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TRANSCRIPTIONAL REGULATION OF DIFFERENTIATION, SELECTIVE TOXICITY AND ATGCAAAT BINDING OF BISBENZIMIDAZOLE DERIVATIVES IN HUMAN MELANOMA CELLS

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Abstract—To study the relationship between the structure of minor groove ligands and their affinity for specific DNA sequences that regulate gene transcription, three analogues of the A-T-specific DNA minor groove ligands Hoechst 33258 and Hoechst 33342 were synthesized with 5, 8 or 12 carbons in an aliphatic chain attached to the phenolic oxygen of the molecule. There was a striking bimodal relationship between toxicity to HeLa cells and the lipophilicity of the five analogues, toxicity being low for the compounds with a free hydroxyl (Hoechst 33258) or a 12-carbon substituent, yet high for the 5-carbon analogue. Selective killing of human melanoma cells compared with normal fibroblasts was observed for the Hoechst analogue with a 12-carbon chain attached. Hoechst 33258 itself was selectively toxic for the MM96E melanoma cell line compared with other cell lines, induced a highly dendritic morphology, increased tyrosinase activity and tyrosinase mRNA but decreased the level of gp75 (TRP-1) mRNA; message for a third pigment gene, Pmel-17, was unchanged. Tyrosinase activity was decreased in the resistant A2058 melanoma cell line and transcription was affected to a lesser extent than in MM96E. Expression of gp75 protein and two intermediate filament proteins was inhibited by Hoechst 33258 in MM96E cells. There was no major difference in the amount of ^{125}I-Hoechst 33258 taken up by sensitive and resistant cells. Of the five derivatives studied, the parent drug Hoechst 33258 and the 2-carbon analogue (Hoechst 33342) were found to have the most inhibitory effect on affinity of octamer binding proteins for the ATGCAAAT consensus sequence found in the promoter region of certain genes associated with proliferation and differentiation. In contrast to Distamycin A (also an A-T-specific minor groove ligand), Hoechst 33258 displaced proteins already bound to the octamer motif. The G-C ligand chromomycin A_3 exhibited a different spectrum of cell toxicity and tyrosinase stimulation compared with Hoechst 33258. Chromomycin A_3 but not Hoechst 33258, strongly inhibited the zinc-dependent transcriptional activity of the sheep metallothionein-Ia promoter in reporter gene assays of transfected cells. Since the six metal-responsive elements of the promoter are GC-rich, this provides independent evidence for the sequence-specificity of transcriptional inactivation by one of these drugs in melanoma cells. Overall, the results suggest that Hoechst 33258 acts by inhibiting the transcription of specific genes, cell lines evidently differing in the accessibility to drugs of certain A-T-rich sequences.

Key words: Hoechst 33258; chromomycin A_3; octamer-binding proteins; tyrosinase; sequence specificity; gp75

Regulation of specific gene expression in cancer cells or certain tissues by DNA sequence-specific drugs is an attractive but elusive goal [1] despite the recent identification of many gene regulatory sequences. As a demonstration of the potential of transcriptional regulation in a cell-free system, the A-T-specific minor groove ligand, Distamycin A, was found to inhibit *in vitro* binding of a nuclear protein (Oct-1) to the A-T-rich recognition motif ATGCAAAT in the human histone H2B promoter. Such activity could conceivably account for the antineoplastic and antiviral properties of the compound [2], although the drug was unable to disrupt the protein–motif complex once formed. There have been few studies where DNA ligands could be shown to induce specific toxicity or differentiation changes in the intact cell.

Hoechst 33258 (H-OH) is a synthetic *N*-methylpiperazine derivative (Fig. 1) developed initially for use as an anthelmintic agent but has activity against L1210 and P388 leukaemias in mice [3]. The bright fluorescent complex formed in binding to double-stranded DNA enables it to be used for cell cycle studies [4, 5]. EC cells were differentiated by the ethoxy derivative Hoechst 33342 (H-2C) but not by H-OH [6]. The mechanism of action may include inhibition of chromosomal DNA

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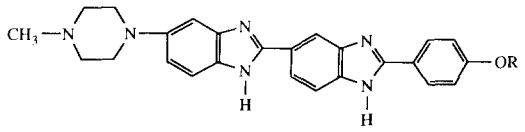

Fig. 1. Structure of Hoechst 33258 derivatives. R = H, H-OH (Hoechst 33258); R = ethyl, H-2C (Hoechst 33342); R = pentyl, H-5C; R = octyl, H-8C; R = dodecyl, H-12C.

condensation [7], prolonging the G2 phase of the cell cycle, and reducing the rate of DNA and RNA synthesis [8, 9]. The drug–DNA complex may prevent unwinding of the double helix during transcription [9], topoisomerase activity may be affected [10] and bacterial excinuclease is inhibited [11], however there is little information available concerning the primary molecular targets responsible for cell arrest.

Binding of H-OH to three A-T base pairs in the minor groove was proposed [12]. The binding interactions between H-OH and the A-T-rich minor groove are the combined results of electrostatic forces, hydrogen bonding and van der Waals contacts. As pointed out by Portugal and Waring [13], one structural feature that may allow Hoechst 33258 to tolerate the presence of a G-C base pair could be the positive charge on only one end of the molecule, in contrast to the purely A-T binding Netropsin which has both ends charged. A single charged end may allow the molecule to move away from the minor groove floor to interact with the G-C base pair. The H-OH molecule has three potential donor sites for formation of hydrogen bonds with the A-T region of the minor groove, the two NH groups on the bisbenzimide structure and the CH_3N^+H group on the piperazine ring. These hydrogen bonds are believed to have primary importance in determining the exact site of binding [12, 14]. Footprinting studies employing DNAse I and micrococcal nuclease gave an estimation of the optimum binding site as at least four A-T base pairs [13, 15], G-C base pairs being rarely protected. A similar conclusion was reached using ^{125}I-labelled drugs [16]. Some degree of G-C binding was found with benzoxazole analogues of H-OH [17].

In addition to Oct-1, which is expressed ubiquitously, two other OBPs* have been detected in human melanoma cells [18, 19]. Termed Oct-M1 and Oct-M2 and possibly reflecting the neural crest origin of melanocytes, expression of these proteins and of brown locus protein (gp75, or TRP-1) was suppressed when the cells were differentiated by treatment with DMSO or butyric acid [19]. A non-consensus octamer element (ATTCAAAT) was recently found in the promoter region of the mouse gp75 gene [20]. The high A-T content of the octamer motif appears to be unusual amongst the known transcriptional control elements, raising the possibility that an A-T selective drug could inhibit cell replication by preventing Oct-1 activation of histone transcription as well as other gene systems activated by Oct-1. Such an agent could also affect differentiation in melanocytic cells by interfering with the action of Oct-M1 and Oct-M2, should these proteins be involved directly or indirectly in the regulation of gp75 or other pigment and neural crest genes. We have found that H-OH and four of its alkyl ether derivatives have selective effects on cell survival, transcription, OBP affinity for the octamer motif, and differentiation markers in human melanoma cells; the GC binding drug chromomycin A_3 was used as control. Independent evidence for sequence-specific inhibition of transcription by chromomycin A_3 in melanoma cells was obtained using a reporter gene.

* Abbreviations: OBP, octamer-binding protein; EMSA, electrophoretic mobility shift assay.

MATERIALS AND METHODS

Synthesis of Hoechst 33258 derivatives. Hoechst 33258 (H-OH) and the ethoxy derivative 33342 (H-2C) were purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The pentoxy derivative H-5C [2′-(4;-pentoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bis-1H-benzimidazole] was prepared by heating H-OH (101.6 mg, 0.16 mmol), 1-bromopentane (72.4 mg, 0.48 mmol), anhydrous potassium carbonate (1 g) and activated 4 Å molecular sieve (1 g) in anhydrous DMSO (30 mL) in the dark at 100–110° for 2 hr. The reaction mixture was diluted with ethyl acetate (100 mL), washed with water at pH 8, the product extracted into water at pH 4 and returned to ethyl acetate at pH 8. After evaporation of the solvent the crude product was chromatographed on a column of LiChroprep Si 60 silica gel in methanol:ethyl acetate:triethylamine (100:500:3). H-5C was obtained as a light yellow solid, m.p. 175–182°. M^+ 495, 438, 424, 410, 396, 353, 339, 325. ^{1}H NMR (DMSO-d_6): 1.04 (t, CH_3, 3H, J = 6.9 Hz), 1.52 (m, CH_2, 4H), 1.89 (m, $OCH_2\underline{CH_2}$, 2H), 2.98 (s, NCH_3, 3H), 3.35 (s, CH_2, 4H), 4.22 (t, OCH_2, 2H, J = 6.3 Hz), 7.33 (d, Ar-H, 2H, J = 8.4 Hz), 7.35 (s, Ar-H, 1H), 7.47 (d, Ar-H, 1H, J = 9.0 Hz), 7.85 (d, Ar-H, 1H, J = 9.4 Hz), 8.07 (d, Ar-H, 1H, J = 8.6 Hz), 8.39 (d, Ar-H, 1H, J = 10.0 Hz), 8.43 (d, Ar-H, 2H, J = 8.7), 8.81 (s, Ar-H, 1H, J = 8.7 Hz), 11.09 (bs, NH, 2H) ppm.

The H-8C derivative [2′-(4-octoxyphenyl) -5-(4-methyl-1-piperazinyl) -2,5′-bis-1H-benzimidazole], prepared as above using 1-bromo-octane, was obtained as a light yellow solid, m.p. 258–264°. M^+ 537, 480, 466, 437, 353, 339. ^{1}H NMR (DMSO-d_6): 1.00 (t, CH_3, 3H, J = 6.8 Hz), 1.41 (m, CH_2, 10H), 1.89 (m, $OCH_2\underline{CH_2}$, 2H, J = 6.8 Hz), 2.99 (s, NCH_3, 3H), 3.35 (d, $\underline{CH_2}$, 4H, J = 8.0 Hz), 3.62 (d, CH_2, 4H, J = 14.5 Hz), 4.24 (t, OCH_2, 2H, J = 6.5 Hz), 7.34 (s, Ar-H, 1H), 7.36 (d, Ar-H, 2H, J = 8.0 Hz), 7.49 (d, Ar-H, 1H, J = 9.3 Hz), 7.87 (d, Ar-H, 1H, J = 8.9 Hz), 8.12 (d, Ar-H, 1H, J = 8.6 Hz), 8.48 (d, Ar-H, 3H, J = 8.7 Hz), 8.67 (s, AR-H, 1H), 11.13 (bs, NH, 2H) ppm.

The H-12C derivative, [2′-(4-dodecoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bis-1H-benzimidazole] was obtained as a light yellow solid, m.p. 154–156°. M^+ 593, 522, 508, 424. ^{1}H NMR (DMSO-d_6): 1.00 (t, CH_3, 3H, J = 6.3 Hz), 1.40 (m, CH_2, 18H),

1.90 (m, OCH_2CH_2, 2H), 2.41 (s, NCH_3, 3H), 3.29 (s, CH_2, 4H), 4.21 (t, OCH_2, 2H, $J = 6.0$ Hz), 7.10 (d, Ar-H, 1H, $J = 7.8$ Hz), 7.28 (d, Ar-H, 2H, J = 8.3 Hz), 7.29 (s, Ar-H, 1H), 7.62 (m, Ar-H, 1H), 7.86 (m, Ar-H, 1H), 8.15 (s, Ar-H, 1H), 8.30 (d, Ar-H, 2H, $J = 8.4$ Hz), 8.46 (m, Ar-H, 1H), 12.77 (bs, NH, 1H), 13.16 (bs, NH, 1H) ppm. The infrared and ultraviolet spectra of the derivatives were consistent with that of H-2C.

Cell culture. The human melanoma lines MM96E [21] and A2058 [22] were derived from metastatic malignant melanoma. The HeLa line was derived from a human cervical tumour. The NFF strain of human fibroblasts [23] was established from a neonatal foreskin. Cells were maintained at 37° in 5% CO_2/air in RPMI medium 1640 (Commonwealth Serum Laboratories, Australia) containing streptomycin (100 μg/mL), penicillin (100 IU/mL), Hepes (3 mM) and 5% (v/v) foetal calf serum. Chromomycin A_3 was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Cell survival assays were performed using 96-well microtitre plates; 2000 cells in 100 μL of culture medium were added to each well and incubated overnight. Drugs were then added at different concentrations to duplicate wells. When the controls were nearly confluent (about 5–7 days), cell growth was visually assessed under a microscope and then quantitated by [^{3}H]thymidine incorporation. Cells in each well were labelled by incubating at 37° with 50 μL of 2 μCi/mL [^{3}H-*methyl*]thymidine (Amersham International plc, Amersham, U.K.) in fresh culture medium for 4 hr. The medium was then discarded and the attached cells washed with PBS to remove excess [^{3}H]thymidine. The cell monolayer was detached with trypsin and washed onto a glass fibre mat with water using a LKB Wallac 1295-001 cell harvester. Liquid scintillation counting was performed in the LKB Wallac 1205 Betaplate liquid scintillation counter (Bromma, Finland).

Dose–response curves were plotted on a log–log scale for survival (calculated as fraction of control cpm) versus drug concentrations. Survivals were compared on the basis of the D_{37}, which is the dose required to reduce the survival to 37%. D_{37} is the dose which statistically causes one lethal event per cell on average. Four to six replicate experiments with different passages of cells were carried out for each drug and cell line to confirm the reproducibility of the assay.

^{125}I-H-OH synthesized using Na^{125}I, Iodogen and H-OH, essentially according to a previous method [24] was incubated with 10^6 cells in culture medium for 1 hr. Samples were removed at various times, washed three times with cold PBS and counted.

EMSA assay. Nuclear extracts were prepared from cells in exponential growth phase, essentially as described [19]. Nuclear extract (2 μL) containing approximately 2 μg of nuclear protein (as determined by Bradford assay) was incubated for 20 min at room temperature with 8 μL of binding mix [25] containing 3 μg of poly d(I-C). ^{32}P-labelled H2B consensus octamer probe (0.5 ng) [19] was incubated for 15 min at room temperature with drug in a total volume of 5 μL, the dilution prepared in a similar way as that for the cell survival assay but calculated on a micromolar basis. After 15 min, the binding solution with nuclear extracts added was aliquoted to each tube of probe–drug mixture. The mixtures were incubated for a further 15 min before fractionation on 6% polyacrylamide gels. Mutant octamer dpm8 probe [25] was used as a negative control DNA. Separated bands were detected by a PhosphorImager, recorded with Image-Quant software (Molecular Dynamics, U.S.A.), and the digitized data further manipulated with Excel (Microsoft, U.S.A.); gel pictures were prepared using Charisma (Micrografx, U.S.A.).

Tyrosinase assays. Cells were lysed in 50 mM phosphate, pH 6.8, containing 1% Triton X-100, centrifuged (5000 *g* for 10 min) and triplicate 15 μL aliquots incubated with 8 mM dopa (150 μL) in the above buffer in a microtitre plate at room temperature. The increase in absorbance at 490 nm was followed with an ELISA reader over 50 min, and the initial rate used to compare enzyme activities.

Northern blotting. RNA was isolated by lysis of cells in 4 M guanidinium thiocyanate [26], separated on 1.2% agarose gels, blotted onto Hybond-N membrane and hybridized with ^{32}P-labelled probes as described previously [27]. The human gp75 cDNA was excised by EcoR1/PvuII digestion of pHTα2 and isolation of 1.1 and 1.7 kb fragments [28], and human tyrosinase probe was generated using the 1.6 kb EcoRI insert of pMel34 [29]. A 518 bp polymerase chain reaction fragment generated from MM96E cDNA using an amplimer pair ATGGATCTGGTGCTAAAAAG and CCAATGCTCAGCCCAGACAC designed against the human pMel17 gene sequence [30] was cloned into the SmaI site of pBS and isolated as a HindIII/EcoRI fragment from a clone referred to as pBSme117. 18S rRNA was quantitated using an oligonucleotide MOE complementary to the mouse sequence

5′-dCATGGTAGGCACGGCGACTACCATC-3′

end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase and hybridization performed in 20% formamide solution as described [27].

Western blotting. Cell proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose and blotted with monoclonal antibody supernatants as described previously [19]. IFA recognises all classes of intermediate filaments [31] and B8G3 reacts with gp75 [19]. Antibody against human GAPDH was raised by i.p. immunization of BALB/c mice with antigen (Sigma) in Freund's complete adjuvant.

Deviation of a reporter construct and transfection of MM96 cells. The shuttle vector p294MetM3 was constructed from p294 (kindly provided by Dr B. Sugden via Dr T. Sculley), itself containing the Epstein–Barr virus (EBV) plasmid origin of replication (*ori P*, sequence coordinates 7338–9517 of the B95-8 EBV genome [32]), the EBNA-1 gene (which regulates episomal replication and maintenance; coordinates 107930–110493 of EBV) driven by the immediate-early cytomegalovirus promoter [33], the hygromycin resistance gene [34], expressed from the herpes virus thymidine kinase promoter and 3′-untranslated sequences [35], and

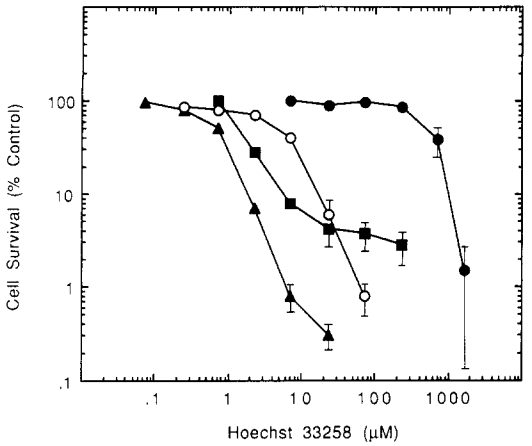

Fig. 2. Dose–response of cell survival after continuous treatment with H-OH (Hoechst 33258) (■) NFF; (●) HeLa; (▲) MM96E; (○) A2058. Points are means ± SE for four to six separate experiments.

bla gene/col E1 sequences for replication and selection in *Escherichia coli*. The sheep metallothionein-Ia (sMT-Ia) promoter (as a segment of 861 bp) [36] and the 3′ polyadenylation signal from the sheep growth hormone gene (2.219 kb) [37, 38] were obtained from the plasmid pBSmetSGH (kindly provided by K. Ward). This plasmid was manipulated such that a unique Bam HI site was positioned between the promoter and the 3′- untranslated region without any intervening coding sequence, and the entire segment (promoter plus 3′ regulatory region; 3.08 kb) cloned as a blunt-ended fragment into the unique Bam HI site of p294 (rendered blunt-ended enzymatically). This destroyed the original p294 Bam HI site, resulting in a new unique Bam HI site from the pBSmetSGH-derived fragment. A Bam HI fragment encompassing the entire coding region for *E. coli* β-galactosidase was subsequently inserted into this construct (p294Met30) at the corresponding unique site. The β-galactosidase (*lac* Z) gene itself was previously manipulated in the following manner: a Bam HI fragment from pMC1871 [39] containing all but the first seven *lac* Z codons was mutagenized [40] to alter the 5′ region. This provided an initiation codon in a favourable local sequence environment as defined by Kozak [41], and introduced a number of convenient restriction sites. In addition, the EcoRI site present near the 3′ end of the lac Z coding sequence was removed by site-directed mutagenesis such that the expressed protein sequence was not altered (GAA TTC changed to GAG TTT). The final construct, where β-galactosidase expression was controlled by the metallothionein promoter and the growth hormone 3′ polyadenylation signals, was termed p294MetM3.

MM96L cells (a late passage of MM96E) were transfected with p294MetM3 under conditions of electroporation previously found to be optimal in transient-expression assays (500 μF, 230 V; 0.8 mL of cells, 5×10^6/mL suspended in Hepes-buffered RPMI 1640 without serum during the electroporation treatment). Forty-eight hours after transfection, cells bearing the p294MetM3 plasmid were selected with hygromycin B at 100 μg/mL for 8 weeks until a completely resistant pool of cells was isolated; subsequently it was shown that these cells (MM96L-gal) had a very low endogenous level of β-galactosidase production, but produced high levels of activity detectable at 4 hr and peaking at 24 hr following treatment with 100 μM $ZnSO_4$. For enzyme activity cells were frozen and thawed three times and assayed essentially as described [39], in microtitre plates in triplicate. Protein was determined as described previously [19].

RESULTS

Cell survival

The range of drug concentrations that could be used was limited by solubility at neutral pH; precipitation of H-12C in culture medium being observed at 168 μM. The dose–response curves (Fig. 2) of Hoechst 33258 (H-OH) in different cell types as summarized by the D_{37} values (Table 1) showed that the drug was selectively toxic to MM96E compared with NFF fibroblasts and A2058 cells. Proliferation of these cell lines was more sensitive to H-OH than was sperm motility, which was inhibited by 168 μM H-OH but not by 32 μM [42]. MM96E cells became highly dendritic during treatment. HeLa cells were the most resistant to H-OH, the D_{37} being 300-fold greater than that of NFF fibroblasts. There was no difference in the uptake of ^{125}I-H-OH by HeLa, A2058, or MM96E cells when incubated with the drug for 1 hr (results not shown).

Table 1. Toxicity of Hoechst 33258 (H-OH) and its alkoxy homologues in cultured human cells

	D_{37} (μM)				
Cell lines	H-OH	H-2C	H-5C	H-8C	H-12C
NFF	1.09 ± 0.23 (4)	0.49 ± 0.25 (5)	2.39 ± 0.10 (5)	2.33 ± 0.2 (5)	>168
HeLa	337 ± 42 (3)	5.63 ± 0.35 (4)	2.29 ± 0.06 (5)	5.50 ± 0.96 (4)	>168
MM96E	0.53 ± 0.10 (6)	0.66 ± 0.10 (4)	2.52 ± 0.09 (5)	2.09 ± 0.05 (4)	57 ± 10 (4)
A2058	5.12 ± 0.86 (4)	0.54 ± 0.10 (4)	2.38 ± 0.01 (4)	2.09 ± 0.05 (4)	3.26 ± 0.35 (4)

Values are mean ± SE; number of separate experiments in parenthesis.

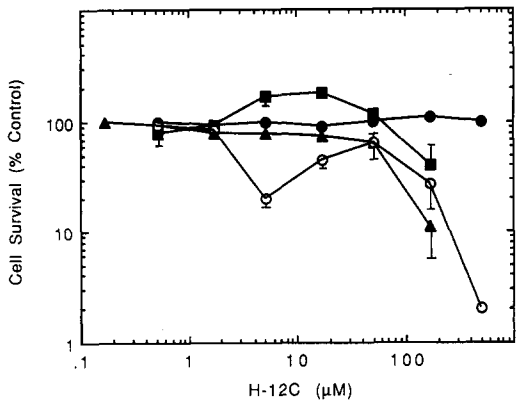

Fig. 3. Dose–response of cell survival after treatment with H-12C. (■) NFF; (●) HeLa; (▲) MM96E; (○) A2058. Points are means ± SE for four separate experiments.

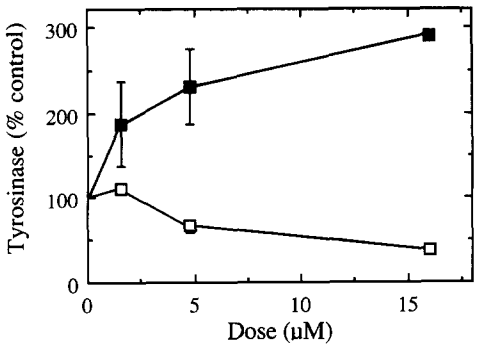

Fig. 4. Effect of a 3-day treatment with H-OH on tyrosinase activity in MM96E (■) and A2058 (□) cells. Points are means ± SE of two to four experiments.

The ethoxy derivative H-2C was less selective, showing no difference between melanoma cells and fibroblasts and HeLa being only 5-fold more resistant (Table 1). The dose–response of the latter cell line appeared to be similar to the 5 μM level of H-2C which differentiated EC cells [6]. The H-5C and H-8C compounds exhibited no selectivity, the D_{37} values for all cell lines being in the 2–5 μM range (Table 1). The apparent D_{37} of the most hydrophobic analogue studied, H-12C, was approximately 20-fold lower for A2058 cells than for MM96E, due to a unique biphasic dose–response with a minimum at 5.6 μM followed by recovery to nearly 100% survival at higher doses (Fig. 3). This was found consistently in four separate experiments. At the highest achievable dose of 168 μM, both melanoma cell lines showed lower survival levels than HeLa and fibroblasts.

The G-C binding drug chromomycin A_3 was highly toxic, as found previously in human tumour cell lines [43], but showed little selectivity against any particular cell line (Table 2).

Differentiation markers

An H-OH dose–response study of tyrosinase (dopa oxidase) activity in cells treated for 3 days revealed a marked difference between the two melanoma cell lines. Activity was substantially increased in MM96E cells, even at doses too low to have significant toxicity, whereas activity in A2058 cells, constitutively lower than in MM96E, was decreased (Fig. 4). In an additional experiment, these opposite effects on tyrosinase activity were maintained at 56 μM (245% of control for MM96E and 12% for A2058). No such differences were found using chromomycin A_3, which increased tyrosinase activity in both cell lines but only at a highly toxic dose (Table 2).

To determine whether regulation of tyrosinase activity and gp75 expression occurred at the level of RNA production, total RNA was extracted from treated cells and hybridized on a northern blot with ^{32}P-probes for three pigment genes. When normalized to the internal control (ribosomal RNA) MM96E cells exhibited a significant increase in tyrosinase message and a decrease in gp75 message, compared with A2058 (Fig. 5). This was not due to a general

Table 2. Effect of chromomycin A_3 on survival and tyrosinase activity in human tumour cells

Cell line	D_{37} (nM)	Tyrosinase activity (3-day treatment) Dose (nM)	% Control
HeLa	3.1	NA	NA
MM96E	3.5	0.67	49
		6.7	120
		67	200
A2058	3.7	0.67	103
		6.7	79
		67	250

NA, not applicable.

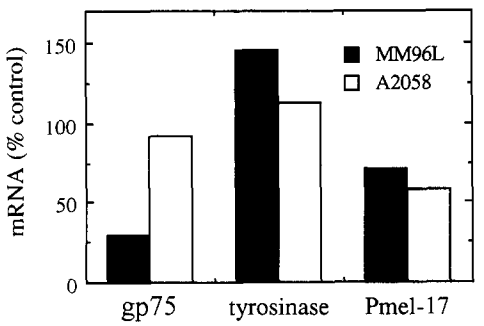

Fig. 5. mRNA of pigmentation genes in cells treated with 1.7 μM H-OH for 3 days. Cpm of bands in northern blots of total RNA were determined with the PhosphorImager and expressed as % of untreated cells. RNA loadings were normalized against a ribosomal RNA probe and glyceraldehyde-3-phosphate dehydrogenase, applied to the same filters.

loss of transcriptional activity because the β-actin and GAPDH messages were not affected. It should be noted that constitutive expression of tyrosinase activity and mRNA for tyrosinase and gp75 was approximately 10-fold higher in MM96E compared with A2058.

Expression of a tyrosinase-related protein gp75 was found by western blotting to be inhibited by H-OH in MM96E (Fig. 6) but not in A2058 cells (not shown). Simultaneous blotting with IFA antibody, reactive with all classes of intermediate filaments, revealed a band at 55 kDa, presumably vimentin [31], and a more diffuse band at 66 kDa. Both of these proteins were suppressed by H-OH treatment. The fact that equal amounts of protein were loaded in each lane was confirmed by reprobing the filter with GAPDH antibody. IFA immunoreactivity of similar molecular weight bands in A2058 and HeLa cells was not affected by treatment with 1.7, 5.6 or 17 μM H-OH (not shown).

Inhibition by Hoechst 33258 analogues of the binding of Oct factors to the octamer sequence

To examine the effects of H-OH on the formation of complexes between A-T-rich sequences and transcription factors, EMSA analysis was performed on nuclear protein extracts of A2058 cells in the presence of increasing amounts of H-OH (Fig. 7). A labelled DNA probe containing a mutated octamer sequence (AAGGAAAG) showed no retarded activity on a polyacrylamide gel, with free probe migrating to the bottom of the gel (lane 1). The H2B gene consensus octamer sequence (ATGCAAAT) gave three bands in addition to unbound probe (lane 2), the slowest migrating band corresponding to Oct-1 [44] and the two faster migrating complexes previously designated as Oct-M1 and Oct-M2 found in melanocytic cells [19]. When the H2B probe was mixed with increasing levels of H-OH before addition to the cell extract, the amount of complex formation of each of the Oct-factors decreased proportionately to their original levels with untreated probe (lanes 3 to 8). At the highest dose (100 μM) some activity remained in the loading slot, presumably due to aggregation or precipitation of the probe. The linear response of the imaging system and lane volume measurement of activity using the ImageQuant program enabled reproducible calculation of the proportion of probe bound to the Oct-factors. Comparison of the proportion of Oct-factors bound at different drug concentrations showed that binding of all three factors was affected similarly (Fig. 8).

Similar results were obtained for the other four Hoechst 33258 analogues, except that precipitation was more pronounced at the higher doses. Since the binding of all three factors was inhibited to similar extents for a particular drug, the dose–responses are shown for the overall level of inhibition (Fig. 9). These results indicated that, as found for toxicity in HeLa cells, H-5C and H-8C were less effective than either H-OH and H-2C or H-12C. Chromomycin A_3 was less effective than H-OH or H-2C.

The strong binding of H-OH to the H2B probe was further demonstrated by its ability to disrupt preformed OBP–probe complexes (Fig. 10).

Inhibition of sMT-Ia promoter activity by DNA ligands

The sheep metallothionein gene promoter has six G-C-rich presumptive metal regulatory elements (consensus CTNTGCRCNCGGCCC; 76% G-C). Electroporation of the vector (Fig. 11) into MM96 cells and subsequent selection with hygromycin produced clones that contained high levels of β-galactosidase activity when cultures were treated with 100 μM $ZnSO_4$ for 6–24 hr. Treatment of relatively dense cultures of MM96L-gal cells with drug for 1 hr followed by addition of $ZnSO_4$ for a further 6 hr revealed a strong inhibitory response for chromomycin A_3 at doses of 10–30 times the D_{37} value but not for Hoechst 33258 even at doses up to 300 times the D_{37} (Fig. 12). No cellular toxicity was observed for either drug during this treatment period.

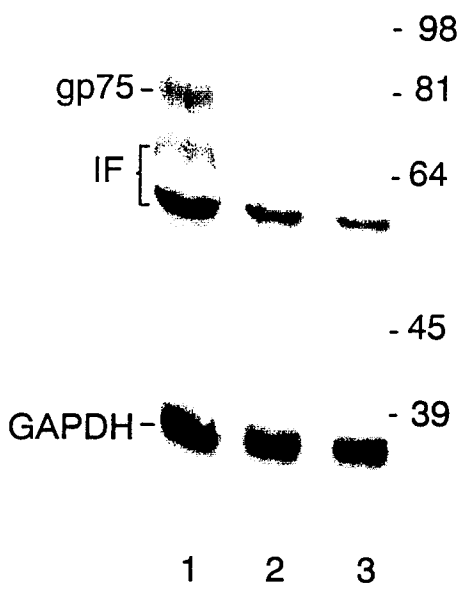

Fig. 6. Western blot of cell proteins with a mixture of gp75, intermediate filament (IF) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies, after treatment with H-OH for 3 days. Lane 1, control; lane 2, 1.7 μm H-OH; lane 3, 5.6 μm H-OH.

DISCUSSION

The extensive literature on the DNA affinity of Hoechst 33258 and its application in cell biology offer little definitive evidence as to the molecular basis of toxicity or, as found in this study, ability to regulate differentiation. Increasing the lipophilicity of H-OH at the phenolic oxygen produced major and unpredictable shifts in toxicity in different cell types, presumably due to subtle differences in the hydrophobic environment of critical A-T-rich target sequences. The rapid inhibition of DNA synthesis by H-2C compared with H-OH [45] suggests that the addition of an ethyl ether may introduce

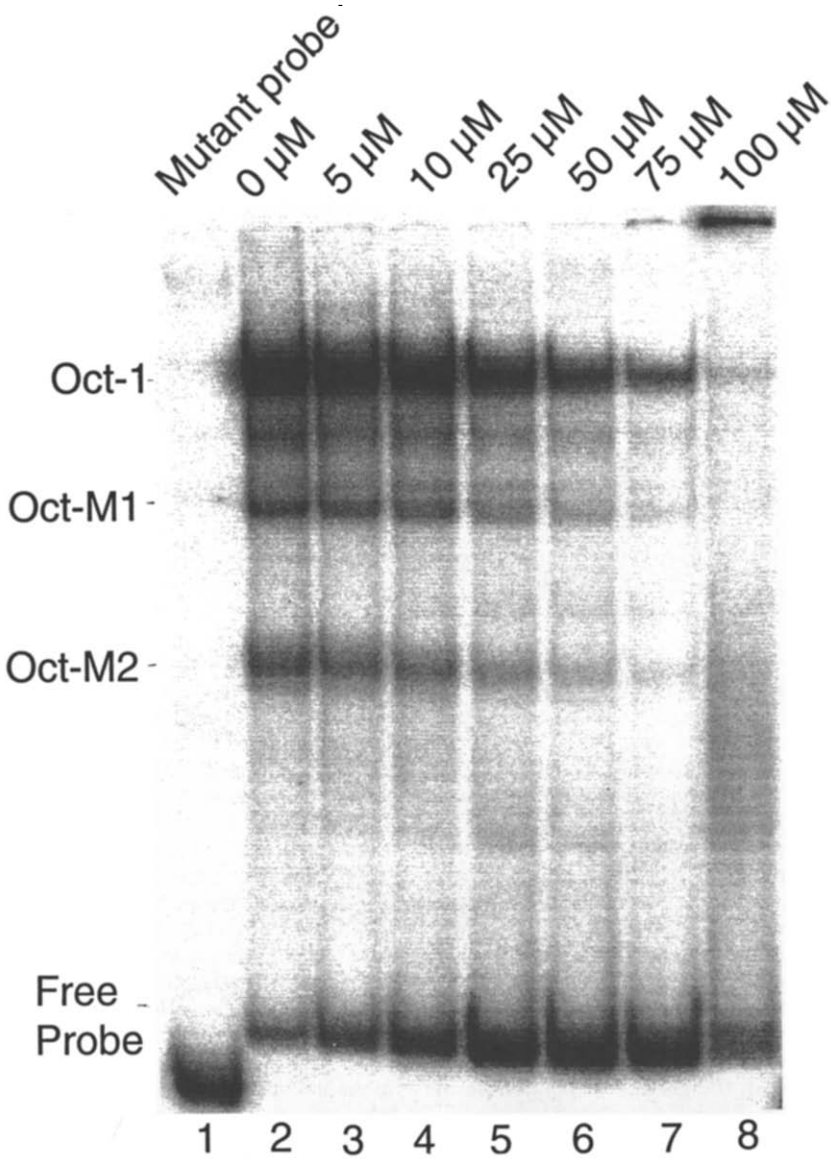

Fig. 7. Octamer-binding proteins in a nuclear extract of A2058 cells, detected by EMSA as complexes with ^{32}P-labelled octamer probes. Preincubation of probe with H-OH inhibited binding. Lane 1, dpm 8 mutant probe; lanes 2–8, H2B octamer probe with the amount of H-OH added indicated at the top.

qualitative changes in the mechanism of action of these drugs. The relatively strong binding of H-12C to the octamer motif indicates the feasibility of using a flexible linker to connect other sequence reading moieties to these analogues. The biphasic response of the A2058 melanoma cell line may result from H-12C acting as a surfactant at concentrations up to the first cell survival minimum, thus enhancing the uptake of growth inhibitory serum lipids, then forming lipid-free micelles at higher concentrations before becoming directly toxic at 168 μM. Alternatively, uptake of the drug itself may be dependent on the degree of micelle formation in the culture medium.

The lack of affinity of H-OH for RNA [46] and protein, the basis for a *Mycoplasma* detection assay [47] and confirmed by the present cell lines, indicates that DNA is the relevant target for this drug. H-OH did not inhibit transcription or translation in general because the mRNA and protein levels of GAPDH were unchanged. Possible effects on DNA–protein interactions therefore require consideration. In principle, any process dependent on DNA–protein interaction for its control or enzymatic function such as gene transcription, replication of DNA, DNA repair, recombination, DNA supercoiling, packaging or chromosomal attachment to the nuclear matrix may be affected by H-OH. The effect of Hoechst analogues on topoisomerases has already been noted [48]. It is interesting to note that Oct-1 has been

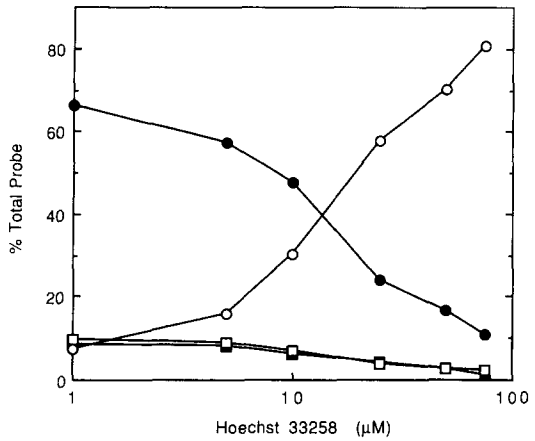

Fig. 8. Quantitative representation of data in Fig. 6, showing dose-dependent inhibition of octamer binding activities by H-OH in nuclear extracts of A2058 cells. (●) Oct-1; (□) Oct-M1; (■) Oct-M2; (○) unbound probe.

shown to be involved in viral DNA replication [49] and may also be involved in cellular DNA replication. Functional inactivation of the Pit-1/GHF1 transcription factor, related to Oct-1, using antisense oligonucleotides has been shown to result in inhibition of cell proliferation [50]. Since the uptake of H-OH and presumably the amount bound to DNA was similar in each cell line, the functional differences elicited by the drug might stem from differences in accessibility to a small but critical subset of A-T-rich regions of DNA. The varied responses found in this study suggests that a number of independent targets are involved, one being the ATGCAAAT or similar motifs. H-OH could be expected to be more active for octamer binding in the intact cell than in the gel shift assay, where the large excess of poly d(I.C) needed to stop non-specific binding of proteins to the labelled DNA competes with the labelled probe by virtue of its structural resemblance to the A-T minor groove. Relevant to potential applications of H-OH, the drug was found to displace OBPs from the DNA motif, in contrast to Distamycin A which binds to a 5 bp sequence and is more potent than H-OH in interfering with topoisomerase II activity [10].

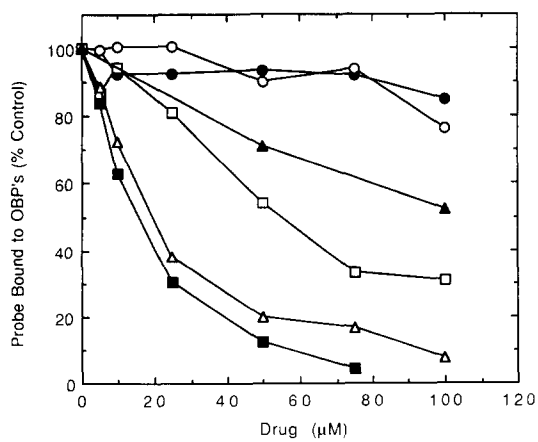

Fig. 9. Dose–response for inhibition of the binding of Oct factors by a sequence-selective drugs. (△) H-OH; (■) H-2C; (●) H-5C; (○) H-8C; (□) H-12C; (▲) chromomycin A_3.

Transcriptional control by H-OH of differentiation in melanoma cells could be inferred from the decrease in gp75 message. The role of gp75 in pigmentation has yet to be determined, but has been described as a catalase [51] or as a tyrosinase of low activity [52]. Gp75 expression is very sensitive to inhibition by differentiating agents [53]. Due to the complexity of possible regulatory elements in the tyrosinase and gp75 promoters and the present lack of assays for pigmentation-specific transcription factors one can only speculate that both positive and negative regulation of cell growth and differentiation arises from alteration in the accessibility of A-T-rich motifs. Such sequences, which could perhaps be isolated by affinity for appropriate H-OH analogues, evidently do not include the TATA box because expression of some genes was not affected.

In addition to suppression of gp75, loss of intermediate filament proteins was found to be a consequence of H-OH treatment. This is consistent with the induction of dendritic morphology in MM96E cells, typical of neural differentiation, and may have significance for therapy because enhanced expression of such proteins is associated with metastatic potential in human melanoma [54]. In contrast, 5 μM H-2C induced the differentiation of teratocarcinoma stem cells with enhanced synthesis of intermediate filaments [6]; H-OH had no effect.

Of the many gene products that could either directly or indirectly influence accessibility of H-OH to DNA, high mobility group proteins HMG-I are possible candidates. HMG-I is a group of non-histone chromosomal proteins abundant in neoplastic, undifferentiated cells and in rapidly proliferating cells. They bind to the minor groove of A-T-rich sequences including the octamer element in the human immunoglobulin light chain promoter and compete with H-OH [55]. Such proteins might therefore regulate gene expression directly as transcriptional activators or by altering the accessibility of A-T-rich regions through changing the conformation of DNA. Given the multiple changes that accompany neoplastic transformation, differences in HMG-I expression may well arise and govern the response of a particular cell to H-OH.

The G-C ligand chromomycin A_3, compared with H-OH as a drug of different sequence specificity, was a much less effective inhibitor of OBP binding than H-OH. In relation to cell function, the concentration of chromomycin A_3 required to inhibit binding of OBPs to the ATGCAAAT motif was 10,000-fold higher than the cellular D_{37}, whereas H-OH required doses of only 1–20-fold greater than the D_{37} to be effective. Further, chromomycin A_3 showed a much different spectrum of cell toxicity and induction of tyrosinase compared with H-OH. Overall, the results suggest that H-OH has some

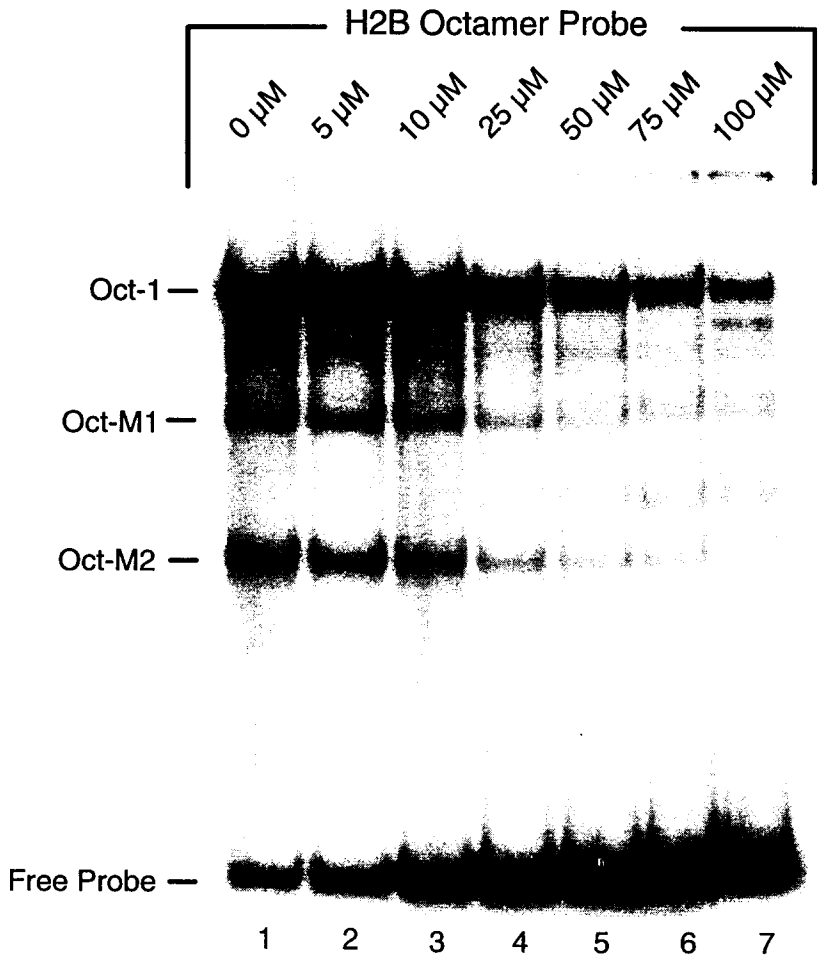

Fig. 10. Disruption of OBP–DNA complexes by H-OH. Nuclear extract was incubated in binding mix with ^{32}P-labelled H2B probe for 20 min, then H-OH was added and the mixture analysed by EMSA.

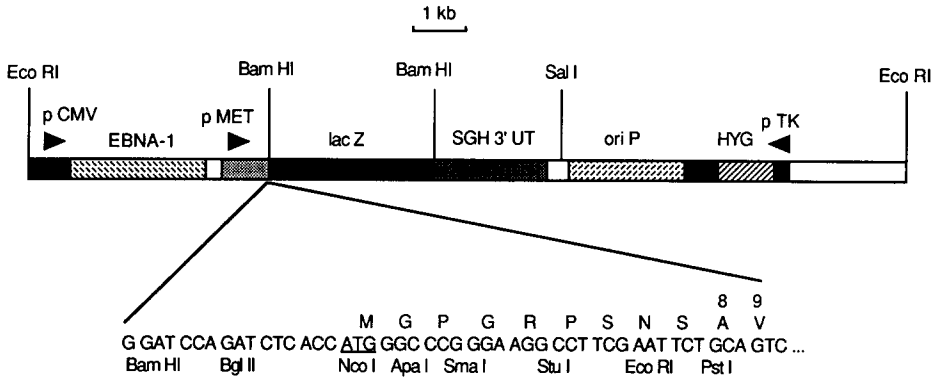

Fig. 11. Reporter construct for transfection of MM96 cells. Structure of p294MetM3 (16.578 kb) shown linearized at a (non-unique) EcoRI site 5′ to the cytomegalovirus immediate-early gene promoter (pCMV). Other abbreviations: EBNA-1, Epstein–Barr virus nuclear antigen 1 gene; pMET, sheep metallothionein-Ia promoter; lacZ, modified *E. coli* gene encoding β-galactosidase; SGH 3′ UT, sheep growth hormone gene and 3′ untranslated sequences; ori P, Epstein–Barr virus plasmid origin of replication; HYG, hygromycin B resistance gene (hygromycin phosphotransferase); pTK, herpesvirus thymidine kinase promoter and 3′ regulatory region. Unshaded segments are derived from pBR322; the largest contiguous segment (3′ to the TK promoter in the above diagram) contains the ampicillin resistance (*bla*) gene and the col E1 origin of replication. The sequence shown below the plasmid diagram corresponds to the 5′ end of the modified *lacZ* gene with the beginning of the encoded protein. Numbers above the amino acid sequence correspond to positions within the wild-type β-galactosidase protein sequence.

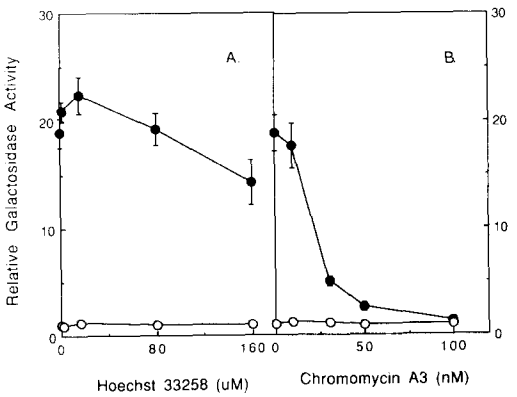

Fig. 12. Inhibition of metallothionein promoter activity in MM96L-gal cells by DNA sequence-specific drugs. Cells were treated with drug for 1 hr, then 100 μM $ZnSO_4$ was added, and the cells harvested after a further 6 hr for determination of β-galactosidase activity relative to controls (no drugs or $ZnSO_4$). (○) No $ZnSO_4$; (●) plus $ZnSO_4$. Points are means ± SE of two to five experiments.

specificity for binding to A-T-rich elements that regulate the expression of certain genes.

The reporter assays with the sMT-Ia promoter, containing GC-rich metal responsive elements, provided direct and independent evidence that chromomycin A_3 inhibits transcription in melanoma cells on a sequence-specific basis. The glucocorticoid elements are not active in this promoter [36]. The striking and complementary differences between the effects of chromomycin A_3 and H-OH on sequences with 76% G-C or 75% A-T, respectively, indicate that only modest deviations from equal G-C/A-T content might be sufficient to confer gene selectivity on such drugs. In due course, it will be of interest to determine positional effects within these sequences and use cell lines such as MM96L-gal to screen new drugs for promoter specificity.

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REFERENCES

1. Hurley LH and Boyd FL, DNA as a target for drug action. *Trends Pharmacol Sci* **9**: 402–407, 1988.
2. Broggini M, Ponti M, Ottolenghi S, D'Incalci M, Mongelli N and Mantovani R, Distamycins inhibit the binding of OTF-1 and NFE-1 transfactors to their conserved DNA elements. *Nucleic Acids Res* **17**: 1051–1059, 1989.
3. Lammler G, Herzog H, Saupe E and Schutze HR, Chemotherapeutic studies on *Litomosodoides carini* infection of *Mastomys natalensis*. 1. The filaricidal action of 2,6-bis-benzimidazoles. *WHO Bull* **44**: 751–756, 1971.
4. Noguchi PD, Johnson JB and Browne W, Measurement of DNA synthesis by flow cytometry. *Cytometry* **1**: 390–393, 1986.
5. Visser JWM, Vital staining of haemopoietic cells with the fluorescent bis-benzimidazole derivatives Hoechst 33342 and 333258. *Flow Cytometry* **4**: 86–90, 1980.
6. Steuer B, Breuer B and Alonso A, Differentiation of EC cells *in vitro* by the fluorescent dye Hoechst 33342. *Exp Cell Res* **186**: 149–157, 1990.
7. Pjura PE, Grzeskowiak K and Dickerson RE, Binding of Hoechst 33258 to the minor groove of B-DNA. *J Mol Biol* **197**: 257–271, 1987.
8. Hirschberg J, Lavi U, Goitein R and Marcus M, The pleiotropic effects of 33258-Hoechst on the cell cycle in Chinese Hamster cells *in-vitro*. *Exp Cell Res* **130**: 63–72, 1980.
9. Dubey DD and Raman R, Effects of Hoechst 33258 on different cell cycle events: inhibition of synthetic activities in bone-marrow cells of the mole rat *Bandicota bengalensis*. *Exp Cell Res* **149**: 419–432, 1983.
10. Woynarowski JM, Sigmund RD and Beerman TA, DNA minor groove binding agents interfere with topoisomerase II mediated lesions induced by epipodophyllotoxin derivative VM-26 and acridine derivative *m*-AMSA in nuclei from L1210 cells. *Biochemistry* **28**: 3850–3855, 1989.
11. Selby CP and Sancar A, Noncovalent drug-DNA binding interactions that inhibit and stimulate (A)BC excinuclease. *Biochemistry* **30**: 3841–3849, 1991.
12. Mikhailov MV, Zasedatelev AS, Krylov AS and Gurskii GV, Mechanism of the "recognition" of A-T pairs in DNA by molecules of the dye Hoechst 33258. *Mol Biol (USSR)* **15**: 541–554, 1981.
13. Portugal J and Waring MJ, Assignment of DNA binding sites for 4′,6-diamidine-2-phenylindole and bisbenzimide (Hoechst 33258). A comparative footprinting study. *Biochim Biophys Acta* **949**: 158–168, 1988.
14. de C. T. Carrondo MAAF, Coll M, Aymami J, Wang AHJ, van der Marel GA, van Boom JH and Rich A, Binding of a Hoechst Dye to d(CGCGATATCGCG) and its influence on the conformation of the DNA fragment. *Biochemistry* **28**: 7849–7859, 1989.
15. Jorgenson KF, Varshney U and van de Sande JH, Interaction of Hoechst 33258 with repeating synthetic DNA polymers and natural DNA. *J Biomolec Struct Dynam* **5**: 1005–1023, 1988.
16. Murray V and Martin RF, Sequence specificity of ^{125}I-labelled Hoechst 33258 damage in six closely related DNA sequences. *J Mol Biol* **203**: 63–73, 1988.
17. Rao KE and Lown JW, Molecular recognition between ligands and nucleic acids: DNA binding characteristics of analogues of Hoechst 33258 designed to exhibit altered base and sequence recognition. *Chem Res Toxicol* **4**: 661–669, 1991.
18. Cox PM, Temperley SM, Kumar H and Golding CR, A distinct octamer-binding protein present in malignant melanoma cell. *Nucleic Acids Res* **16**: 11047–11056, 1988.
19. Sturm RA, Bisshop F, Takahashi H and Parsons PG, A melanoma octamer binding protein is responsive to differentiating agents. *Cell Growth Diff* **2**: 519–524, 1991.
20. Lowings P, Yavuzer U and Goding CR, Positive and negative elements regulate a melanocyte-specific promoter. *Mol Cell Biology* **12**: 3653–3662, 1992.
21. Pope JH, Morrison L, Moss DJ, Parsons PG and Mary R, Human malignant melanoma cell lines. *Pathology* **11**: 191–195, 1979.
22. Todaro GJ, Fryling C and De Larco JE, Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor

receptors. *Proc Natl Acad Sci USA* **77**: 5258–5262, 1980.
23. 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, 1987.
24. Townsend E, Moni R, Quinn R and Parsons PG, Reversible depigmentation of human melanoma cells by halistanol trisulphate, a novel marine sterol. *Melanoma Res* **1**: 349–357, 1991.
25. Sturm R, Baumruker T, Franza BR, Jr and Herr W, A 100 kd HeLa cell octamer binding protein (OBP100) interacts differently with two separate octamer-related sequences within the SV40 enhancer. *Genes Dev* **1**: 1582–1599, 1987.
26. Chomczynski P and Sacchi N, Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
27. Sturm RA, Cassady JL, Das G, Romo A and Evans GA, Chromosomal structure and expression of the human ATF1 locus encoding the Oct-1 protein. *Genomics* **16**: 333–341, 1993.
28. Cohen T, Muller RM, Tomita Y and Shibahara S, Nucleotide sequence of the cDNA encoding human tyrosine related protein. *Nucleic Acids Res* **18**: 2807, 1990.
29. Kwon BS, Haq AK, Pomerantz SH and Halaban R, Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc Natl Acad Sci USA* **84**: 7473–7477, 1987.
30. Kwon BS, Chintamaneni C, Kozak CA, Copeland NG, Gilbert DJ, Jenkins N, Barton D, Francke U, Kobayashi Y and Kim KK, A melanocyte-specific gene, Pmel17, maps near the silver coat colour locus in mouse chromosome 10 and is in a synthetic region on human chromosome 12. *Proc Natl Acad Sci USA* **88**: 9228–9232, 1991.
31. Pruss RM, Mirsky R and Ruff MC, All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell* **27**: 419–428, 1981.
32. Sugden B, Marsh K and Yates J, A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein–Barr virus. *Mol Cell Biol* **5**: 410–413, 1985.
33. Stinski MF and Roehr TJ, Activation of the major immediate early gene of human cytomegalovirus by *cis*-acting elements in the promoter-regulatory sequence and by virus-specific *trans*-acting components. *J Virol* **55**: 431–441, 1985.
34. Gritz L and Davis J, Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **25**: 179–188, 1983.
35. Yates J, Warren N, Reisman D and Sugden B, A *cis*-acting element from the Epstein–Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc Natl Acad Sci USA* **81**: 3806–3810, 1984.
36. Peterson MG and Mercer J, Structure and regulation of the sheep metallothionein Ia gene. *Eur J Biochem* **174**: 417–424, 1986.
37. Byrne CR, Wilson BW and Ward KA, The isolation and the characterization of the ovine growth hormone gene. *Aust J Biol Sci* **40**: 459–468, 1987.
38. Shanahan CM, Rigby NW, Murray JD, Marshall JT, Townrow CA, Nancarrow CD and Ward KA, Regulation of expression of a sheep metallothionein 1a-sheep growth hormone fusion gene in transgenic mice. *Mol Cell Biol* **9**: 5473–5479, 1989.
39. Shapira SK, Chou J, Richaud FV and Casadaban MJ, New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lac* Z gene sequences encoding an enzymatically active carboxy-terminal portion of β-galactosidase. *Gene* **25**: 71–82, 1983.
40. Vandeyar MA, Weiner MP, Hutton CJ and Batt CA, A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. *Gene* **65**: 129–133, 1988.
41. Kozak M, The scanning model for translation initiation: an update. *J Cell Biol* **108**: 229, 1989.
42. Yelian FP and Dukelow WR, Effects of a DNA-specific fluorochrome, Hoechst 33258, on mouse sperm mobility and fertilising capacity. *Andrologia* **24**: 167–170, 1992.
43. Singh B and Gupta RS, Species-specific differences in the toxicity and mutagenicity of the anticancer drugs mithramycin, chromomycin A_3 and oligomycin. *Cancer Res* **45**: 2813–2820, 1985.
44. Sturm RA, Das G and Herr W, The ubiquitous octamer binding protein Oct-1 contains a POU domain with a homeobox subdomain. *Genes Dev* **2**: 1582–1599, 1988.
45. Suciu D, Reproductive death of Chinese hamster V79 cells after exposure to chemical inhibitors of DNA synthesis. *Int J Biochem* **23**: 1245–1249, 1991.
46. Rieber MS and Rieber M, A Hoechst H33258 agarose plate assay for the estimation of nanogram DNA levels without RNA interference: applications in PCR and in estimations of plasmid and cytoplasmic DNA. *Nucleic Acids Res* **18**: 1918, 1990.
47. Chen TR, *In situ* detection of mycoplasma contamination in cell culture by fluorescent Hoechst 33258 stain. *Exp Cell Res* **104**: 255–262, 1977.
48. Beerman TA, McHuch MM, Sigmend R, Lown JW, Rao KE and Bathini Y, Effects of analogs of the DNA minor groove binder Hoechst 33258 on topoisomerase II and I mediated activities. *Biochim Biophys Acta* **1131**: 53–61, 1992.
49. Verrijer CP, Kal AJ and Van der Vliet PC, The DNA binding (POU) domain of transcription factor oct-1 suffices for stimulation of DNA replication. *EMBO J* **9**: 1883–1888, 1990.
50. Castrillo J-L, Theill LE and Karin M, Function of the homeodomain protein GHF1 in pituitary cell proliferation. *Science* **253**: 197–199, 1991.
51. Halaban R and Moellmann G, Murine and human *b* locus pigmentation genes encode a protein (gp 75) with catalase activity. *Proc Natl Acad Sci USA* **87**: 4809–4813, 1990.
52. Jimenez M, Muloy WL and Hearing VJ, Specific identification of an authentic clone for mammalian tyrosinase. *J Biol Chem* **264**: 3397–3403, 1989.
53. Takahashi H and Parsons PG, *In vitro* phenotypic alteration of human melanoma cell induced by differentiating agents: heterogeneous effects on cellular growth and morphology enzymatic activity and antigen expression. *Pigment Cell Res* **3**: 223–232, 1990.
54. Hendrix MJC, Seftor EA, Chu YW, Seftor REB, Nagle RB, McNaniel KM, Leong SPL, Yohem KM, Leibovitz AM, Meyskins FL, Conaway DH, Welch DR, Liotta LA and Stetler-Stevenson W, Coexpression of vimentin and keratins by human melanoma tumor cells: correlation with invasive and metastatic potential. *J Natl Cancer Inst* **84**: 165–174, 1992.
55. Reeves R and Nissen MS, The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. *J Biol Chem* **265**: 8573–8582, 1990.