to give 37% survival or one lethal hit per cell) was interpolated from a plot of percent control cpm versus dose.

For determination of tyrosinase, FACS analysis, and L-[^{35}S]methionine label determination, 15-cm plates were washed twice with ice-cold PBS (phosphate-buffered saline, pH 7.2), harvested by scraping into 10 ml of PBS, and recovered by centrifugation. Tyrosinase (dopa oxidase) activities were determined by sonication of cells in lysis buffer (50 mM sodium phosphate buffer, pH 6.8, containing 0.1% Brij 35), approximately 200 μl per 10^7 cells. The resulting mixture was centrifuged in a microfuge for 10 min and a 10–20-μl aliquot of the supernatant added to 100 μl of a mixture of 7.6 mM dopa and 50 mM phosphate buffer, pH 6.8, in the well of a microtiter plate. The increase in absorbance at 490 nm was read at 2-min intervals in an ELISA reader (Model EL310, Bio-Tek Instruments).

For measurement of melanin, the lysate from 2×10^7 cells in 1 ml lysis buffer was sonicated for 30 sec and, after the addition of 4 ml of heptane containing 1% octanol, the two phases were mixed end over end for 5 min and centrifuged at 3000 rpm for 10 min. Melanin content was assayed by solubilizing the dark pellet at the bottom of the aqueous layer in 0.5 ml Soluene 350 (Packard Instruments) at 56°C for 15 min followed by dilution with 1 ml toluene. The absorbance was read at 405 nm and gave a linear response to cell number and dopa-melanin (Sigma). The procedure was also carried out with nonmelanized cells (HeLa) to show that absorbance at this wavelength from cellular components other than melanin was negligible.

Monoclonal Antibodies MoAb 1C11, 5C12, and 2B7 were obtained by immunization of BALB/c mice with a purified human tyrosinase fraction as the antigen [10] and were isotyped with a Mouse typer kit (Biorad, Richmond CA) as IgG_1. Ascites was obtained from BALB/c mice primed with Pristane and injected intraperitoneally with hybridoma cells. MoAb 1C11 and 5C12 have been described previously [10,11]. MoAb 2B7 bound active tyrosinase on affinity purification to give a molecular weight antigen on SDS-PAGE identical to 5C12. The antigen recognized by MoAb 4F5 is unrelated to tyrosinase and was used as a positive control for recognition by the secondary antimouse antibody.

Flow Cytometry Equal aliquots of log-phase melanoma cells (usually 1×10^6) were fixed in 50% ice-cold methanol for 10 min, washed in cold PBS, and incubated with PBS-diluted monoclonal supernatants at 37°C for different times. MoAb dilutions and incubation times were optimized for each antibody to give the strongest fluorescent signal without increasing nonspecific binding to HeLa cells, previously tested as negative to all antibodies except 4F5. After the incubation with primary antibody, the cells were washed twice as before, centrifuged, and incubated at room temperature for 30 min in affinity-purified sheep antimouse Ig-FITC (Silenus, Melbourne, Australia) at a dilution titrated for maximum specific fluorescence, typically between 1 in 100 and 1 in 500. Cells were washed twice with cold PBS, resuspended in cold PBS, and kept on ice. For simultaneous detection of MoAb and DNA content, propidium iodide (50 μg/ml) was added after FITC readings. RNase (100 μg/ml) and Triton X-100 (0.2%) in PBS were added just before cell DNA analysis to minimize degradation of antibody fluorescence. Cells were analyzed with a Becton Dickinson FACS IV (argon ion laser) operated at 488 nm for excitation and the emission gated to separate the two colors with narrow band pass filters. MoAb labeling was quantitated as the percentage of cells above background fluorescence, the latter determined with HeLa cells (antimelanoma primary and FITC secondary antibodies) and MM96E cells (secondary antibody only).

Production of Affinity Resin For attachment to Tresyl Sepharose (Pharmacia, Uppsala, Sweden), concentrated solutions of the same MoAb as used in flow cytometry were purified from ascites or hybridoma supernatant (McEwan and Parsons, submitted for publication), dialyzed against the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl), and reacted according to the manufacturer's instructions. Antibody was ensured to be in excess by monitoring the UV spectra after dialysis of spent binding solution and by dot blotting for detection of Ig. Nordimaprit-Sepharose was prepared similarly, using 20 mg nordimaprit and 1 g Tresyl Sepharose in 5 ml coupling buffer.

^{35}S-Methionine Labeling and Affinity Purification Human melanoma cells were grown to half confluence in normal FCS-supplemented medium, and then for 30 h in methionine and cysteine-depleted serum-free medium that contained ^{35}S-methionine (approximately 0.1 mCi per 10^7 cells). ^{35}S-methionine was present only for the last 4 h of the 30-h period and the label was chased by returning to complete medium. During all changes of medium, drug doses were maintained. Cells were harvested by scraping into ice-cold PBS and pelleted in a bench centrifuge. The pellet was lysed by a 30-second sonicator pulse in lysis buffer (50 mM phosphate buffer–0.5% Brij 35, pH 6.8; 1 ml per 10^7 cells) and 500 μl of the lysate were then added to IgG-derivatized Tresyl Sepharose (prepared from 20 mg dry powder). Sepharose and lysate were rotated gently on a blood wheel for 30 min at room temperature and the Sepharose was pelleted and washed 4 times with 2 ml of 0.5 M phosphate (pH 6.8). The pellet was suspended in 1.5 ml Instagel liquid scintillation cocktail (Packard Instruments, Downers Grove, IL) and counted for ^{35}S in a β counter.

Cell Lysate Fractionation Lysate from nordimaprit-treated cells (1 ml lysis buffer per 10^7 cells) was separated into two different molecular weight fractions by centrifuging most of the solution through a 10-kDa cut-off filter (Amicon). Maximum recovery of retained protein was ensured by back washing the membrane on the underside with 200 μl of 50 mM phosphate buffer, pH 6.8, and centrifuging the membrane assembly upside-down so that absorbed proteins were flushed off the membrane. Under these conditions the membrance bound less than 0.5% total CPM of ^{35}S-methionine–labeled lysate compared to nearly 3% retained without back washing. Both the retained (upper) and filtrate (lower) fractions were tested for inhibition of tyrosinase activity by addition of aliquots (20–50 μl) to a control cell lysate immediately prior to addition of dopa substrate. Lysate from untreated cells was similarly fractionated and tested.

RESULTS

Modulation of Tyrosinase Activity by Histamine Agonists and Antagonists Preliminary experiments showed that a range of histamine agonists and antagonists applied at nontoxic doses altered tyrosinase activity (dopa oxidase) in the human melanoma cell line MM96E. Mepyramine, an H_1 antagonist, increased tyrosinase activity, whereas dimaprit, an H_2 agonist, inhibited the enzyme (Table I). These rapidly acting but opposite effects could be seen as soon as 4 h after addition of the drugs and were maximal at approximately 7 d, when inhibition by dimaprit (40 μg/ml) caused complete loss of tyrosinase activity. Subsequent investigation showed that the strongest inhibitor of tyrosinase was nordimaprit, a lower homologue of dimaprit (Table I). Because dopa and tyrosine increase tyrosinase and melanin levels in MM96E cells [8], nordimaprit was used in conjunction with dopa to determine whether such stimulation could be prevented. Nordimaprit blocked dopa-induced stimulation of tyrosinase (Table I), preventing melanization and subsequent cell death (data not shown). Dose responses of cell survival demonstrated that the major drugs used in this study were not toxic at the concentrations needed to affect the melanogenic pathway (Table II). In addition, the nordimaprit dose response for clon-

Table I. Effect of Histamine-Active Compounds on Tyrosinase (Dopa Oxidase) Activity in MM96E Cells

Drug	Dose (μg/ml)	Treatment Time (d)	Tyrosinase Activity (% control)
Ranitidine	100	4	142 ± 9[a]
Mepyramine	50	5	151 ± 8
Imidazole	50	4	107 ± 9
Imidazole	300	4	131 ± 8
Impromidine	50	4	125 ± 10
Dimaprit	25	4	21 ± 4
Dimaprit	50	5	11 ± 3
Nordimaprit	25	4	3.4 ± 0.7
N-Methyldimaprit	25	4	97 ± 6
Dopa	40	1.5	158 ± 9
Nordimaprit	25	1.5	25 ± 3
Dopa +	40	1.5	84 ± 10
Nordimaprit	25		

[a] Mean ± SD (n = 3–6) for the rate of increase in A490 during the first 10 min of the reaction.

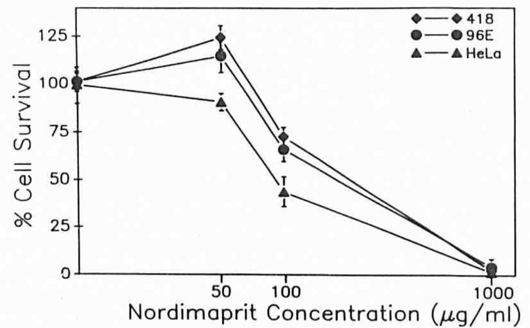

Figure 1. Nordimaprit dose responses of cell survival, determined by [^{3}H] thymidine incorporation of colonies formed after 6 d of continuous treatment and expressed as a percentage of controls. *Points,* mean ± SD (n = 3).

ogenic survival of MM96E cells (Fig 1) showed that the levels used in these experiments (20–50 μg/ml) had a slight positive effect on cell growth. Higher doses did not discriminate between MM96E (lightly pigmented melanoma), MM418 (heavily pigmented melanoma), or HeLa (nonmelanoma) in toxicity.

It was possible that inhibited tyrosinase activity in a cell lysate did not reflect inhibition in the intact cell, where compartmentalization may protect the target from intracellular inhibitors. Therefore, MM418, a constitutively melanizing cell line, was cultured in medium containing a nontoxic level of nordimaprit. Similar inhibition of tyrosinase activity was demonstrated because the cells, while proliferating as rapidly as the controls (doubling time approximately 40 h), became completely depigmented during 14-d treatment with nordimaprit (20 μg/ml). Tyrosinase activity was therefore inhibited in whole cells as well as in the disrupted cell lysate.

The H_2 antagonist, cimetidine, proved to be a strong stimulator of tyrosinase and antagonized the inhibitory effect of nordimaprit (Fig 2), thus confirming involvement of the H_2 receptor. N-methyldimaprit, having a methyl substitution on the thiourea amino group and synthesized as a chemical control for the H_2 receptor [12,13], had only 0.2% of the activity of histamine in gastric acid secretion measurements. At 150 μg/ml, N-methyldimaprit alone had no effect on tyrosinase activity but blocked the inhibition induced by treatment with 20 μg/ml nordimaprit (Fig 3). Tyrosinase activity in the latter case showed a slight lag, which was also found in early stages of inhibition by nordimaprit alone (6–24 h) and which increased with time of exposure to nordimaprit (data not shown).

Other agents active at the H_2 receptor were also tested. Ranitidine (H_2 antagonist) caused a partial stimulation but required approximately double the dose to achieve the same increase in tyrosinase as cimetidine (Table I). Histamine itself had no effect on tyrosinase activity up to 200 μg/ml; a slight increase in tyrosinase rate was suggested at higher doses (results not shown). Imidazole caused a 30% increase in tyrosinase at 300 μg/ml over 4 d but at 50 μg/ml the slight increase was not statistically significant (Table I). Neither imidazole nor impromidine counteracted the inhibition of nordimaprit as powerfully as cimetidine and even at 200 μg/ml the former drugs did not prevent 25 μg/ml nordimaprit from causing a 45% inhibition of tyrosinase over 4 d.

Flow-Cytometric Analysis of Tyrosinase and Related Antigens Determination of cell number and DNA fluorescence confirmed that mepyramine and nordimaprit were not toxic at the doses used and had no consistent effect on cell-cycle progression of cells treated for 24 h (results not shown). Detected by immunofluorescence, the tyrosinase antigens 5C12 and 2B7 were found to be depleted by nordimaprit, whereas 1C11, a related melanosomal antigen, remained constant (Fig 4). Nordimaprit decreased the 2B7 form more quickly during the earlier stages of inhibition than 5C12 but at 7 d no antibody reactivity to either form could be found. Mepyramine consistently increased the levels of 5C12 to a greater extent than 2B7 at all time points measured (Fig 4). HeLa controls, which were treated similarly, showed no expression of any of the antigens. It is worth noting that the tyrosinase rate measured at 4 d with a similar dose of nordimaprit (Table I) was approximately 4% of control, whereas the antigen level as measured by 2B7 antibody reactivity was approximately 40% of control and 70% by 5C12. These results suggest that nordimaprit both inhibits tyrosinase activity and decreases its production.

Table II. Survival of MM96E Melanoma Cells After 6-d Treatment with Drugs

Drug	D_{37} (μg/ml)
Imidazole	>1000
Histamine	>1000
Mepyramine	280
Cimetidine	260
Dimaprit	460
N-Methyldimaprit	>1000
Nordimaprit	250

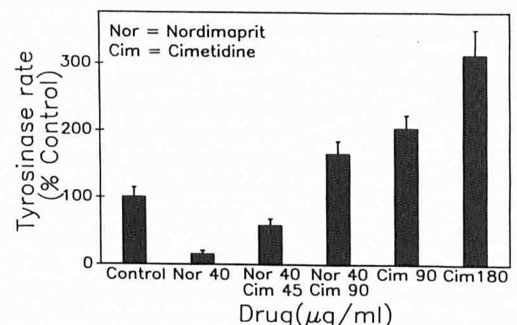

Figure 2. Inhibition of tyrosinase activity in lysates of MM96E cells by nordimaprit and antagonism by cimetidine. The cells were treated for 66 h. *Bars,* mean ± SD (n = 3).

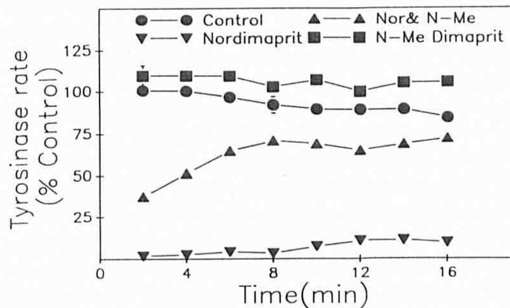

Figure 3. Inhibition of tyrosinase activity in MM96E cells by treatment with 20 μg/ml nordimaprit for 72 h is prevented by simultaneous treatment with 150 μg/ml N-methyldimaprit. The enzyme rate was calculated from the rate of absorbance increase at each time, zero representing addition of dopa substrate to the cell lysate. *Points,* mean ± SD (n = 3).

Turnover of Tyrosinase Antigens Labeled with ^{35}S-Methionine In an experiment where MM96E cells were treated with 30 μg/ml nordimaprit for 48 h, cell proteins were labeled at 26–30 h by a 4-h pulse with ^{35}S-methionine to determine the rate of antigen synthesis after exposure to nordimaprit for approximately one cell-cycle period. The use of purified antibody covalently linked to Sepharose ensured that the relevant antigen was the only protein retained by the MoAb affinity columns [10], thus avoiding the non-specific binding of normal immunoprecipitation procedures. Two hours after pulse labeling, nordimaprit-treated cells showed a 60% increase in labeling of the 2B7 and 5C12 antigens compared to controls (Fig 5). Protein labeling depends on the intracellular specific activity of methionine and on the rate of translation. As all proteins are synthesized from a common methionine pool, and the total incorporation was the same in treated cells as in controls at each time point (results not shown), it follows that the rate of synthesis of the 5C12 and 2B7 antigens was higher in nordimaprit-treated cells than in controls. The incorporation of antibody-bound isotope 5 h after removal of label was higher than at 2 h, presumably due to the time required for newly labeled protein to acquire the carbohydrate epitopes recognized by these MoAb. Antigen labeling was then similar in control and treated cells but subsequently declined much more rapidly in nordimaprit-treated cells compared with controls. The data therefore showed an increased turnover rate of tyrosinase antigens relative to controls following nordimaprit treatment, the half life decreasing from approximately 20 h to 4–6 h. The amount of radioactivity incorporated by the 2B7 antigen was less than 5C12.

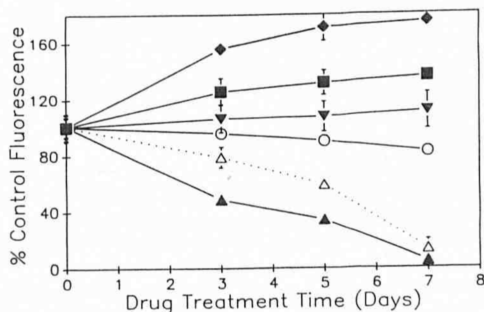

Figure 4. Temporal effect of 30 μg/ml nordimaprit (○, △, ▲) and 50 μg/ml mepyramine (◆, ■, ▼) on the expression of MM96E melanosomal antigens, as judged by the proportion of immunoreactive cells measured by flow cytometry. MoAb 5C12: △, ◆. MoAb 2B7: ▲, ■. MoAb 1C11: ○, ▼. *Points,* mean ± SD (n = 4–6).

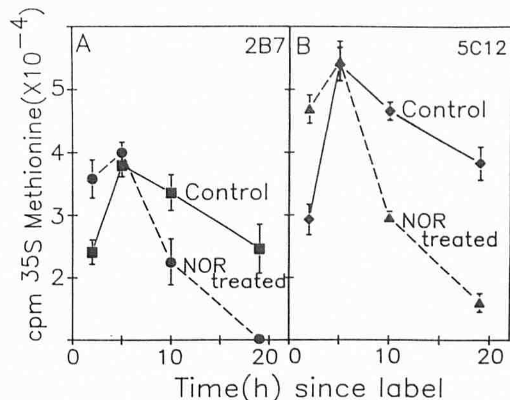

Figure 5. Synthesis and loss of ^{35}S-methionine–labeled tyrosinase in MM96E cells treated continuously with 30 μg/ml nordimaprit for 48 h and labeled for 4 h with ^{35}S-methionine 26 h after commencement of treatment. The horizontal axis refers to the last 18 h of treatment, carried out in the absence of ^{35}S-methionine. *A,* label bound to MoAb 2B7-Sepharose; *B,* MoAb 5C12-Sepharose. *Points,* mean ± SD (n = 3).

Long-Term Effect of Nordimaprit Treatment MM418 cells treated for 13 weeks with nordimaprit (25 μg/ml) proliferated at the same rate as controls and changed from black, heavily melanized cells to nonmelanized tyrosinase-negative cells no longer binding MoAb 2B7 or 5C12. Unlike other demelanizing treatments such as dithiothreitol (unpublished) and DU-24565 [8], nordimaprit-treated MM418 cells recovered tyrosinase activity extremely slowly. Thus, 28 weeks after removal of nordimaprit they had not regained normal melanization, tyrosinase activity, or antigen levels (Fig 6). Tyrosinase enzyme activity showed a lag time of 8 min in its reaction with dopa, 12 weeks after nordimaprit was removed (Fig 7). At 16 min, the reaction rate was nearly equal to the control. Because the lag in initial activity does not reflect the overall activity of the enzyme, the total absorbance change over 16 min was used as a measure of enzyme rate in Fig 6*A*, giving changes similar to the increase in melanin and 5C12 antigen. New protein formed as measured by ^{35}S-methionine incorporation into MoAb-bound antigen revealed a significant difference between the 5C12 and 2B7 populations, with 2B7 being slower to recover (Fig 6*B*).

Nordimaprit-Treated Cell Lysate Inhibited Tyrosinase Activity in Control Lysate Experiments were carried out to determine whether nordimaprit directly inhibited tyrosinase or was metabolized to form an inhibitor in the cells. Nordimaprit added

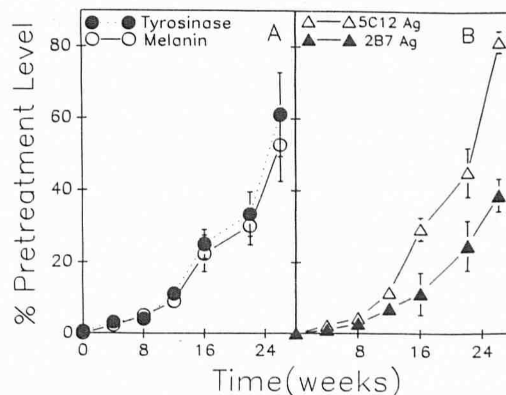

Figure 6. Recovery of melanin, tyrosinase activity, and antigenicity in MM418 cells previously demelanized by treatment with 30 μg/ml nordimaprit for 13 weeks. *Points,* mean ± SD (n = 3).

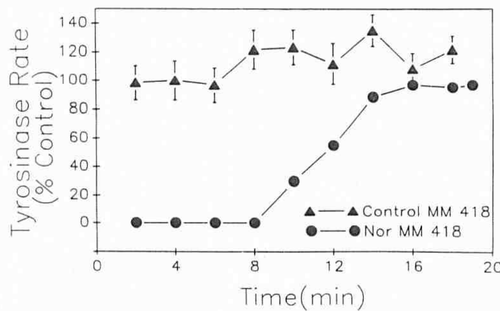

Figure 7. Lag in the tyrosinase activity of MM418 cells (Nor) after 12 weeks recovery from nordimaprit treatment (30 μg/ml for 13 weeks). *Points,* mean ± SD (n = 3).

directly to MM96E cell lysate or to mushroom tyrosinase had no observable effect on enzyme rate (Fig 8*A*). Lysate from nordimaprit-treated cells (25 μg/ml) for 72 h) when added to an equal volume of control cell lysate inhibited tyrosinase activity for at least 8 min, but evidently was not in sufficient excess to sustain inhibition beyond this time when the difference was no longer statistically significant (Fig 8*B*). An attempt to define the molecular weight of the inhibiting agent(s) by centrifuging the treated lysate through a 10-kDa cut-off membrane showed a similar distribution of inhibiting activity in the supernatant and lysate (Fig 8*B*).

DISCUSSION

This investigation showed a consistent response of tyrosinase activity to H_2 agonists and antagonists in human melanoma cells, the most dramatic and specific effect being the complete depigmentation of a constitutively melanizing cell line by nordimaprit. This phenotypic stability has not been shown previously by tyrosinase inhibitors and thus H_2 agonists may be useful agents for investigating long-term control of pigmentation. Using two separate epitopes in two independent techniques, production of mature tyrosinase was shown to be decreased at some point yet to be determined, without affecting the synthesis of the melanosomal antigen 1C11. Further evidence for H_2 receptor involvement was provided by the opposing actions of the agonist nordimaprit and the antagonist cimetidine and by the lack of effect of N-methyldimaprit alone on tyrosinase activity while giving a weak blockade of the inhibition by nordimaprit. Nordimaprit was considered to be a much weaker agonist than dimaprit [12]. In the present system, the relative strengths were reversed but dimaprit was still a very effective inhibitor of melanization.

Nordimaprit treatment of melanoma cells induced an inhibitory activity sufficient to partially inhibit tyrosinase in control cell lysate. The inhibitor may also be responsible for the lag in tyrosinase activity seen in MM418 cells released from long-term nordimaprit treatment and in the early stages of nordimaprit treatment, probably indicating that the formation of the inhibitor precedes the inhibition of tyrosinase synthesis and increased tyrosinase degradation. The lag indicates delayed release from inhibition but it is unclear whether this is due to an inhibitor or to the metabolism of immature tyrosinase, perhaps by a protease to release the active form. If the inhibiting agent shown in the two fractions of the nordimaprit-treated lysate is approximately 10 kDa, then equal partition across the filtration membrane could be expected. Reduced thioredoxin (11.5 kDa) inactivated mushroom tyrosinase in vitro, producing a tyrosinase with increased lag, and bound tyrosinase with rapid inhibition of enzyme activity [14]. Thioredoxin is therefore a candidate for the nordimaprit-induced inhibitor.

This is the first study of the synthesis and degradation of human tyrosinase by MoAb-affinity methods. Such studies are complicated by the nature of the epitope recognized because the half-life measured will depend on the rates of synthesis, maturation, and degradation of the epitope. Murine tyrosinase has been shown to have a half-life of approximately 10 h [15,16]. The maximum incorporation of ^{35}S-methionine into tyrosinase at 5 h is in agreement with the finding of 4 h for synthesis of murine tyrosinase [15]. The half-life of approximately 20 h is double these values but decreased to 4–6 h in nordimaprit-treated cells and therefore should contribute to inhibition of tyrosinase. Finally, after long-term treatment the MoAb cannot recognize any antigen, indicating that synthesis of tyrosinase is interrupted. The tripartite interference by nordimaprit in tyrosinase activity parallels to a certain extent the association of high tyrosinase turnover with constitutively low enzyme activity in cultured melanocytes [17] and suggests a co-ordinated, inverse relationship between enzyme activity and degradation.

H_2 receptor stimulation of cyclic AMP synthesis has recently been demonstrated in two human melanoma cell lines [18]. Histamine action could be antagonized by ranitidine and cimetidine but not by H_1 antagonists. No tyrosinase assays were performed and no change in cell metabolism or appearance was observed in that study. As histamine did not inhibit tyrosinase in the present work it is possible that these longer term effects may require the use of synthetic agonists, particularly in tissue culture. Histamine produced in the skin is metabolized rapidly [19] and is broken down rapidly in tissue culture [20]. The conversion of histamine to methylimidazole-acetic acid by N-methyl transferase and monoamine oxidase could lead to an imidazole-like stimulation of tyrosinase [21], thus opposing inhibition of tyrosinase by histamine via the H_2 receptor. Imidazole stimulation, however, requires concentrations approximately 10 times those affecting tyrosinase through the H_2 axis (Table I and [21]). Increased tyrosinase immunofluorescence has been reported in human melanocytes exposed to 900 μg/ml of histamine for 48 h [22] and could possibly be explained by this high dose, giving rise to stimulation by imidazole metabolites. A level of histamine too low to exert an imidazole-like response (100 μg/ml for 24 h) gave a 37% stimulation of tyrosinase activity in human melanoma cells; no synthetic histamine agonists or antagonists were used to mimic or block the effect on tyrosinase [23]. Further, in contradiction with another study [17] and observations in this laboratory (unpublished), αMSH stimulated tyrosinase activity in the particular melanoma cell line used [23], suggesting that it may have unusual properties. H_1 receptors may be involved in the migration and proliferation of melanoma cells [24].

Increased histamine levels in serum have been associated with tumor development for over a decade [25,26], and antitumoral ef-

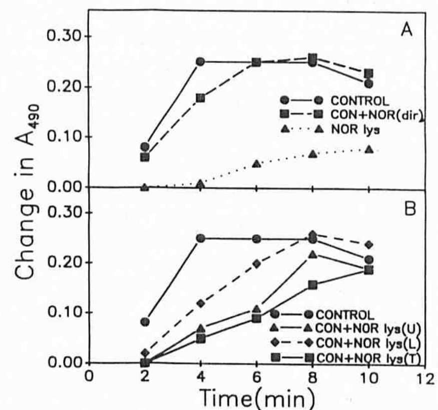

Figure 8. Tests for direct inhibition of tyrosinase by nordimaprit *(A)* and for inhibitors induced by nordimaprit *(B)*. *A*, Effect on tyrosinase activity in control MM96E cell lysate (CON) of 25 μg/ml nordimaprit (NOR dir), compared with the activity in lysates of cells treated with 25 μg/ml nordimaprit for 72 h (NOR lys). *B*, Inhibition of tyrosinase in control lysate by addition of lysate from treated cells. Cells were treated with 25 μg/ml nordimaprit for 3 d and total lysate (T) or lysate separated by ultrafiltration into >10 kDa (U, upper) or <10 kDa (L, lower) fractions was mixed with control lysate (CON). *NOR* lysates alone had no tyrosinase activity (not shown). *Points,* means of triplicates (all SD < 10%).

fects [27–29] including antimelanoma responses [30–33] have been achieved by treatment with histamine receptor antagonists and agonists. Melanoma patients were reported to have depressed histamine levels in their blood [33]; no other studies confirming or explaining this finding are available. Dimaprit has demonstrated antitumor activity in fibrosarcoma-bearing mice [34]. Given that the toxic effects of melanin and its by-products lead to terminal differentiation, it is more probable that the growth of melanoma would be enhanced by H_2 agonists. Indeed, our constitutively melanizing cell line, MM418, appeared to proliferate faster and reached a higher density during nordimaprit treatment.

The proximity of mast cells [36,37] to melanocytes [38] and the increases in histamine, prostaglandins, and arachidonic acid in human skin following ultraviolet irradiation [39] invite speculation that histamine inhibits melanization and/or promotes proliferation in the early stages of its release but stimulates melanogenesis following conversion to the more stable imidazole metabolites.

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