Biochemical Pharmacology, Vol. 53, pp. 1719–1724, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00016-6

Tumor Selectivity and Transcriptional Activation by Azelaic Bishydroxamic Acid in Human Melanocytic Cells

P. G. Parsons,† C. Hansen,† D. P. Fairlie,‡ M. L. West,‡ P. A. C. Danoy,† R. A. Sturm,§ I. S. Dunn,‖ J. Pedley† and E. M. Ablett†*

†Queensland Cancer Fund Laboratories, Queensland Institute of Medical Research, Herston, Australia 4029; ‡Centre for Drug Design and Development, §Centre for Molecular and Cell Biology and ‖Pathology Department, The University of Queensland, Brisbane, Australia 4072

ABSTRACT. Azelaic bishydroxamic acid (ABHA), a potent differentiating agent for lymphoid cells, was selectively toxic for 5 human tumor cell lines and transformed human melanocytes and keratinocytes (dose for 37% survival, D_{37}, 30–100 μg/mL) compared with normal cells (melanocytes, fibroblasts; $D_{37} > 300$ μg/mL). Dendritic morphology was the only indicator found for increased differentiation, markers for the pigmentation pathway being unchanged or inhibited by ABHA. In contrast to hexamethylene bisacetamide and azelaic acid, ABHA significantly increased the HIV LTR, SV40 and *c-fos* promoter activities during a 24 hr treatment. Metallothionein promoter activity was enhanced by 5 hr treatment with ABHA in a sensitive melanoma cell line (MM96L) but was inhibited in a more resistant line (HeLa); *c-fos* promoter activity was inhibited in HeLa during this time. Transcription from a p53 binding response element was inhibited in MM96L by a 24 hr ABHA treatment but enhanced in HeLa. ABHA may represent a structural prototype for designing more potent and selective anti-melanoma agents. BIOCHEM PHARMACOL 53;11:1719–1724, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. differentiation; human melanoma; selective toxicity; transcription; azelaic bishydroxamic acid

Hexamethylene bisacetamide (HMBA; Fig. 1) is an effective differentiating agent in various murine and human leukemic and solid tumor cell lines [1]. It has produced remissions in a clinical trial but suffers from rapid deacylation *in vivo* and side effects such as thrombocytopenia, acidosis and neurotoxicity [2]. In murine erythroleukemic cells, HMBA induces arrest in G1, promotes translocation of protein kinase C (PKC) from the cytosol to the membrane, decreases c-myb, c-myc and p53 protein levels and increases *c-fos* mRNA [2, 3]. A transient increase in hypophosphorylated retinoblastoma protein pRB was found 12 hr after treatment, followed by enhanced production of the hyperphosphorylated form ppRB during the next 2–3 days [4, 5]. A recently reported group of structurally-related but more potent differentiating agents induce differentiation in murine erythroleukemic cell lines, human promyelocytic cells (HL-60), and human colon carcinoma cells [6]. Azelaic bishydroxamic acid (ABHA) and the flexible analogues of suberic acid bisdimethylamide were up to a hundred-fold more active than HMBA. Melanocytes and melanoma cells express a range of differentiation markers related to pigment synthesis, including tyrosinase, melanin and the tyrosinase-related protein-1 (TRP-1) [7, 8]. During a comparison of the effects of azelaic acid, HMBA and the nine carbon derivative ABHA on these markers, we found that ABHA was unique in being selectively toxic for transformed cells, in parallel with activation of the transcription of certain genes involved in signal transduction.

MATERIALS AND METHODS

Cell Culture

The origins of the human melanoma cell lines MM96E, MM96L (subclones of MM96), MM418c1 and MM418c5, the human cervical tumor line HeLa and the ovarian tumor line JAM [9, 10] and the spontaneously transformed keratinocyte line HaCat [11] have been described previously. NFF were neonatal skin fibroblasts. Normal human melanocytes from foreskins were cultured in 100 μg/mL, 12-O-tetradecanoylphorbol 13-acetate and 6 μg/mL cholera toxin. The mel-SV line of immortalised human melanocytes was obtained after infection of melanocytes with an SV40-adenovirus 5 hybrid virus kindly provided by Prof. P. Gallimore. Cultures were grown at 37°C in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% fetal calf serum (FCS) as described [10].

*Corresponding author: Dr. P. G. Parsons, Queensland Institute of Medical Research, Herston, Queensland, Australia, 4029. Tel. 61 7 3362 0316; FAX 61 7 3362 0107.

Abbreviations: HMBA, hexamethylene bisacetamide; ABHA, azelaic bishydroxamic acid; PBS, phosphate-buffered saline, pH 7.3, pRB, retinoblastoma protein; PKC, phosphokinase C; TRP-1, tyrosinase-related protein-1.

Received 3 September 1996; accepted 26 December 1996.

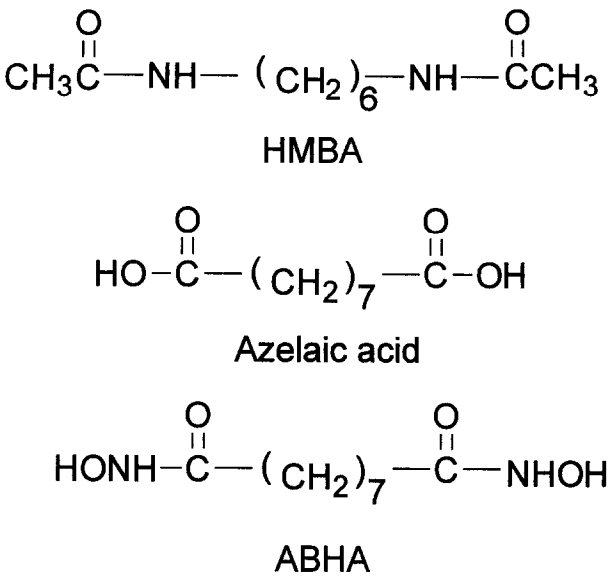

FIG. 1. Structures of drugs used in this study.

Cell survival was determined by counting the increase in number (hemacytometer) of 25000 cells seeded in 24-well plates (16 mm diameter wells) and treated with drug for 24 hr, washed twice and incubated for 3 doubling times (6 days for NFF, melanocytes and MM418; 3 days for the other cell lines).

In the functional tests described below, treated cells were compared with controls on the basis of equal cell number or protein, the latter determined by addition of bichinchoninic acid reagent (Pierce Chemical Co, USA) to triplicates in a microtitre plate and determining the absorbance increase at 570 nm. Bovine serum albumin was used as the standard.

Synthesis of ABHA (azelaic bishydroxamic acid)

To a solution of azelaic acid (nonanedioic acid; 1.0 g; 5.3 mM) in dimethylformamide (25 mL) was added triethylamine (2.68 g; 3.7 mL; 26.5 mM), hydroxylamine hydrochloride (0.77 g; 11 mM and benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (6.2 g; 14 mM) and the reaction mixture stirred under nitrogen at room temperature for 12 hr. The mixture was then diluted with water (100 mL) and lyophilised to yield the crude product as a thick syrup. This material was redissolved in a mixture of acetonitrile (4 mL) and water (16 mL), filtered, and chromatographed by HPLC (Waters deltapak C18, 15 mm, 40 × 100 mm, flow 20 mL/min, eluant 20% acetonitrile/80% water/0.1% trifluoroacetic acid). The purified material was lyophilised to yield a white powder (0.92 g; 80%), which was identical by ^{1}H NMR spectroscopy and electrospray mass spectrometry with that previously reported [6].

Tyrosinase Activity and Pigmentation Antigens

Tyrosinase (dopa oxidase) activity was measured by the oxidation of L-dopa as described [7]. Immunoblotting was conducted using B8G3 mouse monoclonal antibody supernatant against TRP-1 [7] or HMB-45 mouse antibody (diluted 1 in 250), followed by alkaline phosphatase-conjugated anti-mouse antibody and was quantitated by a Molecular Dynamics laser densitometer with ImageQuant software.

Analysis of Transcriptional Regulation Using Luciferase Transfectants

Clones of MM96L and HeLa cells stably transfected with reporter plasmids were used to determine the effect of ABHA on promoter activities. The *c-fos* promoter construct was kindly provided by Dr. M. Waters and Dr. C.-M. Chen, and the HIV LTR containing plasmid by Dr. D. Hume. Construction of the TRP-1 promoter has been described [8]. The SV40 promoter/enhancer construct was obtained from Promega. For construction of a reporter containing a p53 response element, a duplex oligonucleotide containing a strong palindromic p53 recognition site [12] was cloned as a blunt-ended fragment (5'CCGTCT GGACATGCCCGGGCATGTCCTCTCC) into the (blunt) Ec1 136II site of the enhancer-probe vector pGL2-promoter (Promega). The structure of plasmids containing a single inserted oligonucleotide was confirmed by automated DNA sequencing [13] with primers allowing reading of both strands of the insert. Each of the above response elements was coupled to a luciferase reporter gene. Stably transfected cell clones were picked after cotransfection with a Neo resistance plasmid and selection with 400 μg/mL Geneticin. Cells were seeded (50000/well) in black microtitre plates with clear, cell culture grade bottoms and incubated with drug for 6 or 24 hr. The medium was then replaced with 20 μl of Promega luciferase assay reagent and luminescence counting performed immediately *in situ* with a Packard Top Plate instrument.

Metallothionein Transcriptional Activity

MM96L and HeLa cells were transfected by electroporation with the p294MetM3 plasmid containing the sheep metallothionein Ia promoter and β-galactosidase followed by selection of stably transfected clones with hygromycin [10]. For reporter assays, cells were seeded in microtitre plates (5×10^4/well) and treated next day. Medium was removed and β-glactosidase activity was measured in an ELISA reader at 570 nm using chlorophenol red galactoside as the substrate, essentially as previously described [10].

RESULTS

Morphology and Cell Survival

Within 12 hr of commencing ABHA treatment (10 μg/mL), cells revealed characteristic morphological changes. The pigmented line, MM418c5, and the amelanotic melanoma line MM96E altered from spindle shape to markedly elongated cells with long processes. HeLa morphology

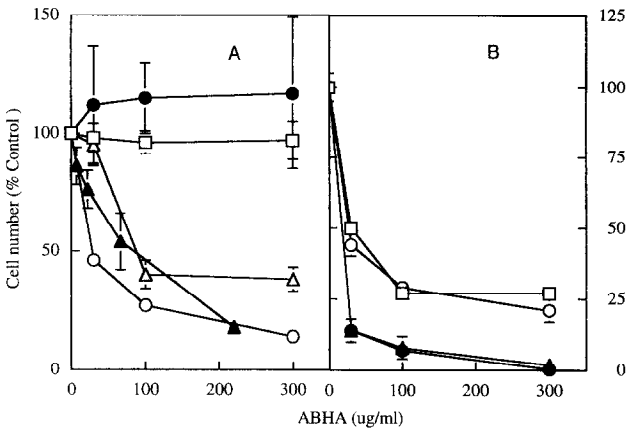

FIG. 2. Inhibition of cell growth by ABHA, 3 doubling times after a 24 hr dose. A. ●, melanocytes; □, NFF; △, HaCat; ▲, Mel-SV; ○, MM418c1. B. □, HeLa; ○, A2058; ●, MM96L; △, JAM. Points are means ± SD (N = 3).

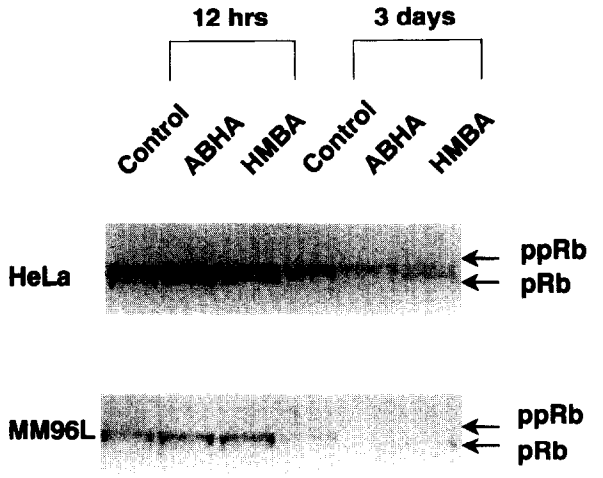

FIG. 3. Loss of hyperphosphorylated RB in cells treated with ABHA (30 μg/mL) or HMBA (1000 μg/mL) for 12 hr or 3 days. ppRB, hyperphosphorylated RB (115 kDa); pRB, hypophosphorylated RB (105 kDa).

changed from cuboidal to a more elongated, spindle-shape, with many cells possessing distinct processes. Cells ceased to proliferate after several days treatment but there were no signs of overt toxicity. Both long and short term treatment of cells revealed that ABHA was 100-fold more potent for inhibiting growth than HMBA or azelaic acid (results not shown). Melanoma cells were considerably more sensitive than HeLa and a higher proportion became arrested in G1 (results not shown). Treatment for 24 hr was about 10-fold less effective than for 6 days, but the former treatment time was further explored because of the limited exposure period anticipated *in vivo*. Compared on the basis of cell growth for 3 doubling times following a 24 hr treatment, ABHA was found to be highly selective against tumor cell lines, spontaneously transformed keratinocytes and SV-40 transformed melanoctyes compared with fibroblasts and melanocytes (Fig. 2). Selectivity also extended to HeLa, which although being the most resistant tumor line tested, was more sensitive than the normal cells.

Expression of Pigmentation Markers and pRB

Tyrosinase activity, a major enzyme in melanin synthesis, was assayed in MM96E and MM418c5 after 72 hr treatment, by which time activity had reached a minimum. High levels of ABHA caused marked inhibition of tyrosinase activity in both cell lines (Table 1); HMBA at an equitoxic concentration was less effective. Expression of the melanosomal antigens TRP-1 and HMB45, determined by Western blotting, was greatly reduced by ABHA and HMBA, the latter being more effective than ABHA in the pigmented MM418c5 cells (Table 1). Azelaic acid at an approximately equitoxic dose had little effect.

During the first 12 hr of ABHA treatment the ppRB level (hyperphosphorylated pRB) was not affected but a small amount of pRB (hypophosphorylated form) could be detected (Fig. 3). After prolonged treatment of MM96L the ppRB was lost, but pRB remained in both ABHA- and HMBA-treated cells. The loss of ppRB was less marked in HeLa cells than in MM96L.

ABHA-induced Transcriptional Changes Determined by Reporter Genes

Cell clones stably transfected with a range of reporter constructs were used to directly test the effect of differentiating agents on specific gene promoters related to control of the cell cycle and in several instances to compare the

TABLE 1. Effect of 3-day drug treatment on pigmentation markers in human melanoma cell lines

Cell line	Agent	Dose (μg/mL)	Antigenic expression (% of control)		Tyrosinase activity (% control)
			TRP-1	HMB-45	
MM96E	Azelaic acid	2,000	54	81	NT*
	HMBA	1,000	3.2	0.4	107 ± 7†
	ABHA	30	9.1	7.7	44 ± 4
MM418c5	Azelaic acid	2,000	75	47	NT
	HMBA	1,000	7.2	48	141 ± 12
	ABHA	30	27	160	86 ± 4

* NT, not tested. † Mean and SD of triplicates.

TABLE 2. Transcriptional regulation of luciferase-linked reporter constructs in MM96L and HeLa

DNA Motif	Drug	Dose (μg/mL)	Induction (% Control)*			
			MM96L		HeLa	
			6 hr	24 hr	6 hr	24 hr
p53 response element	ABHA	30	73	36	61	172
	ABHA	100	68	25	76	202
	HMBA	1000	84	93	74	73
	Azelaic acid	2000	97	194	59	30
c-fos promoter	ABHA	30	101	639	31	388
	ABHA	100	103	459	34	580
	HMBA	1000	171	270	240	118
	Azelaic acid	2000	121	61	58	77
HIV LTR	ABHA	30	88	233	133	320
	ABHA	100	96	332	98	448
	HMBA	1000	121	104	113	55
	Azelaic acid	2000	99	87	77	38
SV40 promoter	ABHA	30	131	237	NT†	NT
	ABHA	100	130	352	NT	NT
	HMBA	1000	90	48	NT	NT
	Azelaic acid	2000	88	48	NT	NT
TRP-1 promoter	ABHA	30	42	32	NT	NT
	ABHA	100	41	28	NT	NT
	HMBA	1000	47	39	NT	NT
	Azelaic acid	2000	44	91	NT	NT

* Means of triplicates. † NT, not tested.

transcriptional effects of ABHA in sensitive (MM96L) and relatively resistant cells (HeLa) (Table 2). The results were confirmed with a second cell clone. ABHA treatment for 24 hr resulted in elevation of *c-fos* and SV40 promoter activities, whereas equitoxic levels of HMBA and azelaic acid had lesser effects or were inhibitory. All drugs inhibited the TRP-1 promoter, with 6 hr or 24 hr treatment.

In some instances the sensitive MM96L cells gave different responses to ABHA compared with HeLa. MM96L showed inhibition of p53-activation in a 24 hr treatment. *c-Fos* promoter activity was strongly inhibited in HeLa but not in MM96L, following a 6 hr treatment with ABHA.

The zinc-induced activity of the metallothionein promoter in 6 stably transfected, mixed clones of MM96L cells (MM96L-gal) was highly sensitive to ABHA, enhancement being detected after a 5 hr treatment with 1 μg/mL of drug (Fig. 4). The dose response of 6 mixed HeLa-gal clones showed an inverse response, being inhibited to 40% of the control at 10 μg/mL ABHA (Fig. 4). Similar trends were found when transiently-transfected cells were used. When MM96L-gal cells were treated with 10 μg/mL ABHA for 24 hr, washed and then induced with zinc for 5 hr, increased activity (181 ± 13%) was also obtained, indicating that ABHA was not acting by transporting zinc into the cells. The ABHA enhancement of zinc activity was not abrogated by exposure to the PKC inhibitors calphostin C (0.5 μg/mL) or bisindolyl maleimide (1 μg/mL).

DISCUSSION

As anticipated from studies in other cell types [2, 6], ABHA was considerably more potent to human melanoma cells than HMBA. More surprising, however, was the high degree of selectivity for tumor cell lines and transformed cells compared with normal cells. This difference did not result simply from differences in cell cycle time. Furthermore, the ABHA-sensitive tumor line MM96L showed major differences from the more resistant HeLa cells in the transcriptional activation of certain genes, particularly metallothionein.

Enhanced dendritic morphology was the only evidence found for induction of differentiation by ABHA. For melanoma cells, this may relate to their neural crest origin. In the pigmentation pathway, loss of the TRP-1 and

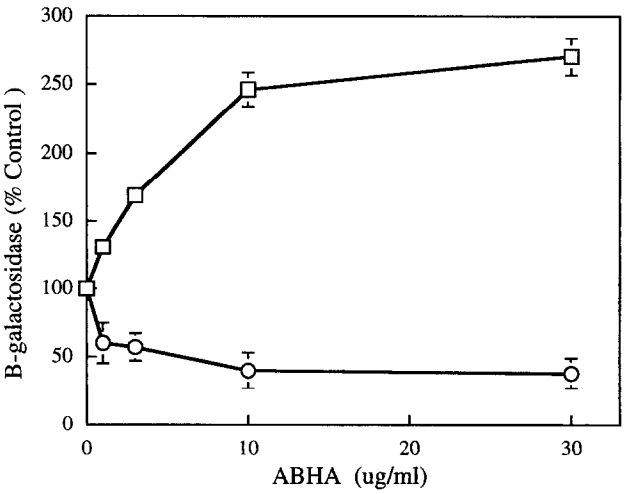

FIG. 4. ABHA dose response for activation of the sheep metallothionein Ia promoter in the presence of 100 μM $ZnSO_4$. □, MM96-gal; ○, HeLa-gal. Points are mean and SD of triplicates.

HMB-45 proteins and a decrease in tyrosinase activity indicated that ABHA acted as a dedifferentiating agent. That this occurs at least in part at the transcriptional level was suggested by loss of TRP-1 reporter activity and, in previous studies of HMBA [8, 14, 15], loss of TRP-1 message and protein. The SV40 promoter/enhancer responds to TPA (results not shown), thus an AP-1 site in this construct may be affected by ABHA. The variety of response elements in these promoters and in the HIV-LTR sequence [16], coupled with lack of specificity for activation in sensitive cells, indicates that ABHA may exert many effects which are not associated with cell selectivity.

HMBA caused a decrease in p53 protein in murine cells [3], along with S phase proteins, evidently as part of differentiation. The level of p53 was not directly determined in this study but the reporter assays indicated that ABHA was more effective than HMBA in depressing the functional activity of p53, and was selective for the sensitive melanoma cell line. A difference between MM96L and HeLa in the MAP kinase pathway [16] could be inferred from the specific early decrease in *c-fos* transcriptional activity in the latter cell line.

The primary target(s) of ABHA and other members of this class of differentiating agent remain unknown. As a hydroxamic acid, ABHA has the ability to chelate zinc and other metal ions but this is unlikely to explain the metallothionein promoter response on the basis of assisting uptake of zinc from the culture medium because treatment with ABHA before zinc induction was also effective. The metallothionein reporter activation by ABHA in melanoma cells may result from changes in chromatin structure as suggested for the much less potent differentiating agent butyrate [17], enhancing transcription of a range of genes including *c-fos* but repressing TRP-1 and genes activated by p53. Demethylation of DNA enhances metallothionein promoter activity in certain cells [18] but was precluded in this study by the rapid transcriptional response to ABHA and its insensitivity to 5-azacytidine (results not shown). The metallothionein promoter response parallels the survival difference between HeLa and melanoma cells and while not necessarily involved in selective inhibition of cell growth may help to identify the type of molecules targeted by ABHA. This promoter contains GC-rich motifs resembling Sp-1 binding sites, thus one or more steps in such a signal transduction pathway may be aberrant in melanoma cells. Metallothionein itself may play several roles in cellular signalling including metal ion homeostasis [19] and regulation of PKC [20], perhaps leading to alteration in regulators of the cell cycle.

As found in murine erythroleukemia cells treated with HMBA, ABHA induced a small elevation of pRB in melanoma cells treated for 12 hr. More significantly, and consistent with the observed G1 block in MM96L cells, pRB persisted at later times whereas ppRB was lost, in contrast to murine erythroleukemic cells where ppRB levels increased [5]. Cyclin-dependent kinase inhibitor p21 (WAF-1) activity is associated with pRB hypophosphorylation and is induced by p53-dependent and -independent pathways, which may be aberrant in melanoma and other transformed cells [21].

Overall, the results suggest that in addition to greatly increased potency, ABHA has a more specific range of cellular targets than the differentiating agents studied previously, resulting in selectivity for transformed human cells. The identification here of metallothionein transcription activation as an early marker of this selectivity may facilitate the discovery of structures with improved action against melanoma.

This work was supported by the Queensland Cancer Fund and the National Health and Medical Research Council.

References

1. Reuben RC, Khanna PL and Gazitt Y, A new group of potent inducers of differentiation in murine erythroleukemia cells. *Proc Natl Acad Sci* **72:** 1003–1006, 1975.
2. Marks PA, Richon VM, Kiyokawa H and Rifkind RA, Inducing differentiation of transformed cells with hybrid polar compounds: A cell-cycle dependent process. *Proc Natl Acad Sci USA* **91:** 10251–10254, 1994.
3. Richon VM, Ramsay RG, Rifkind RA and Marks PA, Modulation of the c-*myb*, c-*myc* and p53 mRNA and protein levels during induced murine erythrleukemia cell differentiation. *Oncogene* **4:** 165–173, 1989.
4. Richon VM, Rifkind RA and Marks PA, Expression and phosphorylation of the retinoblastoma protein during induced differentiation of murine erythroleukemia cells. *Cell Growth Diff* **3:** 413–420, 1992.
5. Kiyokawa H, Richon VM, Rifkind RA and Marks PA, Suppression of cyclin-dependent kinase 4 during induced differentiation of erythroleukemia cells. *Mol Cell Biol* **14:** 7195–7203, 1994.
6. Breslow R, Jursic B, Yan ZF, Freidman E, Leng L, Ngo L, Rifkind RA and Marks P, Potent cytodifferentiating agents related to hexamethylenebisacetamide. *Proc Natl Acad Sci* **88:** 5542–5546, 1991.
7. Takahashi H and Parsons PG, *In vitro* phenotypic alteration of human melanoma cells induced by differentiating agents: Heterogeneous effects on cellular growth and morphology, enzymatic activity and antigenic expression. *Pigm Cell Res* **3:** 223–232, 1990.
8. Sturm RA, O'Sullivan BJ, Thomson JAF, Jamshidi N, Pedley J and Parsons PG, Expression studies of pigmentation and pou-domain genes in human melanoma cells. *Pigm Cell Res* **7:** 235–240, 1994.
9. McEwan M, Parsons PG and Moss DJ, Monoclonal antibody against human tyrosinase and reactive with melanotic and amelanotic melanoma cells. *J Invest Dermatol* **90:** 515–519, 1988.
10. Wong SSC, Sturm RA, Michel J, Zhang X-M, Danoy PAC, McGregor K, Jacobs JJ, Kaushal 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.
11. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A and Fusenig NE, Normal keratinisation in a spontaneously immortalised aneuploid human keratinocyte cell line. *J Cell Biol* **106:** 761–771, 1988.
12. Funk WD, Pak DT, Karas RH, Wright WE and Shay JW, A

transcriptionally active DNA-binding site for human p53 protein complexes. *Mol Cell Biol* **12:** 2866–2871, 1992.
13. Taylor GO and Dunn IS, Automated cycle sequencing of PCR templates: relationships between fragment size, concentration and strand renaturation rates on sequencing efficiency. *DNA Sequencing* **5:** 9–15, 1994.
14. Vijayasaradhi S, Doskoch PM, Wolchok J and Houghton AN, Melanocyte differentiation marker gp75, the brown locus protein, can be regulated independently of tyrosinase and pigmentation. *J Invest Dermatol* **105:** 113–119, 1995.
15. Vijayasaradhi S, Doskoch PM and Houghton AN, Biosynthesis and intracellular movement of the melanosomal membrane glycoprotein gp75, the human b (brown) locus product. *Exp Cell Res* **196:** 233–240, 1991.
16. Edwards DR, Cell signalling and the control of gene transcription. *Trends Pharm Sci* **15:** 239–244, 1994.
17. Liu J, McKim JM Jr, Liu YP, Klaassen CD, Effects of butyrate homologues on metallothionein induction in rat primary hepatocyte cultures. *In Vitro* **28A:** 320–326, 1992.
18. Biard DS, Cordier A and Sarasin A, Establishment of a human cell line for the detection of demethylating agents. *Exp Cell Res* **200:** 263–271, 1992.
19. Hamer DH, Metallothionein. *Ann Rev Biochem* **55:** 913–951, 1986.
20. Ou CZ and Ebadi M, Pineal and retinal protein kinase C isoenzymes: Cooperative activation by calcium and zinc metallothionein. *J Pineal Res* **12:** 17–26, 1992.
21. Vidal MJ, Loganzo F, de Oliveira AR, Hayward NK and Albino AP, Mutations and defective expression of the WAF1 p21 tumor-suppressor gene in malignant melanomas. *Melanoma Res* **5:** 243–250, 1995.