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Induced Differentiation of Erythroleukemia Cells by Hexamethylene Bisacetamide: A Model for Cytodifferentiation of Transformed Cells

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There is considerable evidence that malignant transformation need not eliminate the potential for a cell to express its developmental capabilities. This review explores the process whereby polar compounds, hexamethylene bisacetamide (HMBA) in particular, induce murine erythroid leukemoid cells (MELC) to express the differentiated erythroid phenotype, including hemoglobin production and cessation of cell division. This is a multi-step process which, although the mechanisms of action of HMBA are not yet fully understood, is amenable to experimental definition and analysis. Early effects, including changes in protein kinase C activity, in ion transport, and in expression of certain nuclear proto-oncogenes, have been examined in relation to the onset of terminal cell differentiation. This experimental experience has formed the context for initiating preliminary clinical studies designed to examine the pharmacology of HMBA and to explore its potential for modifying the natural history of cancer.

Introduction

Characterization of inducer-mediated alterations in the phenotype of transformed cells, employing hexamethylene bisacetamide (HMBA)-induced murine erythroleukemia (MEL) cell differentiation as a model, has provided considerable understanding of the changes involved in this complex, multistep process (*1–3*). These investigations have provided, in part, a basis for considering the use of cytodifferentiation agents to overcome the maturation defect commonly associated with cell transformation, and to induce loss of proliferative capacity, as an approach to therapy of various tumors (*4–8*).

Studies of HMBA-induced MEL cells have generated a considerable body of knowledge about the process by which a proliferating population of precursor cells, with the potential for expressing a differentiated phenotype (in this case an erythroid cell phenotype) withdraws from the cell division cycle and expresses those genes characteristic of the differentiated phenotype. However, our understanding of the process is far from complete. We are only beginning to define the mechanisms by which these cells receive proliferative or differentiation signals from their environment and the ways in which these signals are transduced to alter nuclear events, such as DNA replication and specific gene expression.

This review focuses on HMBA-induced MEL cell differentiation to the terminal erythroid phenotype. It will summarize studies of inducer action during the early, latent period and the later, irreversible alterations involving expression of genes for products characteristics of the differentiated erythroid phenotype. We will also review studies with cell lines in culture that have provided a basis for phase I clinical trials with HMBA as a potential cancer therapy.

Potential of Transformed Cells to Differentiate

The polar compound, HMBA, can circumvent the virus-induced block of MEL cell development and cause these cells to differentiate with loss of proliferative capacity (*8,9*). Thus, transformation does not eliminate the poten-

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tial of MEL cells to express their genetic program of erythroid differentiation (*1–3*). Additional evidence that tumor cells are not necessarily irreversibly blocked with respect to differentiation derives from studies of a number of systems. For example, compounds structurally related to HMBA, as well as a number of other agents, can induce the expression of differentiated characteristics in MEL cells, as well as a variety of different transformed cells *in vitro* (Table 1) (*1–3,8–32*). This concept is also supported by the behavior of temperature-sensitive transforming viruses that block normal development at permissive temperatures but permit expression of the differentiated phenotype at nonpermissive temperatures (*33,34*). Other studies that support this hypothesis concern malignant teratocarcinoma cells that, implanted into the inductive environment of the early mouse embryo, differentiate and contribute to the normal organs of the chimeric mouse (*35*).

HMBA-Induced MEL Cell Commitment to Terminal Differentiation

MEL cells are virus-transformed erythroid precursors, apparently blocked in development at a stage comparable to that of the erythroid-colony forming cell, the CFUe (*2,3*). They can be passaged in culture indefinitely while retaining their potential for expression of the differentiated erythroid phenotype (*8*). Friend and colleagues (*8*) first demonstrated that MEL cells in culture with dimethyl sulfoxide are induced to express that differentiated phenotype. Subsequently, we investigated the relationship between the structure and activity of inducing compounds (*36*). It was found that the polar group was critical to inducer activity. Further studies identified a new group of potent inducers, the polymethylene bisacetamides, of which HMBA is among the most potent (Fig. 1)

Table 1. Transformed cells that can be induced to differentiate by HMBA to other polar compounds (*1–26*).

Cell source	Cell type
Cell lines	
Mouse	Erythroleukemia
	Teratocarcinoma
	Hepatic tumor
	Neuroblastoma
Rat	Mammary tumor
	LB myeloblast
Canine	Kidney epithelial carcinoma
Human	HL-60 promyelocytic leukemia
	Melanoma
	Colon carcinoma
	Bladder carcinoma
	Glioblastoma multiforma
	T cell
	B cell
Primary cell cultures	
Human	Colon carcinoma
	Bladder carcinoma
	Acute myeloid leukemia
	Melanoma

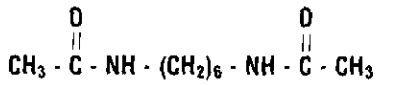

FIGURE 1. Hexamethylene bisacetamide (HMBA).

(*9,37,38*). Other agents have also been shown to be effective, to a greater or lesser extent, as differentiation inducers (*1–3*).

Culture of MEL cells with HMBA initiates expression of a developmental program similar to that observed during normal terminal erythroid differentiation. HMBA-induced differentiation of MEL cells is not, however, identical to that of normal erythropoiesis (*1–3*). For example, erythropoietin, the hormone involved in maintaining the viability and proliferation of normal erythroid precursor cells, is neither required for, nor stimulates, proliferation of MEL cells. HMBA-induced differentiation of MEL cells leads to relatively few cells that actually reach the nonnucleated, mature erythroid stage of development. Inducer-mediated differentiation of MEL cells is not associated with elimination of the viruses.

The kinetics of inducer-mediated commitment of MEL cells to terminal cell division is consistent with a stochastic or probabalistic process (*39,40*). Upon addition of HMBA to MEL cells, such as line 745-DS19 (a clone derived in our laboratory from the cell line originally developed by Charlotte Friend), there is a latent period of approximately 10 to 12 hr during which there is no detectable commitment to terminal differentiation. Commitment is defined as the capacity of cells to express characteristics of the erythroid-differentiated phenotype, including loss of proliferative capacity, upon removing them from HMBA (*39,40*). This early or latent period is followed by a period during which an increasing proportion of the population express characteristics of terminal differentiation (Fig. 2).

HMBA-mediated MEL cell terminal differentiation is a multistep process (*41,42*). During the early period, the inducer initiates a number of metabolic changes that precede irreversible commitment. Among these changes are alterations in membrane permeability that involve sodium, potassium, and calcium flux (*43–45*), alterations in membrane fluidity (*46,47*), changes in cell volume, a transient increase in cyclic AMP concentration (*48–49*), a prompt increase in membrane associated protein kinase C (PKC) activity, the appearance in the cytosol of a Ca^{2+} and phospholipid-independent form of PKC, presumably generated by proteolytic cleavage of membrane-bound PKC (*50*), and a change in expression of a number of genes, including c-*myb*, c-*myc*, c-*fos*, and p53 gene (*51–54*). During this early period, as indicated above, commitment (as assayed by the capacity of cells to express terminal differentiation despite removal of the inducer) is not detected (Fig. 3).

Upon becoming committed, morphological and chemical changes occur in MEL cells that are similar to normal erythroid terminal cell differentiation. These include the coordinated expression of a number of genes, including genes for the α_1 and β_{maj} globin, the heme synthetic en-

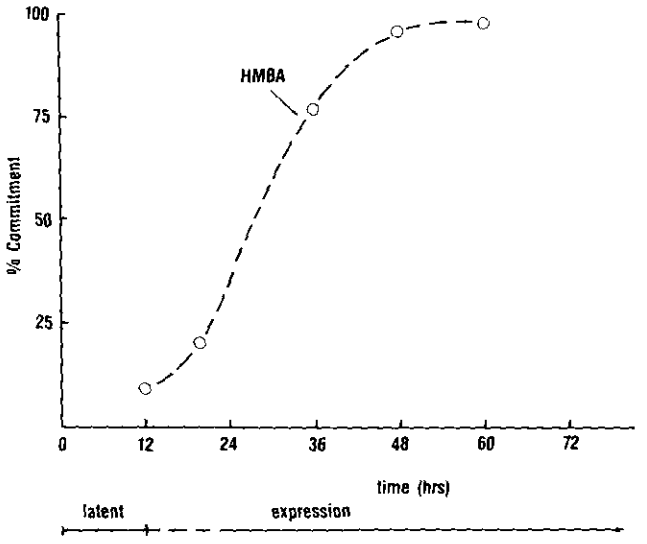

FIGURE 2. HMBA-induced MEL cell commitment to terminal cell division and hemoglobin accumulation. Commitment was assayed by transfer of cells from suspension culture with HMBA to Petri dishes (2.5 cm in diameter) containing a semi-solid medium (1.8% methylcellulose) without inducer. Cells were plated to assure that each was single. The plates were incubated for 5 days at 37°C and scored for colony size and benzidine reactivity. A cell was scored as committed if it yielded a colony of ≤ 64 cells that was benzidine reactive.

zymes and erythroid specific membrane proteins, as well as suppression of DNA replication and suppression of rRNA synthesis (*1–3*). From studies with variant MEL cells, blocked at different steps in the inducer-mediated developmental pathway, evidence has accumulated that the coordinated expression of these inducer-mediated changes in gene expression does not necessarily involve an obligatory sequential series of steps (*55,56*). For example, with variant cell line 745A-DR10, HMBA induces accumulation of α_1 and β_{maj} globin without initiating commitment to terminal cell division (*55*). Likewise, hemin induces MEL cells to accumulate globin mRNA but fails to induce commitment to terminal cell division (*56–58*).

Modulation of Gene Expression

During the early period, HMBA induces a modulation in expression of a number of genes, among which are c-*myb*, c-*myc*, c-*fos*, and p53 (*51–54*). These genes control products that are localized in the nucleus and may be involved in the regulation of gene expression and cell replication (*59–63*). However, the specific action of the protein products of these genes is not known. In MEL cells in culture with HMBA, the level of c-*myc* mRNA decreases markedly within the first hour of culture. A decrease in c-*myb* mRNA level occurs within 1 to 4 hr, whereas c-*fos* mRNA, which is almost undetectable in uninduced MEL cells, begins to accumulate within about 4 hr of onset of culture, reaching a maximum by about 18 to 24 hr. p53 mRNA levels do not change appreciably in MEL cells in culture with HMBA for at least 48 hr. With continued exposure to HMBA, further modulation of the c-*myb*, c-*myc*, and c-*fos* genes occur. The level of c-*myc* mRNA rises back toward that of uninduced cells by about 12 hr and remains there for at least the ensuing 36 hr, while the elevated level of c-*fos* mRNA remains essentially unchanged.

By comparison, by 12 hr, the level of c-*myb* mRNA has increased almost to control levels, from its early nadir, then falls again to an undetectable level by 24 to 36 hr, where it remains for the duration of culture with inducer. That inducer-mediated suppression of c-*myb* mRNA may be an important factor in the recruitment of cells to commitment is suggested by several lines of evidence: a) continued suppression of c-*myb* mRNA level (but not that of

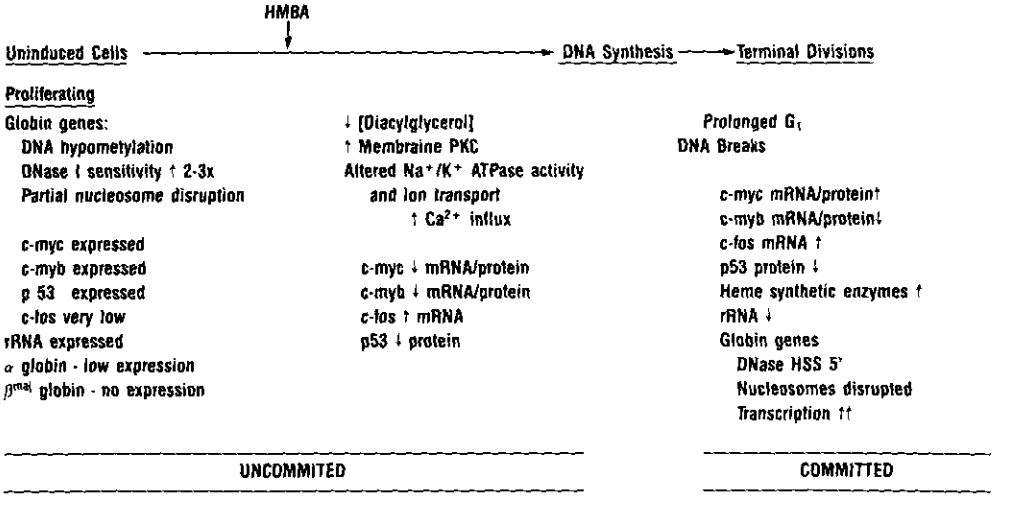

FIGURE 3. Schematic representation of model of HMBA effects in inducing MEL cells to terminal differentiations (for details see text). Modulation in metabolic function and gene expression is indicated as follows: (↓) decreased; (↑) increased. The early, latent period is indicated as "uncommitted" and is approximately 12 hr in duration for MEL cell line, 754A-DS19, in culture with 5 mM HMBA. Recruitment of MEL cells to the committed state is detectable by 12 to 14 hr, and the proportion of cells committed increases, in a stochastic manner, to levels of >95% by 48 to 60 hr in culture with HMBA. The horizontal list of various characteristics gives an indication of the sequence of events in relation to onset of culture with HMBA, but time in culture is not represented in a linear scale.

c-*myc*) is characteristic of progressive recruitment of MEL cells to terminal cell division; b) hemin, which initiates globin mRNA accumulation, but not commitment, does not suppress c-*myb* mRNA; c) dexamethasone inhibits HMBA-induced commitment and inhibits the late (> 12 hr) suppression of c-*myb* mRNA, but does not modify the changes in c-*myc* or c-*fos* mRNA or p53 protein that occur in response to HMBA alone.

Recent studies in our laboratories (*64*) have shown that the level of the c-*myb* and c-*myc* proteins parallel the changes in c-*myb* and c-*myc* mRNA, respectively, with the exception that the c-*myb* protein remains elevated at 24 hr when the c-*myb* mRNA begins to fall to almost undetectable levels and then the protein level falls markedly. The level of p53 protein decreases to very low values between 4 and 8 hr in culture with HMBA and then remains low, even though, as noted already, p53 mRNA levels do not decrease appreciably during this time. These studies suggest that HMBA-mediated modulation of the c-*myc* and c-*myb* proteins is exerted primarily by regulation of c-*myc* and c-*myb* mRNA accumulation, whereas the level of p53 protein is modulated by mechanisms acting at the level of translation or posttranslation.

The early modulation of expression of c-*myb*, c-*myc*, and c-*fos* gene expression appears to be under control mechanisms different from those involved in the regulation of the alterations in expression of these genes occurring during the period of recruitment of cells to commitment to terminal differentiation (*53*). This is suggested by the finding that agents that inhibit HMBA-induced MEL expression of the differentiated phenotype, such as the steroid dexamethasone, or the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), do not alter the HMBA-induced changes in gene expression during the early period, but will block HMBA-induced suppression of c-*myb* during the later period (*53,64*).

Evidence that the c-*myc* product has a role in differentiation has been supported by studies showing that MEL cells stably transfected with plasmid containing c-*myc* coding sequences, which are constitutively expressed, fail to differentiate in response to dimethyl sulfoxide (*65*). Such results, taken together with studies cited earlier, suggest that there may be a particular time during the course of HMBA-induced MEL cell differentiation when changes in expression of the *myc*, *myb*, *fos*, and p53 genes are critical to the multistep process that leads to expression of the terminal differentiated phenotype.

Modulation of Expression of Globin Genes

In our laboratory, several features of DNA and chromatin structure in the globin gene domains have been examined in relation to inducer-mediated modulation of expression of the α- and β-globin genes (*66–70*). We have reported that certain structural patterns of chromatin and DNA, commonly associated with actively transcribed genes, are, in fact, detected in the α_1- and β_{maj}-globin gene domains of uninduced MEL cells despite the relative inactive transcription of these genes. These include a pattern of DNA hypomethylation of both genes, an increase of 2- to 3-fold in the sensitivity of the globin gene DNA domains to DNase I digestion compared with genes that are not transcribed in erythroid cells (*71*). There is disruption of the normal nucleosome pattern across the α_1- and β_{maj}-globin gene domain. Whereas HMBA causes no evident change in the pattern of methylation of DNA in the globin gene domains (*71*), the inducer does cause certain changes in chromatin structure, including appearance of DNAse I hypersensitive sites within 100 base pairs 5′ of both the α_1- and β_{maj}-genes (*66,67,71*) and the appearance of another DNAse I hypersensitive site about 3000 base pairs upstream of the cap site of the β_{maj}-globin (*72*). HMBA initiates additional nucleosome disruption, limited to the transcribed regions of the α_1- and β_{maj}-globin gene domains (*73*). Time-course studies on the appearance of alterations in chromatin in MEL cells induced by HMBA indicate that these changes, which are detectable within 12 to 24 hr (that is, within approximately 1 to 2 cell cycles), precede the onset of accelerated transcription. Studies with variant cell lines, such as 745A-R1, which are resistant to HMBA-mediated induction, provide further evidence that changes in chromatin structure precede onset of accelerated transcription of the globin genes (*68*). In these cell lines, HMBA induces certain limited changes in chromatin structure, but not the complete pattern observed in HMBA-sensitive cell lines such as 745A-DS19. Taken together, the present data indicate that HMBA causes selective and dramatic increases in the rate of transcription of globin genes that are dependent upon a number of specific changes in chromatin configuration within the globin gene domains. These changes in globin gene related chromatin structure and globin gene transcription are part of the late modulation in gene expression associated with HMBA action on MEL cells.

Mechanism of Action of HMBA

A working model for the effects of HMBA on MEL cells is presented in Figure 2. The mechanism of action of HMBA is not known. There is evidence that HMBA and related polar compounds cause a significant decrease in the levels of phosphatidylinositol metabolites, including inositol triphosphate and diacylglycerol, within 2 hr of onset of culture (*74*). Diacylglycerol activates the Ca^{2+} and phospholipid dependent PKC activity (*75*). The tumor promoter TPA, which activates PKC and for which PKC is a specific receptor, can suppress HMBA-induced differentiation in MEL cells (*76,77*). We have recently reported evidence that PKC activity has a role in inducer-mediated modulation of late gene expression in MEL cell differentiation, based on the following findings (*50*): a) HMBA induces the formation of a soluble, proteolytically activated form of PKC that is catalytically active in the absence of Ca^{2+} and phospholipid; b) the protease inhibitor, leupeptin, blocks formation of this activated form of the kinase and inhibits HMBA-induced MEL cell hemoglobin accumulation; c) TPA, which causes depletion of total PKC activity, inhibits HMBA-induced MEL cell terminal dif-

ferentiation but does not prevent the inducer-mediated early modulation in expression of c-*myc*, c-*myb*, and p53 genes; d) MEL cells depleted of PKC activity by TPA are resistant to induction by HMBA; e) removal of TPA leads to restoration of MEL cell sensitivity to HMBA, correlated with progressive reaccumulation of PKC activity. If, as suggested, PKC activation has a role in the pathway of HMBA-induced modulation of gene expression and consequent differentiation and loss of capacity for cell division, it may involve the phosphorylation of proteins critical to the regulation of gene expression and DNA replication.

There is evidence for other inducer-mediated events that may also be involved in the pathway leading to expression of the differentiated phenotype in MEL cells. These include single strand breaks in DNA (*78–80*), changes in configuration of chromatin structure (*81,82*), and expression or suppression of specific oncogenes (as described above) (*51–65*). Recently, it has been shown, using the technique of cell fusion, that MEL cell differentiation may involve a synergistic effect of two distinct intracellular reactions (*83*), one originating from the inhibition or cessation of DNA replication by diverse agents, and the other involving a reaction triggered specifically by inducing agents such as HMBA. The former reaction appears not to be specific to MEL cells, whereas the latter is specific to MEL cells and inducible by HMBA. The induced activity is transient and inhibited by TPA (*84,85*). Partial purification of the protein factors responsible for these reactions has been reported (*86,87*). It is not yet clear how these protein factors interact or how they may trigger erythroid differentiation of inducer-treated MEL cells.

Phase I Clinical Trials and Pharmacological Studies with HMBA

The evidence that HMBA can overcome the developmental block associated with transformation in a number of cell lines has provided the basis for evaluating this agent in treatment of human neoplastic disease. HMBA has proved to be the most potent inducer of differentiation of a number of transformed cell lines, as well as several primary human cancer cells grown *in vitro* (Table 1). HMBA has been evaluated in the National Cancer Institute Division of Cancer Treatment Tumor Screen Program and showed no therapeutic benefit to animals bearing several murine leukemic and solid tumors and possessed little or no cytotoxic activity against human mammary, colon, and lung xenografts in nude mice (*88*). These animal models, however, did not replicate the *in vitro* conditions that appear to be critical for induced differentiation of transformed cell lines, in particular, the optimal concentration, which is about 5 mM for almost all of the sensitive cell lines, and the requirement for the continuous presence of the inducer over a relatively prolonged period for recruitment of transformed cells to differentiation and loss of proliferative capacity.

At least three phase I clinical studies of HMBA have now been performed (at the University of Maryland Cancer Center, at the Johns Hopkins Oncology Center, and at the Memorial Sloan-Kettering Cancer Center) (*89–93*). These initial phase I studies of HMBA examined the toxicity and pharmacology of the agent administered as a 5-day, continuous infusion schedule repeated every 3 weeks, escalating from a starting dose of 4.8 g/m^2 per day to 40.0 g/m^2 per day. Toxicities consisting of renal insufficiency, hyperchloremic metabolic acidemia/acidosis, and central nervous system toxicity characterized by agitation and delerium limited dose escalation. Whereas these symptoms were all reversible upon cessation of the drug, the level of administration, which was not associated with undesirable side effects (24 g/m^2/day), yielded average concentrations of the agent ranging only between about 1 to 1.5 mM. More recently, a 10-day continuous infusion, administered every 4 weeks, was evaluated in a phase I trial (*92*, C. Young, unpublished observations). In these studies, the maximum tolerable dose was approximately 20 g/m^2 per day in order to avoid thrombocytopenia, which was the most prominent dose-limiting toxicity on the 10-day schedule. At this dose, the mean steady-state HMBA concentration among different patients ranged from a low of 0.31 to a high of 3.4 mM.

If effective concentrations of HMBA or some other active polar compound can be achieved clinically, the potential for inducing proliferating neoplastic cells to express differentiated functions and decrease their rate of cell division has important implications for cancer therapy. It is also possible that such agents could prove useful in treatment of premalignant lesions. Even the ability to decrease the body load of cancer cells by the use of a differentiation-inducing agent could prove valuable when used in conjunction with cytotoxic drugs. The *in vitro* studies summarized in this review suggest that to evaluate HMBA or similar agents for their potential therapeutic effectiveness it will be necessary to achieve an optimal *in vivo* concentration of the agent; to maintain that concentration over a prolonged period; and to have markers that can be evaluated for confirming the biological effect of the agent in modulating the cancer cell phenotype.

Summary

Considerable evidence has accumulated indicating that transformation does not necessarily destroy the differentiation potential of tumor cells. One striking example is the ability of polar compounds, such as HMBA, to induce leukemic MEL cells to express the differentiated erythroid phenotype, including cessation of proliferation. While the mechanism of action of HMBA has not yet been fully defined, it is clear that inducer-mediated differentiation is a multistep phenomenon. Among the early effects of the inducer are a decrease in diacylglycerol concentration; an increase in membrane-bound PKC activity; the appearance of Ca^{2+} and phospholipid independent PKC activity in the cytosol; alterations in ion transport, and modulation in expression of a number of genes including c-*myb*, c-*myc*, c-*fos*, and the p53 protein. HMBA-mediated commitment to irreversible terminal differentiation is first detected at about 12 hr and continues in a stochas-

tic fashion until over 95% of the population are recruited to a state of irreversible commitment to terminal differentiation and terminal cell division by 48 to 60 hr. Commitment is associated with persistent suppression of c-*myb* gene expression and elevated levels of c-*fos* mRNA, while c-*myc* mRNA returns to the level in uninduced cells. By 36 to 48 hr, transcription of the globin genes has increased by 10- to 30-fold, whereas transcription from the rRNA genes is suppressed.

The *in vitro* studies with HMBA induction of MEL cells provide a basis for considering polar agents, such as HMBA, for clinical therapy of human cancers. Based on these *in vitro* studies it is likely that, to be effective, HMBA must be administered in doses that achieve adequate concentrations in the serum (probably in the neighborhood of 3 to 5 mM), that these concentrations be maintained over a prolonged period of time, and that the biological effect of the agent be monitored by indices of the particular differentiated phenotype of the cancer being studied.

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