
Inhibition of Melanin Synthesis by Cystamine in Human Melanoma Cells

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In studies to determine whether pigmentation can be regulated physiologically by thiols, human melanoma cells (MM418c5) and melanocytes were found to become depigmented when cultured continuously in 50 μ M cystamine. Cystamine was depleted from the culture medium and the treatment was nontoxic and reversible. Cysteamine, dithiothreitol, and phenylthiourea were less effective, and glutathione, cysteine, and cystine were inactive. Tyrosinase (dopa oxidase) activity was not greatly affected except for induction of a lag period. In contrast, tyrosinase activity in an amelanotic melanoma cell line (MM96L) was rapidly inhibited without consumption of cystamine/cysteamine, in association with the generation of free thiol in the culture medium, and could be enhanced by the cystine transport inhibitor, glutamate. Tyrosinase expressed by a recombinant vaccinia virus was inhibited by cystamine treatment

of MM96L and HeLa cells. Cystamine treatment lowered the degree of cross-linking of the pigmentation antigen gp75/TRP-1 in MM418c5 cells. Tyrosinase protein and mRNA levels in MM418c5 cells were not affected by cystamine. The results show that cystamine at a concentration close to physiologic levels has multiple effects on the melanogenic pathway. In amelanotic cells, tyrosinase has a short half-life and is readily inhibited by cystamine/cysteamine whereas tyrosinase in the more mature melanosomes of the pigmented cell appears to be less accessible to proteolytic and thiol attack. Inhibition of melanin synthesis in the latter cell type may arise to a significant degree from reduction of cystamine to cysteamine, which sequesters quinones. Key words: cystamine/depigmentation/human melanoma cells/melanin/tyrosinase. *J Invest Dermatol* 114:21–27, 2000

A number of pigmentation genes expressed in melanocytes and their promoter sequences have been cloned (del Marmol and Beermann, 1996) but there is still incomplete understanding of how melanin synthesis is regulated *in vitro* or *in vivo*. A knowledge of such mechanisms may allow pigment synthesis to be manipulated, for the purposes of photoprotection or for driving melanoma cells into a state of permanent differentiation. Tyrosinase is a major enzyme in this pathway, converting tyrosine to dopa products for polymerization to melanin (Benedetto *et al*, 1982; Hearing and Tsukamoto, 1991). Being a copper enzyme, tyrosinase is susceptible to inhibition by naturally occurring thiols including glutathione (GSH), cysteine, and proteins with reactive thiols such as thioredoxin (Wood and Schallreuter, 1991; Jimbow *et al*, 1992; Ando *et al*, 1993; Naish-Byfield and Riley, 1998). Alternatively, thiols may react with quinone intermediates (Ito and Prota, 1977) to divert pigment synthesis from eumelanin (black) to pheomelanin (red/yellow). Elevated levels of thiols including GSH have been linked to pheomelanin formation or lack of pigment in humans

(Benedetto *et al*, 1982) and in the tabby syndrome in mice (Robertson and Blecher, 1987). The depigmenting activity reported for high-dose, local application of the naturally occurring disulfide cystamine (Chavin and Schlesinger, 1966; Bologna *et al*, 1995) and for cysteaminyphenols (Pankovich *et al*, 1990) appears to arise from the destruction of melanocytes, due to a reaction with molecules which are not necessarily specific to pigment synthesis (Parsons *et al*, 1991). Little consideration has been given to the possibility that cystamine and its reduced monomer cysteamine may regulate melanization in viable cells at the physiologic level.

Inside the cell, cystamine/cysteamine may interact with many proteins via disulfide exchange reactions, leading for example to the mobilization of zinc ions from metallothionein (Maret, 1995). Inhibition of GSH synthesis (Bologna *et al*, 1995), transglutaminase (Birckbichler *et al*, 1981), monoamine oxidase and glucose-6-phosphate dehydrogenase (Terada, 1994), and activation of lysosomal proteases (Pisoni *et al*, 1990; Jeitner *et al*, 1998) by cystamine have been reported. Cystamine has been used to relieve cystine accumulation in cystic fibrosis patients (Butler and Zatz, 1984). In cultured cells cystamine promotes the uptake of cystine via a glutamate-sensitive transporter with consequent increased synthesis of glutathione (Issels *et al*, 1988). Cysteamine inhibits human immunodeficiency virus replication (Bergamini *et al*, 1994) and activates neurofibromatosis kB in lymphocytes (Goldstone *et al*, 1995). The effects of cysteamine may vary considerably in different cell types, inducing hsp 27, hsp 90, and heme oxygenase-1, forming peroxidase-positive granules and damaging mitochondria in astrocytes but not in glial cells (Chopra *et al*, 1995).

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Abbreviations: BCIP, 4-bromo-5-chloroindolyl-3-phosphate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); GSH, glutathione; NBT, nitroblue tetrazolium; TRP-1, tyrosinase-related protein-1.

Synthetic β -aminoethyl disulfides structurally related to histamine H2 agonists are reduced to thiols by cultured melanoma cells (Fechner *et al*, 1994), with inhibition of tyrosinase activity and loss of pigment synthesis. Study of the simplest member of this family, cystamine, at nontoxic levels has now shown that it slightly reduces tyrosinase activity in pigmented melanoma cells and inhibits pigment synthesis.

MATERIALS AND METHODS

Cell culture MM96E, MM96L, MM418c5, and HeLa cells (Fechner *et al*, 1994) were grown in RPMI 1640 culture medium supplemented with 5% heat-inactivated fetal bovine serum. Human melanocytes were grown in culture media supplemented with 10% fetal bovine serum, 10 ng per ml 12-*O*-tetradecanoyl-phorbol-13-acetate and 6 ng per ml cholera toxin. All cell lines were adherent monolayers grown at 37°C in a humidified atmosphere containing 5% CO₂/air. Cell lines were tested routinely for *Mycoplasma* using a Hoechst 33245 staining technique (Chen, 1977). Cell survivals were determined by incorporation of ³H-thymidine as previously described (Fechner *et al*, 1994), 5–7 d after commencement of drug treatment.

Tyrosinase (dopa oxidase) activity was determined in cell lysates as described (Fechner *et al*, 1994), except that the substrate solution contained 1 mM dopa, 3 mM 3-methyl-2-benzothiazolinone hydrazone and 0.1% Triton X-100 in 50 mM phosphate buffer, pH 6.8. Melanin was determined by solubilization of cell pellets in Soluene 350 (Fechner *et al*, 1994). Free thiols were detected by adding 50 μ l of 3 mM dithiobisnitrobenzoic acid (DTNB) to 50 μ l of culture medium, or supernatant obtained by lysis of 2×10^6 cells in 1% Triton X-100 in 50 mM phosphate buffer (0.5 ml) followed by centrifugation (9000 $\times g$ for 10 min). After 30 min the absorbance at 415 nm was read on an enzyme-linked immunosorbent assay reader.

Western and northern blotting Whole cell lysates were subjected to western blotting using the 2B7 mouse monoclonal antibody to tyrosinase (McEwan *et al*, 1988) and B8G3 antibody against TRP-1 and immunostaining with alkaline phosphatase secondary antibody and BCIP/NBT substrates as described (Wong *et al*, 1994), except that lysates were not treated with dithiothreitol (DTT). Equal loading was achieved by determination of lysate protein and confirmed in some experiments by Coomassie Blue staining of a gel run in parallel or by reprobing the membrane with monoclonal antibody IFA against intermediate filaments, which in these cells recognized primarily vimentin at 55 kDa (Pruss *et al*, 1981).

Northern blotting of total RNA extracted from MM418c5 cells was carried out with probes for human tyrosinase and β -actin as previously described (Sturm *et al*, 1994).

Reporter assays for transcriptional activity Cells were transfected by electroporation with a construct containing the cytomegalovirus promoter, the SV40 promoter-enhancer, or with the p294MetM3 plasmid containing the sheep MT-1a promoter (Wong *et al*, 1994), each driving expression of the bacterial *lacZ* gene (β -galactosidase). For reporter assays, cells were seeded on to microtiter plates (5×10^4 cells per well) and treated the following day. Media was removed, cells washed with phosphate-buffered saline (0.1 M NaCl, 50 mM phosphate, pH 7.3) and the β -galactosidase activity was measured in an enzyme-linked immunosorbent assay microplate reader (BioRad 3550, Hercules, CA) at 570 nm following incubation with chlorophenol red β -D-galactoside at pH 8.3, essentially as described previously (Wong *et al*, 1994).

The mouse tyrosinase promoter (Beermann *et al*, 1991) of mutTRY1 (kindly supplied by Rosemary Sutton) was subcloned as a 270 bp *Xba*I/*Xma*I fragment into KS2, a modified KS(+) vector (Stratagene, La Jolla, CA) which possessed the polylinker 5'-*Sac*I-*Eco*RI-*Hind*III-*Xba*I-*Xma*I-*Xho*I-*Spe*I-*Kpn*I-3'. The polylinker of the resultant TYRP construct was expanded by the addition of the 142 bp *Eco*RI/*Xba*I fragment from pSL1180 (Pharmacia, Sydney, Australia), after which the tyrosinase enhancer was introduced as a 3.7 kb *Eco*RI/*Sac*I fragment from 3'E (kindly supplied by Susan Porter), yielding the TYREP plasmid. The TE β plasmid was obtained by subcloning into TYREP the *Xho*I/*Spe*I digested β -galactosidase gene of β -gal:KS(+), which had been derived by shuttling the *Pst*I/*Hind*III fragment from pSV β (Promega, Madison, WI) into KS(+). The Te β :pLDV construct was obtained by subcloning the tyrosinase enhancer/promoter/ β -galactosidase transgene (Porter and Meyer, 1994) from TE β as an *Eco*RI (partial)/*Spe*I fragment into pLDV, which is a modified pZero vector (Invitrogen, Groningen, the Netherlands) in which the zeocin resistance cassette (*Spl*I/*Dra*I) has been replaced with the KS(+)

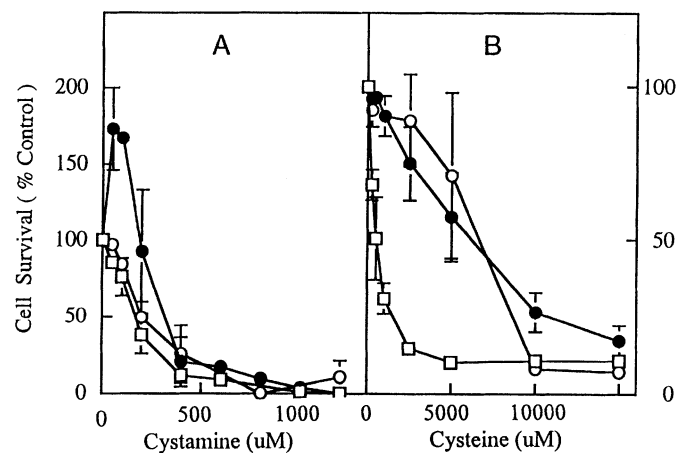


Figure 1. Survival of cells treated for 7 d with cystamine or cysteine. (A) Cystamine; (B) cysteine. ●, MM418c5; □, MM96L; ○, HeLa. Points are mean \pm SD (n = 3)

ampicillin resistance gene (as an end-filled *Bsp*HI fragment). The pSV- β -galactosidase vector containing the SV40 promoter and enhancer was obtained from Promega.

Expression of tyrosinase encoded by vaccinia virus The vaccinia virus construct, where the human tyrosinase gene is activated by the vaccinia p7.5 early late promoter (Yee *et al*, 1996), was kindly provided by Dr. C. Yee. It was grown in CV-1 cells by Dr. Rajiv Khanna and a pool used at 1/100 dilution on cell lines cultured in 96 well plates (10^5 cells per well). After infection for 24 h, the monolayer was washed once with phosphate-buffered saline (0.1 M NaCl, 50 mM phosphate, pH 7.3) and incubated with 100 μ l of 0.1% Triton X-100, 3 mM 3-methyl-2-benzothiazolinone hydrazone and 1 mM dopa in 50 mM phosphate, pH 6.8. When a red color developed (20–60 min), 100 μ l of ethanol was added to inactivate the virus and the mixture was carefully aspirated on to another 96 well plate for absorbance measurement at 490 nm on an enzyme-linked immunosorbent assay reader. The virus titer was determined 24 h after infection of parallel cultures, by immunoperoxidase detection of viral antigen using human immune serum essentially as described for adenovirus (Musk *et al*, 1990).

RESULTS

Depigmentation of melanoma cells and melanocytes by culture in the presence of cystamine Cell survival experiments showed that melanoma and the HeLa cell lines were less sensitive to cystamine than other tumor cell lines (Jeitner *et al*, 1998) and could be cultured continuously with 50–100 μ M cystamine without any detrimental effect on growth, determined by DNA synthesis after 6 d of treatment (Fig 1A). Growth of the pigmented MM418c5 cell line was enhanced by low levels of cystamine, presumably because fewer cells became terminally differentiated. Cysteine was less toxic than cystamine, although the amelanotic melanoma cell line MM96L was found to be considerably more sensitive than the other cell lines tested (Fig 1B). Cystine was nontoxic at the limit of solubility (500 μ M), close to the level in RPMI 1640 medium (262 μ M), and glutathione was nontoxic at 1000 μ M (results not shown).

Long-term culture of the pigmented MM418c5 cells was carried out in the presence of drug to allow dilution of preformed melanin and thus enable inhibition of melanin synthesis to be demonstrated in viable, proliferating cells. Depigmentation was apparent as a light-colored cell pellet compared with the usual black appearance, and confirmed by measurement of melanin content (Table I). As expected for tyrosinase inhibitors (Benedetto *et al*, 1982), phenylthiourea and DTT also inhibited melanin synthesis (Table I). Cystamine was one of the most potent agents tested, compared with cysteamine and other thiols used at the maximum tolerated doses, yet tyrosinase activity was only slightly inhibited. The lack of correlation between constitutive tyrosinase activity and pigment

Table I. Decrease in melanin content and tyrosinase activity after long-term culture of pigmented melanoma MM418c5 cells in the presence of thiol compounds

Compound	Concentration (μM)	Melanin (% control)	Tyrosinase (% control)
Cysteine	1000 ^a	93 \pm 6 ^b	101 \pm 5
Glutathione	1000 ^a	95 \pm 7	93 \pm 6
Phenylthiourea	200 ^c	3 \pm 0.6	44 \pm 5
DTT	650 ^c	2 \pm 0.5	6.5 \pm 0.7
Cysteamine	100 ^c	21 \pm 4	87 \pm 5
Cystamine	50 ^c	5 \pm 2	89 \pm 7
3 d recovery from 50 μM cystamine	0	53 \pm 5	101 \pm 6

^aMaximum dose permitting cell proliferation over a 2 wk period, fresh medium and drug being added twice weekly. The cell numbers increased from $10^5/75 \text{ cm}^2$ flask to $2-3 \times 10^6$.

^bMean \pm SD (n = 3).

^cMinimum dose required for depigmentation.

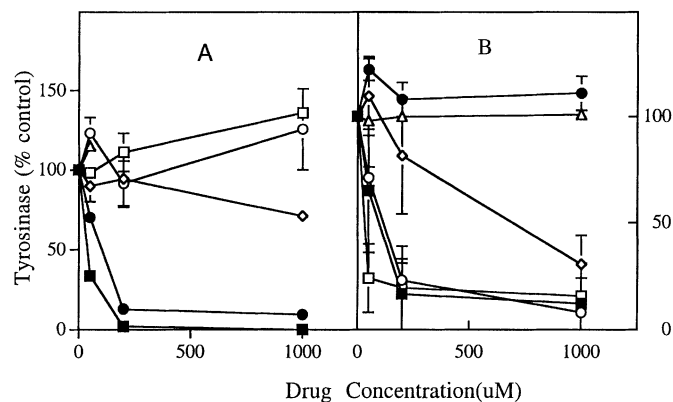


Figure 2. Thiol dose response for inhibition of tyrosinase (dopa oxidase) activity in pigmented human melanoma cells (MM418c5). (A) Activity in cells treated for 3 d. ●, cystamine; ◇, cysteamine; □, GSH; ○, cysteine; ■, DTT. (B) Activity of cell lysate treated directly with thiol before addition of substrate. Symbols as in (A). △, cystine. Points are mean \pm SD (n = 3)

synthesis has been noted previously, including a recent study of human melanoma cell lines (Eberle *et al.*, 1998). A more detailed examination was therefore made of the activity and expression of pigment-related genes in order to understand the action of cystamine in MM418c5 cells. The melanin content of melanocytes fell to $6.3 \pm 2\%$ of controls when cultured for 2 wk in 50 μM cystamine.

The dose-response for inhibition of tyrosinase (dopa oxidase) activity in cells treated for 3 d indicated that cystamine was again more active than cysteamine (Fig 2A) although the converse was true when the drugs were added directly to a cell lysate (Fig 2B). Studied at earlier treatment times, tyrosinase activity was reduced in MM418c5 cells after 8 h of treatment (Fig 3A), but then partly recovered. Inhibition was more marked and prolonged in the amelanotic MM96L line. Examination of the temporal response of the absorbance increase during these assays revealed evidence for participation of free thiols, as a lag period which was particularly noticeable for MM96L (Fig 3B) compared with MM418c5 cells (Fig 3C).

Formation and action of thiols in cultured melanoma cells Preliminary experiments showed that in RPMI 1640 medium, cultures of relatively high cell density (> 50% confluent) rapidly accumulated free thiols, as measured by the DTNB

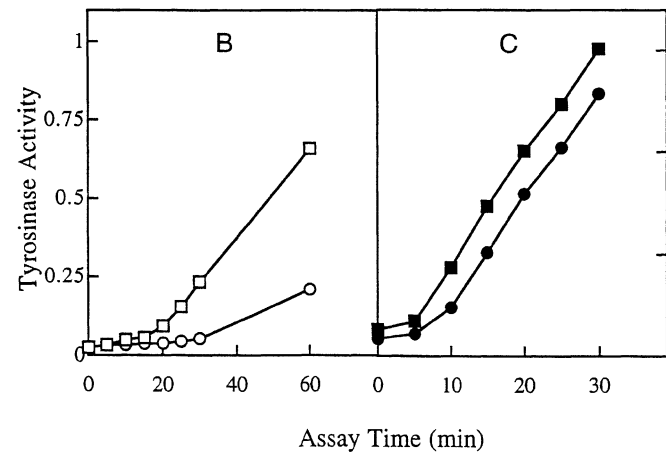
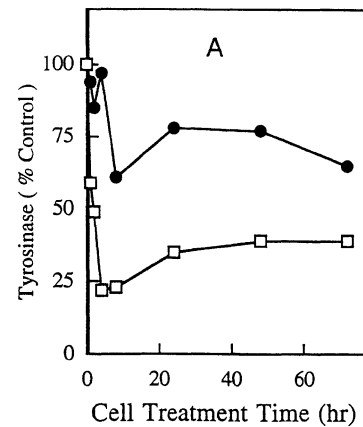


Figure 3. Tyrosinase activity after cystamine treatment of cells. (A) Cells treated with 50 μM cystamine for different times. ●, MM418c5; □, MM96L. (B) Loss of activity (A_{495}) in MM96L cells. □, Control MM96L cells; ○, MM96L treated with 50 μM cystamine for 3 d. (C) Increase in the lag period in MM418c5 cells. ■, Control MM418c5; ●, MM418c5 treated with 50 μM cystamine for 3 d. Points are means of triplicates.

reaction, presumably by cellular reduction of cystine in the medium. The rate and steady-state level of thiol formation varied with cell density, but typically achieved maximum concentration within 24 h (Fig 4). The thiol level was increased in the presence of 50–1000 μM cystamine in MM96L cells (Fig 4A), whereas in MM418c5 cells, 50 μM cystamine had no effect and higher levels produced less thiols than in MM96L cells (Fig 4B).

Culture supernatant from near-confluent cultures of MM418c5 and MM96L showed similar concentrations of thiol 3 d after initiation of the experiment but higher levels were produced by MM96L cells treated with cystamine for this period (Table II). Inclusion of the protein synthesis inhibitor cycloheximide (10 μg per ml) during the last 6 h of treatment had no additional effect. Intracellular thiol content was also similar in the two cell lines, and again was enhanced in MM96L by cystamine treatment, but not in MM418c5 cells. To test for consumption of cystamine from the culture medium, MM418c5 cells were treated with 50 μM cystamine for 3 d, then supernatant was removed and incubated with confluent MM96L cells for 6 h followed by thiol assay with DTNB. Thiols were only 20% higher than in control medium, indicating that very little cystamine or cysteamine remained in the MM418c5 supernatant.

The potential of thiols to regulate tyrosinase was investigated with buthionine sulfoximine, an inhibitor of GSH synthesis, and with glutamic acid, an inhibitor of cystine uptake (Issels *et al.*, 1988). The results showed that tyrosinase activity in two sublines of

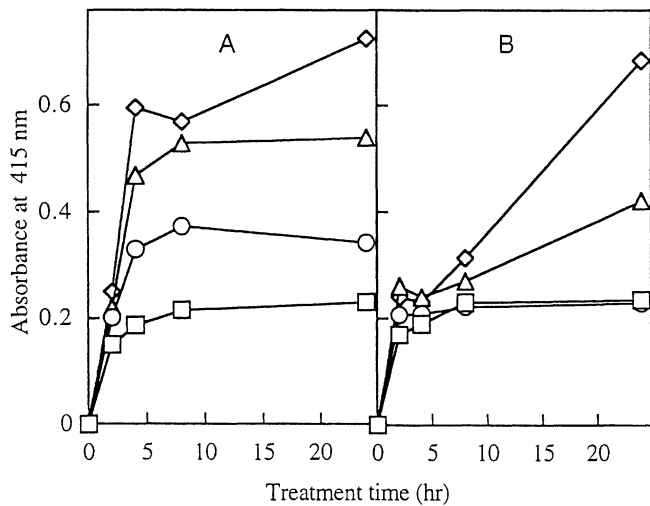


Figure 4. Generation of thiols in culture medium by MM96L or MM418c5 melanoma cells. (A) MM96L melanoma cells; (B) MM418c5 melanoma cells. □, Medium alone; ○, 50 μ M cystamine; △, 200 μ M cystamine; ◇, 1000 μ M cystamine. Points are means of triplicates.

Table II. Thiols in cultured cells and supernatants after treatment with 50 μ M cystamine for 3 d

Cell line	Supernatant SH		Intracellular SH	
	No cystamine	Plus cystamine	No cystamine	Plus cystamine
MM96L	100 ^a (0.105)	273 ± 13	100 (0.168)	214 ± 23
MM418c5	100 ^a (0.120)	94 ± 8	100 (0.213)	82 ± 3

^aPercentage control. A₄₁₀ from DTNB reaction in parentheses. Mean ± SD (n = 3).

MM96 more than doubled during culture with glutamic acid for 3 d; buthionine sulfoximine had no effect on tyrosinase activity in MM96L, MM418c5 or melanocytes (Table III). DTT was inhibitory in all cell types but cystamine at the depigmenting level of 50 μ M was most active in MM96 and had little effect on tyrosinase activity in MM418c5. The constitutive tyrosinase activity in the amelanotic MM96 cells was higher than in MM418c5 cells, presumably because of inhibition by melanin product in the latter.

Regulation of transcriptional activity by cystamine To determine whether tyrosinase was transcriptionally downregulated by cystamine, northern blotting was carried out with a human tyrosinase probe (Fig 5). No change in the mRNA level was found after treatment with cystamine, or with the other inhibitors tested. In addition, a reporter construct containing the mouse tyrosinase promoter driving the β -galactosidase gene was transfected into cells. Normalized for transfection efficiency with a β -galactosidase construct linked to the SV40 promoter-enhancer, MM418c5 cells gave higher reporter activity than MM96L, only low activity being obtained with HeLa cells (results not shown). Treatment with cystamine for 20 h following transfection led to marked inhibition of tyrosinase reporter activity in MM418c5 cells at concentrations as low as 50 μ M (Fig 6); cysteamine was less effective and other thiols were inactive. The activities of the SV40 promoter-enhancer, the cytomegalovirus promoter and the zinc-stimulated metallothionein promoter were inhibited to a maximum of 50%

Table III. Effect of thiols and thiol-regulating agents on tyrosinase activity in human melanocytic cells treated for 3 d^a

Cell line	Tyrosinase activity (% control)			
	Cystamine (50 μ M)	DTT (650 μ M)	BSO (100 μ g per ml)	Glutamic acid (10 mM)
MM96L	12	5	107	204
MM96E	NT	NT	104	275
MM418c5	78	18	107	110
Melanocytes	33	10	96	106

^aNT, not tested; BSO, buthionine sulfoximine.

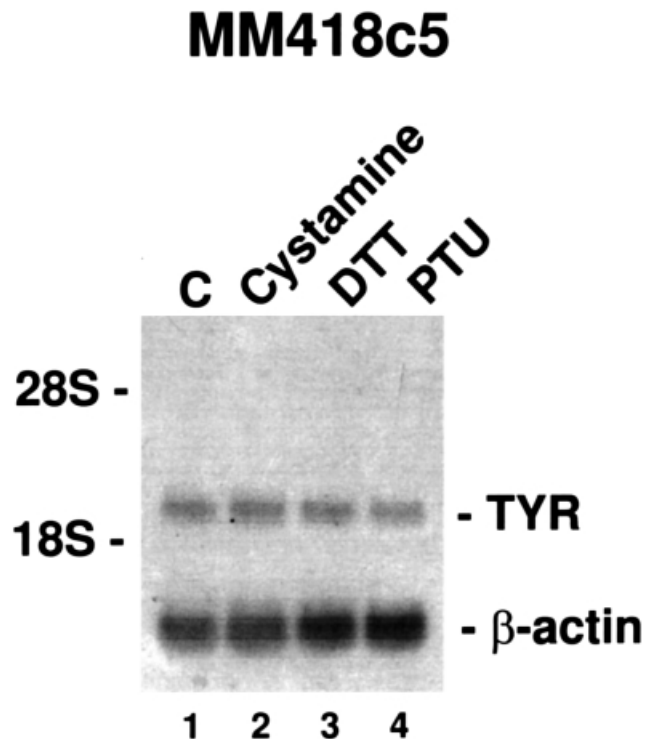


Figure 5. Northern blot of tyrosinase mRNA from MM418c5 cells treated with 400 μ M cystamine, DTT or phenylthiourea for 20 h.

only at 1000 μ M, the highest level of cystamine used (results not shown). A stably transfected cell line (A4/4) in which the metallothionein promoter requires activation by DNA demethylating agents and zinc (Biard *et al*, 1992) was also subjected to treatment with cystamine (50–1000 μ M) followed by induction with 100 μ M ZnSO₄. Unlike 1 μ M 5-azacytidine applied under the same conditions, no reporter activity was obtained, indicating that cystamine treatment does not lead to DNA demethylation (results not shown).

As levels of the pigmentation antigen gp75/TRP-1 are sensitive to a variety of drugs including differentiation agents (Wong *et al*, 1994), western blotting was conducted to determine the effect of cystamine on pigmentation antigens. The level of tyrosinase or TRP-1 protein was not affected (Fig 7). It should also be noted that the high molecular weight TRP-1 band at 155 kDa in MM418c5 cells, assumed to represent a cross-linked form associated with oxidative events of melanin synthesis, was diminished following treatment with 50 μ M cystamine. At the growth inhibitory level of

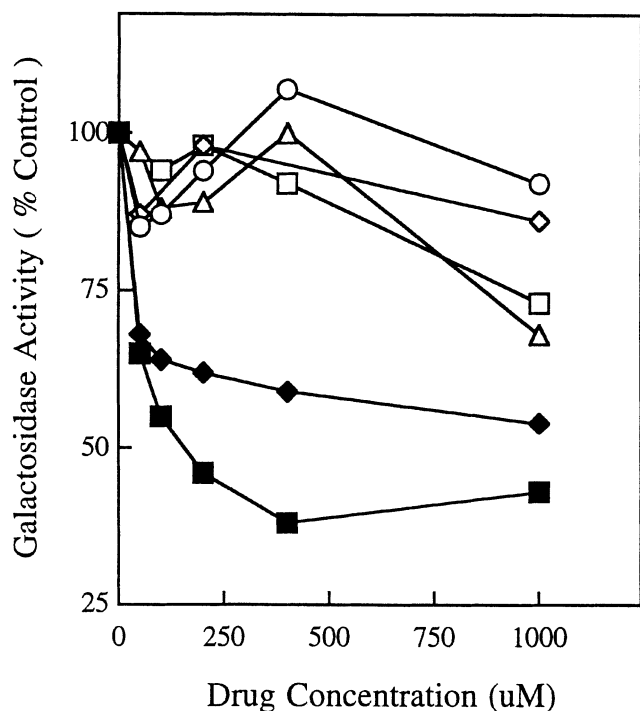


Figure 6. Effect of thiol compounds on transcription of β -galactosidase from the mouse tyrosinase promoter in MM418c5 cells. ■, Cystamine; ◆, cysteamine; □, phenylthiourea; ○, cysteine; ◇, cysteine; △, DTT. Points are means of triplicates.

1000 μ M cystamine, extensive cross-linking was seen as a ladder of high molecular weight bands, presumably caused by oxygen radicals generated during redox cycling of excess cysteamine. The fact that the bands above 80 kDa were abolished by treatment of cell lysates with 2 mM DTT (not shown) was taken to be evidence for oxidative formulation of disulfide cross-links. Cystamine/cysteamine has been shown to generate hydrogen peroxide in cultured cells (Jeitner *et al*, 1998).

Action of cystamine on ectopically expressed tyrosinase

The availability of recombinant vaccinia virus expressing human tyrosinase under the control of the viral promoter (Yee *et al*, 1996) allowed the inhibitory effect of cystamine on tyrosinase activity to be examined independently of its endogenous promoter. Preliminary experiments established that viral replication, as judged by cytopathic effect and quantitation of viral antigen (results not shown), was similar in the cell lines tested and was not altered by cystamine treatment.

Tyrosinase activity was readily detected in infected non-melanoma cells (HeLa cells) and was enhanced in infected MM96L cells (Table IV). No significant increase, however, could be detected in MM418c5 cells after infection. Pretreatment of cells with 50 μ M cystamine 24–72 h before infection did not consistently inhibit subsequent viral expression of tyrosinase activity in any cell line. When cystamine was given simultaneously with virus, however, tyrosinase activity measured 24 h later was found to be inhibited in MM96L and HeLa but not in MM418c5 (Table IV).

DISCUSSION

Cystamine was found to be a potent, nontoxic, and reversible agent for depigmenting human melanoma cells at a concentration close to that in blood (19 μ M) (Lentner, 1984). Normal melanocytes were studied less extensively but also were depigmented under the same conditions. Other naturally occurring thiols such as GSH were either less effective or were toxic to some cell lines. In particular, cysteine was highly toxic to the MM96L melanoma cell line. Autoxidation of cysteine generates toxic radicals (Saez *et al*, 1982),

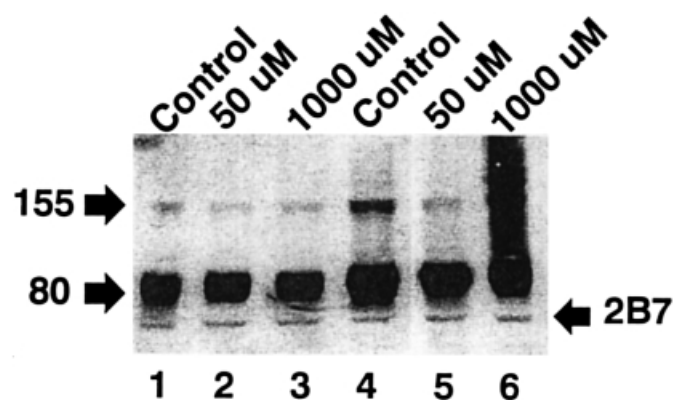


Figure 7. Western blotting of tyrosinase (60 kDa) and TRP-1 (80 and 155 kDa) 3 d after treatment of cells with cystamine. Lanes 1–3, MM96L; lanes 4–6, MM418c5. Antibody for tyrosinase (2B7) gave a band at approximately 60 kDa.

Table IV. Inhibition by 50 μ M cystamine of tyrosinase expressed in cells by recombinant vaccinia virus

Cell line	Tyrosinase activity (A_{490} per h per 10^5 cells)			
	No virus		Plus virus	
	Control	Cystamine	Control	Cystamine
HeLa	0	0	0.137 ± 0.005^a	0.030 ± 0.003
MM96L	0.235 ± 0.03	0.131 ± 0.008	0.375 ± 0.012	0.158 ± 0.025
MM418c5	0.120 ± 0.015	0.085 ± 0.005	0.121 ± 0.020	0.122 ± 0.021

^aMean \pm SD (n = 3).

and a similar situation was inferred to explain the *in vitro* toxicity of N-acetylcysteine (Karg *et al*, 1991) to melanoma cells. MM96L and some other melanoma cell lines are particularly sensitive to killing by oxygen radicals (Parsons and Morrison, 1982), which in endothelial cells increase the uptake of cystine (Miura *et al*, 1992). The redox properties or transport of cystamine/cysteamine therefore appear to be different from cystine/cysteine. Further studies of thiol-regulated melanin synthesis in proliferating cells were centered on cystamine.

Thiol homeostasis in mammalian cells depends on a range of variables including culture conditions and cell type. In this study, measurements of free thiols in culture medium revealed that the levels vary widely during a single passage due to cellular reduction of exogenous cystine, beginning at zero and rising to a steady-state level determined by cell density. Thiol transferases, GSSG reductase and glucose-6-phosphate dehydrogenase are also involved in thiol homeostasis in mammalian cells (Mieyal *et al*, 1991). Spontaneous hydrolysis of glutamine (2.1 mM in RPMI 1640 medium) to glutamic acid during cell culture or storage of medium is another variable that may affect thiol levels and tyrosinase activity, as glutamate inhibits the uptake of cystine (Issels *et al*, 1988), and may have contributed to the enhancement of tyrosinase activity found when mouse B-16 melanoma cells were cultured in the presence of glutamine (Chakraborty *et al*, 1988). The high level of glutamate in the human brain (6 mM; Clarke *et al*, 1989) is likely to enhance tyrosinase activity in the substantia nigra. Addition of 50 μ M cystamine to the medium increased the extracellular thiol level with MM96L cells and the lag period of tyrosinase, presumably through cellular reduction to cysteamine, but little or no increase was found with the pigmented MM418c5 cells. This was unlikely to reflect a major difference in disulfide reductase activity between the two cell

types because extracellular thiols were elevated using higher levels of cystamine with MM418c5 cells.

The mechanism of depigmentation by cystamine may involve several components. Inhibition of tyrosinase activity, an expected action of thiols (Benedetto *et al*, 1982; Mishima *et al*, 1988; Imokawa, 1989), was minimal in the pigmented cells. A significant part of the depigmenting action of cystamine/cysteamine appears to involve reaction with the products of tyrosinase activity and diverting them from pigment synthesis. This hypothesis is consistent with the increase in lag period in the dopa oxidase assay of cell lysate, showing that dopa oxidation products can be sequestered by cystamine, and with the observed loss of cystamine/cysteamine from the culture medium of MM418c5 cells but not with the amelanotic MM96L cells. It also suggests that quinones generated in melanosomes are more accessible to cystamine than to cysteine, as the medium was not depleted of cystine/cysteine (210 μ M in RPMI 1640 medium).

Cystamine had no effect on the protein or message levels of tyrosinase in the pigmented human cell line but downregulated the mouse tyrosinase promoter. Of a number of differences between the human and mouse promoters (Ferguson and Kidson, 1997), the large negative regulatory element in the mouse promoter between -195 and -125 bp is a potential target because of its activity specifically in pigmented cells (Ganss *et al*, 1994). These results raise the possibility that removal of such repression by ultraviolet or α -melanocyte-stimulating hormone, as suggested by Ganss *et al* (1994), could occur by a thiol-dependent mechanism. Cystamine/cysteamine may also change protein structure, as suggested for its inhibition of the assembly of human immunodeficiency virus virions, viral transcription and translation being unaffected (Bergamini *et al*, 1994). Although the role of TRP-1 in human melanogenesis remains unclear (Boissy *et al*, 1998), the loss of TRP-1 disulfide cross-linking found in this study may inhibit the deposition of melanin precursors within the melanosome.

Replication of the recombinant vaccinia virus was not affected by cystamine, thus inhibition of tyrosinase could be followed independently of expression. The lack of an observable increase in tyrosinase activity in infected MM418c5 cells with or without cystamine treatment, however, was not informative. An increase in tyrosinase protein may have occurred and been offset by product inhibition; in support of this possibility, a lightly pigmented clone of MM418 has a much higher tyrosinase activity than MM418c5 (unpublished). In amelanotic melanoma cells and HeLa cells, ectopically expressed tyrosinase was readily inhibited by concomitant incubation of cells with cystamine, suggesting that the enzyme is accessible to the reduced form of the drug.

The sensitivity of tyrosinase in amelanotic cells to inhibition by cystamine follows from previous observations (Le Gros *et al*, 1994; Bologna *et al*, 1995) that there is a different type of tyrosinase in pigmented compared with amelanotic melanoma cells. Enzyme in the latter is unstable (Le Gros *et al*, 1994; Halaban *et al*, 1997) and sensitive to changes in extracellular and intracellular thiol levels, presumably because of being accessible in the endoplasmic reticulum (Halaban *et al*, 1997). Tyrosinase in pigmented cells (MM418c5) on the other hand is relatively stable (Le Gros *et al*, 1994) and insensitive to thiols, presumably resulting from isolation within the more mature melanosome while still allowing access for thiols, particularly cystamine, to conjugate with quinones. Compared with the N-substituted derivatives tested previously (Fechner *et al*, 1994), cystamine/cysteamine is unique in not inhibiting tyrosinase during depigmentation. This study did not address the question of whether the concentration of cystamine in skin is sufficiently high or sustained to influence the melanin pathway, or whether thiols can induce synthesis of pheomelanin. The results, however, suggest that accessibility of tyrosinase to thiols such as cystamine, together with thiol-dependent gene expression, may be factors in controlling the overall pigmentation level.

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