

GROWTH INHIBITION BY SIALOGLYCOPEPTIDES  
FROM THE SURFACE OF BOVINE CEREBRAL CELLS  
AND THEIR INTERACTION WITH  
12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA)

by

Hsin-Hwei Joseph Chou

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To My Parents,  
Brother and Sisters

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## INTRODUCTION

There have been numerous reports describing negative growth regulators very recently. The majority of the work described the isolation and antiproliferative activity of these molecules. This type of work and studies on the functions of cell membranes have provided insight about growth regulation. It becomes clear that cell growth is controlled not only by positive growth factors but also by negative growth factors. However, the biological significance (i.e., the physiological role) of these substances during cell proliferation, differentiation or even the development of the individual species remains to be clarified.

In this thesis, evidence is presented to show that molecules purified from the surface membrane of bovine cerebral cortex cells exhibit inhibitory activity on cell growth. Further evidence is provided to show the potency of the molecules in antagonizing the activity of a phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), a strong tumor promoter.

## I. HISTORICAL REVIEW

Cell-cell communication has been of considerable interest to many biologists. Mammals, in particular, have evolved nervous and endocrine systems which function to transmit signals to distal parts of the organism. Cell-cell interactions between adjacent cells, on the other hand, also exist. For instance, a developing embryo would be the most vivid example of this type. Early observations (Townes & Holfreter, 1955) pointed out preferential aggregation of the embryonic cells with their starting tissue type when cells of ectodermal, mesodermal or endodermal origin from various stages of development were dissociated. The results implied that cells somehow recognized each other. Indeed, cell-cell communication may be fundamental to the coordinated living systems of an organism as a whole.

It would be an insurmountable task to study signal transduction between cells in a whole organism. Studies of this sort are often complicated by a number of factors, such as interactions of different organ systems. It is very difficult to discriminate between these factors under most conditions. To circumvent these problems, one could resort to cell culture as an alternative; it was soon discovered that cell culture offered many advantages. It is only feasible to explore molecular and cell biology when specific type of cells are available in large quantity and grown in defined conditions. Hence the findings can be reproduced by other workers. These advantages of cell culture have outweighed the disadvantages in the development and application of this system over several decades. However, care must be taken

in extrapolating the data from cell culture studies back to the organism.

Earlier observations indicated that primary chick embryo fibroblasts grew in culture as an ordered array of cells (Abercrombie & Heayman, 1954). These cells exhibited a decreased rate of proliferation at higher cell density. Later, Todaro and Green (1963) grew embryo fibroblasts from Swiss 3T3 mice. They observed that these cells would not grow at densities greater than  $6.0 \times 10^4$  cells/ml. Rather, they formed a confluent monolayer. The observations of restriction of growth in dense culture led Stoker and Rubin (1967) to propose the term "density dependent growth inhibition". They observed that saturation density of cells in culture was constant and characteristic of each cell line under optimal conditions. In addition, they emphasized that the term contact inhibition ought to be strictly reserved for arrest of movement and should not be confused with cell division, because the available evidence suggested that inhibition of movement and of growth were unrelated. Several hypotheses, however, are postulated to explain the phenomenon of density dependent growth inhibition and they are not mutually exclusive. It must be pointed out that the biochemistry of growth control of cells involves complex interactions between cells and the environment. Causal and casual consequences of observations are very likely intermingled in forming the hypotheses. In general, there are three major theories as follows:

1. Nutrient Transport Hypothesis: The emphasis is on the transport of nutrients across the cell membrane as the



key components in growth control.

2. Soluble Growth Factor Hypothesis: The focus is on some growth factors or molecules as mediators predominantly in a positive regulatory control.

3. Cell-cell Contact Hypothesis: A concern of the growth control by components of the cell membrane in contact that mediate in a negative manner.

## Nutrient Transport

Cell growth requires increased uptake and/or incorporation of nutrients (e.g., amino acids) into the cells. It is plausible to assume that some of these nutrients play a key role in growth regulation. This theory is simple and no other regulatory molecules are considered. The observations that subconfluent cultures were rich in these molecules whereas confluent cultures lacked these molecules led Holley (1972) to propose that cell growth was under the control of nutrients. Depletion of nutrients at higher cell densities accounted for declined rate of cell proliferation; cells would reach higher saturation density if increased levels of serum, amino acids or other nutrients were provided (Todaro & Green, 1963). These findings supported the important part of nutrients in growth control.

Pardee (1971) provided evidence in support of this theory. It was considered that regulatory molecules altered the structure of the cell membrane. Subsequently, the transport of other molecules, such as amino acids, were changed as well. Growth control was thus subjected to alteration of functions and structures of the cell membrane. However, it is very difficult to pursue the model of nutrient transport. First, it is no easy job to sort out a variety of nutrients in the studies. It is almost impossible to follow one-to-one correspondence between transport of certain nutrients and the consequential activity in the cell at the molecular level. For example, the exact mechanism

involved during the transport of lysine into the cells and the biochemical consequences is not clear. Second, more convincing data have been recently emphasized from studies on growth factors and inhibitors. Holly and others (1980) have described the isolation of a growth inhibitor from BSC-1 cells. Recently, Croy and Pardee (1983) have become interested in studying growth factors and certain key regulatory proteins in controlling cell proliferation. Nonetheless, nutrient transport is, at best, indirectly associated with growth control.

### Soluble Growth Factors

There has been a proliferation of literature on soluble growth factors recently (Feramisco et al., 1985). It is known that cells in culture can be maintained in a resting state for a long period (Pardee et al., 1978). Soluble growth factors, however, stimulate these quiescent cells and initiate DNA synthesis and cell division (Heldin & Westermark, 1984). These agents are hormone-like molecules in function. A number of growth factors have been isolated and purified (reviewed by James & Bradshaw, 1984). The amino acid sequence of some factors has been determined. In addition, cell surface receptors for these factors were identified and characterized. These receptors are responsible for the biological activity of the growth factors.

Tremendous progress has been made in this field. First, a tyrosine-specific protein kinase activity of several growth factor receptors was described (Heldin & Westermark, 1984). Cohen and his colleagues (1982) found

this kinase activity in cell surface membranes from A431 cells, which are rich in epidermal growth factor (EGF) receptors. The kinase activity was also found in receptors for insulin (Kasuga et al., 1982) and platelet-derived growth factor (PDGF) (Ek et al., 1982; Nishimura et al., 1982). Second, antibodies against EGF receptors were able to block EGF binding and induced early and delayed effects of EGF (Schreiber et al., 1981), including DNA synthesis. Fab fragments of the same antibodies induced kinase activity but not DNA synthesis (Schreiber et al., 1981) and clustering of Fab-receptor complexes resulted in induction of DNA synthesis (Schreiber et al., 1982). The most important observations were probably the discoveries that several growth factors have extremely high homology with oncogene products. For example, the sis proto-oncogene encodes the beta-chain of PDGF (Doolittle et al., 1983; Waterfield et al., 1983). The erb-B oncogene product is a truncated form of the EGF receptor (Downward et al., 1984). The fms oncogene product is related to macrophage colony stimulating factor receptor (Sherr et al., 1985). These discoveries will provide us some clues in the quest to understand growth regulation of normal cells and its aberrant form in transformed cells.

Cell proliferation can also be regulated by a "negative growth factor". An example is the substance called chalone. Chalone which are hormone-like molecules of molecular weight 5,000 to 50,000 inhibit DNA synthesis of cells in culture (Houck et al., 1977). Chalone were isolated from different cell types, however, further purification and characterization of these molecules remains to be done.

## Cell-cell Contact

It is conceivable that growth inhibition is due to cell-cell contact between adjacent cells involving receptors. Growth inhibitor, either in secreted form as mentioned previously or membrane-bound form, is involved in cell proliferation. Mild protease treatment of confluent 3T3 cells resulted in another round of mitosis, suggesting that the growth inhibitors were removed in the treatment (Burger, 1970). However, the exact mechanism of action of proteases is not known. Koch (1974) showed that some cell surface molecules removed from HeLa cells exhibited inhibitory activity for protein synthesis. Treatment of subconfluent 3T3 cells with extracts of membrane components from confluent 3T3 cultures inhibited DNA synthesis of the cells (Whittenberger et al., 1978). DNA synthesis of simian virus 40-transformed 3T3 (SV3T3) cells, in contrast, was not affected by the same treatment. Extracts from SV3T3 were not effective in inhibiting DNA synthesis of growing 3T3 or SV3T3 cells. These early discoveries have provided directions to study growth inhibitors more extensively. Various sources and the experimental evidence of a negative growth factor will be discussed.

## Hypothalamus-Pituitary

Hypothalamus and pituitary tissues are good sources of different hormones. Crude extracts of these tissues were fractionated and several fractions exhibited growth inhibitory activity in various cell types (Redding &

Schally, 1982). Boiling of the material did not inactivate the activity whereas treatment with some proteases abolished the inhibitory effect. This substance has not been purified to homogeneity.

### Cerebral Cortex

Growth inhibitors, which were polypeptides in nature and isolated from cerebral cortex cells, were shown to inhibit protein synthesis and cell division. Material from mouse brain (Kinders et al., 1980) and bovine brain (Kinders & Johnson, 1982) shared similar biological activities and the bovine material inhibited cell growth of normal but not transformed cells. The bovine material contained a major species of molecular weight 18,000 and a minor species of 16,000. Very recently, another bovine growth inhibitor was purified as a result of a new purification procedure (Sharifi et al., 1986) sharing many physical and biological properties with the previous bovine inhibitor. The latest purified molecule differed from the previous molecule in several properties. For example, the new inhibitor with pI 3.0, bound to diethylaminoethyl (DEAE) ion exchange column and a Limulus polyphemus lectin column but not to an Ulex europaeus lectin column. The old bovine molecule with pI 8.1, bound to Ulex and carboxymethyl (CM) ion exchange resin, but not to DEAE. Interactions between this growth inhibitor of pI 3.0 and tetradecanoylphorbol ester (Chou et al., 1987), EGF (Bascom et al., 1986) and bombesin (Sharifi et al., in preparation) were explored. Binding of the molecule to the cell surface receptors was studied as well (Bascom et

al., 1986).

#### Liver

Another source of proliferation inhibitor has been isolated from the liver (McMahon et al., 1982). This hepatic growth inhibitor was purified to homogeneity yielding a polypeptide of molecular weight 26,000 and pI 4.65. The active material inhibited growth of liver cells only while hepatoma cells were not inhibited. No other type of cells were tested for growth inhibition.

#### Mammary Gland

A growth inhibitor was purified from bovine mammary gland (Bohmer et al., 1984). This inhibitor was a polypeptide of molecular weight 13,000 and inhibited cell division of Ehrlich ascites mammary carcinoma cells. So far no other cells were tested for growth inhibition; the inhibition was antagonized by insulin, epidermal growth factor or low levels of fetal calf serum.

#### Platelet

Two growth inhibitors isolated from platelets have been described. The partially purified material inhibited endothelial cell division and DNA synthesis (Brown & Clemmons, 1986). The material had a molecular weight of 35,000 to 40,000 and was heat labile. Treatment

with proteolytic enzymes inactivated the biological activity. Interestingly, another molecule purified from platelets was beta-transforming growth factor (beta-TGF) (Assoian et al., 1983). Beta-TGF is a protein composed of two 12,500 subunits linked by disulfide bonds. Beta-TGF inhibited DNA synthesis of normal rat kidney cells (Roberts et al., 1985). Beta-TGF has also been isolated from placenta (Frolik et al., 1983).

### BSC-1 Cells

Growth inhibitors have been isolated from confluent BSC-1 cultures (Holley et al., 1980). The material was active on BSC-1 cells, but did not inhibit the growth of 3T3 mouse embryo fibroblasts or human skin fibroblasts. Recently, it was shown that the growth inhibitor from BSC-1 cells was closely related to beta-TGF (Tucker et al., 1984). In addition, a dual regulation of cell growth was reported for beta-TGF (Roberts et al., 1985). Experiments on Fisher rat fibroblasts transfected with c-myc showed enhancement of colony formation by beta-TGF in one case and decrease in another case. The data were interpreted that the effects of beta-TGF on cells were not a function of the molecule itself, but rather of the entire set of growth factors involved and/or their receptors in response to the treatment.

### 3T3 Cells

Growth inhibitors isolated from quiescent Swiss 3T3 (Hsu & Wang, 1986) in culture media and BALB/c 3T3 in



membrane-bound form (Datta & Natraj, 1980) were reported. These substances, in general, inhibited DNA synthesis of subconfluent cultures. Purification of these molecules remains to be shown.

### Interferon (IFN)

Interferons were first identified by their ability to protect cells from virus infection (Isaacs & Lindenmann, 1957). Early work in this area also showed that these molecules inhibited growth of certain cells (Paucker et al., 1962). Mice injected with tumor cells showed a slower rate of tumor growth when they were simultaneously treated with IFN (Gresser & Bourali, 1970). Different experimental models were used for studying IFN and various degrees of sensitivity to growth inhibition were reported (Clemens & McNurlan, 1985). In general, human gamma-IFN (immune IFN) is a more potent inhibitor of cell growth (Crane et al., 1978) and mouse gamma-IFN synergizes with mouse beta-IFN or alpha-IFN in inhibiting cell proliferation (Fleischmann, 1982). The important discoveries were that IFN treatment can block stimulation of quiescent cells by serum (Sokawa et al., 1977) or by growth factors (Lin et al., 1980).

A mechanistic approach was pursued to elucidate the action of IFN. Several lines of evidence were reported in support of that IFN inhibited DNA synthesis indirectly. First, IFN decreased the activity of DNA polymerase in glioma cells (Lundblad & Lundgren, 1981). As a result, cells were trapped in S phase in the cell cycle and completion of DNA replication is delayed. Second, in

IFN-treated Daudi cells, short Okazaki fragments, which are essential components in discontinuous DNA synthesis, accumulated and were delayed in formation of longer fragments (Moore et al., 1984). IFN is also effective in inhibiting certain activities of the transformed cells. IFN decreases the levels of RNA transcribed from c-Has-ras transfected 3T3 cells (Samid et al., 1984) or from c-has-ras and src genes in human bladder carcinoma cells (Soslau et al., 1984). Expression of c-myc is also affected by the IFN treatment (Kelley et al., 1983). However, it is difficult to pinpoint whether the expression is the cause or effect of growth inhibition (Clemens & McNurlan, 1985).

#### Other Antiproliferative Substances

Growth inhibitors, which are peptide in nature, are only a subset of the family of antiproliferative molecules. Different molecules from senescent and quiescent human diploid fibroblasts were isolated (Pereira-Smith et al., 1985). These molecules which are membrane-associated proteins inhibit DNA synthesis. Interestingly, suppression of tumorigenicity was described in hybrids of normal and oncogene-transformed Chinese hamster embryo fibroblast cells (Craig & Sager, 1985), suggesting an inhibitory mechanism. Growth inhibitor, which is carbohydrate (Castellot et al., 1986) or lipid (Stallcup et al., 1984) in nature, has been described very recently. Further characterization of these molecules, however, remains to be done.

## Dual Control of Cell Growth

The major point of this model is that cell proliferation is controlled by two elements: a positive growth factor and a negative growth factor. Proliferation of normal cells requires exogenous growth factors for optimal growth (Temin, 1970). Transformed cells, on the contrary, are not under the control of exogenous growth factors. That fewer exogenous growth factors are needed for growth accounts for the relatively autonomous nature of transformed cells and is indicative of defective growth control. Previously, it was shown that many human tumor cells produced growth factor-like molecules constitutively (Heldin & Westermark, 1984). It may well be that transformed cells produce and respond to their own "growth factors" (Heldin & Westermark, 1984). Several important experiments confirmed this possibility. High concentrations of bombesin were detected in human small cell lung carcinomas (Moody et al., 1981) and bombesin can support growth of the cells in serum-free medium (Carney et al., 1983). Antibodies specific to bombesin inhibited growth of these cells in vitro and in xenograft form in nude mice (Cuttitta et al., 1985). The responsiveness of cells to growth factors can also explain some actions of growth factors. Some transformed cells are less sensitive to the negative growth factors (Kinders & Johnson, 1982; McMahon et al., 1982). Antagonism between positive and negative growth factors may occur in the culture medium or in binding to the surface receptors or even in the post-receptor signaling pathway (Sporn & Roberts, 1985). Defects at any of these levels may lead to cell transformation. A critical role for the growth inhibitor, however, remains

to be established.

### Effects of TPA on Cells in Culture

Sivak and Van Duuren (1967) showed that croton resin enhanced focus formation of 3T3 cells in culture. TPA was later shown to be the compound with the highest tumor promoting activity in mouse skin (Hecker, 1978). The pleiotropic effects of TPA were documented in diverse cell types in culture (Diamond et al., 1980). Caution must be taken, however. Many of these findings were not necessarily indicative of the promoting activity of TPA. Rather, these studies described the effects of TPA on cell proliferation and differentiation. Fisher et al., (1978) described that TPA increased 2-3 fold the number of transformed foci in rat embryo cell cultures infected with adenovirus type 5. Mondal and others (1976) established the protocol for two-stage transformation in cell culture which was analogous to two-stage carcinogenesis in mouse skin.

O'Brien (1976) described the induction of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis, with a 200-fold increase in activity stimulated by TPA. In addition, the induction of ODC was correlated well with the promoting activity of different doses and non-phorbol tumor promoters in the epidermis. Also, TPA was used to induce ODC activity in Swiss 3T3 cells and similar results were obtained (Butler-Gralla and Herschman, 1983). TPA stimulated DNA synthesis in stationary human fibroblasts and mouse fibroblasts (O'Brien et al., 1979; Mondal et al., 1976).

In particular, synergism between TPA and epidermal growth factor, insulin or fibroblast growth factor in stimulating DNA synthesis has been described (Disker & Rozengurt, 1978). Recently, Niedel et al. (1983) described that protein kinase C was the cell surface receptor for TPA. Phosphoinositol turnover was implied in the signal transduction and diacylglycerol may be the endogenous component which triggers the cascade of signal transduction in response to an extracellular stimulus (Nishizuka, 1984).

TPA enhanced oncogene-induced transformation in C3H 10T1/2 cells and rat fibroblasts (Hsiao et al., 1984; Dotto et al., 1985; Hsiao et al., 1986). The exact mechanism of action of TPA on these cells is not clear. Studies of chemical carcinogenesis, particularly tumor promotion, have revealed some insights very recently. Oncogenes in combination with TPA were used in the study of cell transformation in vitro and tumor development in vivo (Quintanilla et al., 1986; Brwon et al., 1986) and the critical role of TPA in the stage of promotion was substantiated. TPA enhanced the incidence of the transformed phenotype in both systems. These data indeed suggested common pathways of tumor development from viral origin or chemical origin.

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II. INHIBITION OF CELLULAR DNA  
SYNTHESIS BY A BOVINE CELL  
SURFACE SIALOGLYCOPEPTIDE

## ABSTRACT

A sialoglycopeptide, which was isolated from bovine cerebral cortex cell surface, was shown to be a potent inhibitor of DNA synthesis and cell division. The inhibition of DNA synthesis could be measured as early as 5 h after the addition of the molecules to exponentially dividing 3T3 cells. The inhibitory activity was reversible and removal of the sialoglycopeptide led to a burst of DNA synthesis that peaked far above the level of DNA synthesis measured in rapidly dividing control cultures. DNA synthesis could be inhibited for as long as 50 h, although a small proportion of the arrested cells appeared to escape after 40 h. A comparable degree of inhibition was evident when cell numbers in the treated cultures were counted. The sialoglycopeptide also inhibited DNA synthesis of human skin fibroblasts while two mouse myeloma cell lines and mouse LM 22 cells were less sensitive to the inhibitory activity. In contrast to another cell surface inhibitor described previously which could inhibit cell growth if the LM cells were pretreated with gangliosides, pretreatment with ganglioside G<sub>D1a</sub> did not convert LM cells to being sensitive to the sialoglycopeptide in this study.

## INTRODUCTION

Cell proliferation is regulated by a complex series of interaction at the cell surface involving both growth factors and growth inhibitors. Cells propagated in culture become quiescent at confluency (Pardee et al., 1978) and can be stimulated to traverse the cell cycle by a number of mitogenic agents which presumably activate kinases, alter ion fluxes, or activate oncogenes (Heldin and Westermark, 1984; Martin et al., 1984; Sporn and Roberts, 1985). In addition to the stimulation of cell division by growth factors, DNA synthesis and division of proliferating cells can be inhibited by addition of cell membrane fragments and solubilized membrane components (Whittenberger and Glaser, 1977; Whittenberger et al., 1978; Wieser et al., 1985). Although work on cell growth factors has been extensive, relatively few studies on purified growth inhibitors emerged. Purification and characterization of these cell surface components have been difficult even though growth inhibitors have been identified from a wide variety of tissues and cells. Most of these inhibitors appeared to be proteins or glycopeptides and have been identified from mouse and bovine brain (Kinders et al., 1980; Kinders and Johnson, 1982; Charp et al., 1983), liver (McMahon et al., 1982), platelet (Brown and Clemmons, 1986), mammary gland (Bohmer et al., 1986,) and recovered from cell culture media (Holley et al., 1980; Hsu and Wang, 1986). Since most of these inhibitors have not been purified to homogeneity, the sites and mechanism of action remain unclear.

We previously described the isolation and purification of a sialoglycopeptide from bovine cerebral cortex cell surface membrane that was a potent inhibitor of both DNA synthesis and protein synthesis in a variety of cells grown in culture (Sharifi et al., 1986a). The sialoglycopeptide has been purified to homogeneity and shown to have a molecular weight of 18,000 and pI 3.0. The inhibitor decreased both protein and DNA synthesis at the intracellular level; uptake of radiolabelled precursors were not affected. Binding of the sialoglycopeptide to target cells involved specific, high affinity cell surface receptors and the inhibition of protein synthesis was correlated with receptor occupancy (Bascom et al., 1986).

In this communication, we describe the kinetics of inhibition of DNA synthesis of 3T3 cells by the sialoglycopeptide and the reversible nature of the inhibition following its removal. In addition, the sensitivity of several nontransformed and transformed target cells to the inhibitor were compared.

## MATERIALS AND METHODS

### Isolation and purification of bovine sialoglycopeptide

The bovine sialoglycopeptide was purified to homogeneity as described elsewhere (Sharifi et al., 1986a). Briefly, the bovine cerebral cortex cell suspensions were treated with pronase at a final concentration of 0.02 unit/ml (Sigma Chem. Co., St. Louis, MO). The supernatant fluid was collected and precipitated with 95% ethanol. The precipitate was suspended in distilled water and extracted with chloroform/methanol (2/1, v/v) and lyophilized to dryness. Then the material was purified by diethylaminoethyl (DEAE) ion exchange chromatography, wheat germ agglutinin affinity chromatography, size exclusion high pressure liquid chromatography (HPLC) and hydroxyapatite HPLC (Bascom et al., 1986).

### Cell culture

Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) and human foreskin fibroblasts (HSBP) (from Dr. Paul Charp, Oak Ridge National Laboratories, TN) were grown at 37°C in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) plus 10% calf serum (Hazleton, Denver, PA) and maintained in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. Cells were plated at  $5 \times 10^3$  cells/cm<sup>2</sup> in 24-well plates (Costar, Cambridge, MA) and allowed to grow for 24 h. Cells at the density of approximately  $1.0 \times 10^4$  cells/cm<sup>2</sup> were then

incubated with the sialoglycopeptide as described in individual experiments.

Mouse LM 22 cells obtained from Dr. David Rintoul, Kansas State University, were plated and grown under similar conditions to that for 3T3 cells except in Eagle's minimal essential medium (Flow Laboratories, McLean, VA) plus 5% fetal calf serum (KC Biological, Lenexa, KS).

Myeloma X63-Ag8 (Ag8) cells (American Type Tissue Collection, Rockville, MD) and myeloma Fox-NY cells (HyClone Laboratories, Logan, UT) were grown in conditions similar to that for LM cells except 10% fetal serum was added and cells were maintained in a humidified atmosphere of 10% CO<sub>2</sub>/90% air.

#### Measurement of DNA synthesis

After incubation with the sialoglycopeptide, the culture medium was removed and DNA synthesis was measured by incubation with 0.2 ml of medium containing 2.0  $\mu$ Ci of [<sup>3</sup>H]thymidine (Adjusted with thymidine to a specific activity of 0.5 Ci/mmole) (ICN Radiochemicals, Irvine, CA) for 1 h. After incubation the medium was removed and the cells were solubilized in 1 ml of 0.2 N NaOH. Macromolecules were precipitated with 1 ml of 20% trichloroacetic acid (TCA) and 0.1 ml of 1% BSA as a carrier and centrifuged at 2,000 x g for 7 min. The pellets were washed with 20% TCA and the precipitates were collected by centrifugation. The precipitates were suspended in 0.2 ml of 1 N NaOH and 0.1 ml aliquots of the samples were mixed with 2.5 ml scintillation cocktail and



the radioactivity was measured.

Ag8 and Fox-NY cells were grown in suspension and DNA synthesis was measured by adding 7.5  $\mu$ Ci of [ $^3$ H]thymidine in 20  $\mu$ l of medium to the cells and incubated for 1 h after treatment with the sialoglycopeptide. Cells were then transferred to tubes and processed in the same way as for 3T3 cells described above.

#### Determination of cell number

After incubation of 3T3 cells with the bovine sialoglycopeptide, cells were detached by trypsin and diluted in Isoton II (Coulter Electronic Inc., Hialeah, FL). The number of cells was counted with Coulter counter model Z<sub>f</sub> (Coulter Electronic Inc., Hialeah, FL). Inhibition of cell growth was calculated as follows:

(%) Inhibition of cell growth =  $100\% \times$

$$\left( 1 - \frac{\text{increase in cell number in inhibitor group}}{\text{increase in cell number in untreated group}} \right)$$

## RESULTS

To measure the ability of the sialoglycopeptide to inhibit DNA synthesis in exponentially growing 3T3 cells, we incubated the cells with various concentrations of the peptide in complete medium and 2.5% calf serum as described in Materials and Methods. Following a 20-h incubation period, DNA synthesis was determined and compared with DNA synthesis in untreated control cultures by incubation with [ $^3\text{H}$ ]thymidine. A dose-dependent inhibition of DNA synthesis was observed. A 25% decrease of DNA synthesis with 40 ng/ml of the sialoglycopeptide and a maximum of 35% inhibition with 80 ng/ml of the sialoglycopeptide were observed (Fig. 1).

As 3T3 cells were incubated with 40 ng/ml of the sialoglycopeptide for a prolonged period, DNA synthesis of the cells continued to decline until 30-h when it reached approximately 50% of that measured in exponentially growing cell cultures (Fig. 2). The inhibitory activity was shown to be nontoxic and reversible when cells were incubated with 40 ng/ml of the sialoglycopeptide for 20 h. Upon removal of the inhibitor an increase in DNA synthesis was shown at a higher rate than that of untreated exponentially growing 3T3 cell cultures (Fig. 2). Approximately 10 h after removal of the sialoglycopeptide, DNA synthesis of the cells was 150% to 160% of that measured in rapidly dividing 3T3 cell cultures. The kinetics of reversal and the peak of DNA synthesis followed by a sharp decline indicated that a significant fraction of the cell population was arrested

and synchronized by the sialoglycopeptide (Fig. 2).

The kinetics of inhibition of DNA synthesis was demonstrated with 40 ng/ml of the sialoglycopeptide and the decrease in DNA synthesis could be measured throughout 40 h with a single addition of the inhibitor (Fig. 3). By 50 h, however, a small fraction of the cells appeared to escape the inhibition since the rate of DNA synthesis began to increase slightly, even in the presence of the sialoglycopeptide (Fig. 3). The escape of these cells could not be prevented by the addition of a fresh aliquot of the inhibitor, suggesting that the escape was not a result of depletion or inactivation of the sialoglycopeptide initially added.

To be certain that the treatment of the sialoglycopeptide also give rise to mitotic arrest, we incubated exponentially growing 3T3 cells with 40 ng/ml of the peptide. The numbers of cells were counted following 24 h and 48 h of incubation and compared to cultures to which the inhibitor was not added. Cell division was clearly inhibited by the sialoglycopeptide since treated cultures showed a 40% reduction in cell number 24 h after the addition of the sialoglycopeptide (Table 1). Similar to the results in figure 3, an escape of a fraction of the arrested cells was evident after 48 h of incubation with the inhibitor since the reduction in cell number was only 30% of that measured in untreated control cultures (Table 1).

The ability of the sialoglycopeptide to inhibit transformend cell lines and human skin fibroblasts was tested by incubating the cells with 20 ng/ml and 40 ng/ml

of the molecules for 20 h. Human skin fibroblasts were at least as sensitive as 3T3 cells to the sialoglycopeptide. (Table 2). In contrast, transformed cells of Ag8 myeloma and Fox-NY myeloma were less sensitive to the inhibitory activity at both concentrations of the molecules. Mouse LM 22 cells, which are devoid of cell surface gangliosides, also were refractory to the inhibitory activity of the sialoglycopeptide. However, LM cells became sensitive to another cell surface-derived glycopeptide growth inhibitor isolated from bovine cerebral cell surface after these cells were pretreated with gangliosides (McGee et al., 1983; Bascom et al., 1985). In the present study, the pretreatment with ganglioside G<sub>D1a</sub> did not convert the LM cells to a sensitive state when incubated with the sialoglycopeptide (Table 2).

## DISCUSSION

Although there is little question that integral cell surface components play an important role in cell-cell communication and growth control, the hydrophobic nature of many membrane-associated proteins and glycopeptides has made their isolation and purification a difficult task. As a result, biologically relevant cell surface macromolecules, that may function as inhibitors of cell metabolism and division, and their receptors have not been characterized. Most studies concerning growth inhibitors, therefore, have been restricted to the detection and titration of their biologically inhibitory activity (Whittenberger and Glaser, 1977; Lieberman et al., 1981; Bohmer et al., 1984). In this report, we did not starve or deprive the cells of serum throughout the experiments and observed growth inhibition. We want to emphasize that the conditions we used may reflect or relate to the physiological relevance of this growth inhibitor.

Previous studies with this sialoglycopeptide focused on its ability to inhibit protein synthesis in a variety of target cells and it was shown that binding of the sialoglycopeptide to the cell surface receptors was sufficient to mediate inhibition of protein synthesis while the ligand did not have to be internalized (Sharifi et al., 1986b). The present study shows that the sialoglycopeptide is a potent inhibitor of DNA synthesis as well as cell division of nontransformed cells (Fig 1 and Table 1). Similar patterns of inhibition of DNA

synthesis or cell division were described by others as well (Charp et al., 1983; Hsu and Wang, 1986; Brown and Clemmons, 1986). DNA synthesis remained inhibited for at least 40 h when the inhibitor was maintained in the culture medium but removal of the sialoglycopeptide led to recovery of the cells from growth arrest (Fig. 2).

The reversible and nontoxic nature of inhibition of DNA synthesis by the sialoglycopeptide was similar to that observed with another glycopeptide inhibitor previously studied (Charp et al., 1983). One might argue that the burst of DNA synthesis was due to the addition of fresh medium containing serum factors. We rule out this possibility of serum stimulation. First, we could not find any increase in DNA synthesis upon addition of fresh medium to those logarithmically growing cells (control group) in the same data. Second, we could not detect any increase in DNA synthesis by adding fresh medium to logarithmically growing cells at 10 h interval for 30 h (data not shown). However, unlike the glycopeptide inhibitor that arrested cells in G<sub>2</sub> phase of the cell cycle, the kinetics of inhibition suggested that the sialoglycopeptide blocked the cells in the G<sub>1</sub> phase or G<sub>1</sub>/S boundary (Fig. 1 and Fig. 2). Cells remain arrested (measured by cell number) for at least 10 h after removal of the inhibitor (unpublished observation). These cells have generation time about 17 h. Another feature that distinguishes these two inhibitors is associated with the sensitivity of LM cells to these two growth inhibitors. LM cells when pretreated with gangliosides were converted from a resistant state to a sensitive state to the glycopeptide (McGee et al., 1983; Bascom et al., 1985) while LM cells remained refractory to the

sialoglycopeptide after incubated with ganglioside G<sub>D1a</sub> (Table 2).

These results are consistent with the possibility that cell surface components mediate growth regulation through cell-cell contact (Whittenberger and Glaser, 1977; Bielka et al., 1986; Hermann and Grosse, 1986; Wieser and Oesch, 1986) and events that lead to cell transformation may render the cells refractory to the inhibitory molecules (Kinders and Johnson, 1982; Sharifi et al., 1986a). It is not clear that transformed cells lack specific receptors for the sialoglycopeptide or are defective in the process of signal transduction.

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Table 1. Inhibition of cell division by a bovine sialoglycopeptide

Incubation Time	Control Cultures	Sialoglycopeptide Treated	Inhibition
(h)	(number of cells $\times 10^4$ )		(%)
0	$0.8 \pm 0.3$	$0.8 \pm 0.2$	-
24	$1.9 \pm 0.2$	$1.3 \pm 0.2$	40
48	$5.2 \pm 0.1$	$3.9 \pm 0.2$	30

3T3 cells were plated as described in Materials and Methods. Following a 24-h period, 40 ng/ml of the sialoglycopeptide was added to one set of cultures. The cell number was determined in the control and sialoglycopeptide-treated cultures after an incubation period of 24 h or 48 h. Each determination of cell number is reported as an average and range of duplicate cultures.

Table 2. Inhibition of DNA synthesis in Mouse and Human Cell Lines

Cell Line	Sialoglycopeptide	DNA Synthesis
	(ng/ml)	(% of control)
3T3	20	80 $\pm$ 6
	40	75 $\pm$ 5
human fibroblast	20	71 $\pm$ 12
	40	67 $\pm$ 7
X63-Ag8 myeloma	20	96 $\pm$ 6
	40	90 $\pm$ 7
Fox-NY myeloma	20	115 $\pm$ 16
	40	83 $\pm$ 7
LM 22	20	96 $\pm$ 12
	40	99 $\pm$ 6
LM 22 and G <sub>D1a</sub> pretreatment	20	99 $\pm$ 6
	40	91 $\pm$ 3

Each cell line was incubated with the sialoglycopeptide for 20 h and DNA synthesis was compared to similar cultures to which the inhibitor was not added. Ganglioside G<sub>D1a</sub> (3  $\mu$ g/ml) was added to LM 22 cells 3 h prior to incubation with the sialoglycopeptide. The data are reported as the average and range of two independent determinations carried out in duplicate.

Fig. 1. Inhibition of DNA synthesis by a sialoglycopeptide. 3T3 cells were treated with various concentrations of purified bovine sialoglycopeptide after they were allowed to attach for 24 h. Cells were treated for 20 h and incubated with [ $^3$ H]thymidine for 1 h. Cellular DNA was precipitated twice with 20% trichloroacetic acid. DNA was then dissolved in 1 N NaOH and radioactivity was determined by liquid scintillation counting. The data were plotted as percent of DNA synthesis in control cultures receiving 2.5% calf serum plus complete medium and indicated by average and range of duplicate determinations.

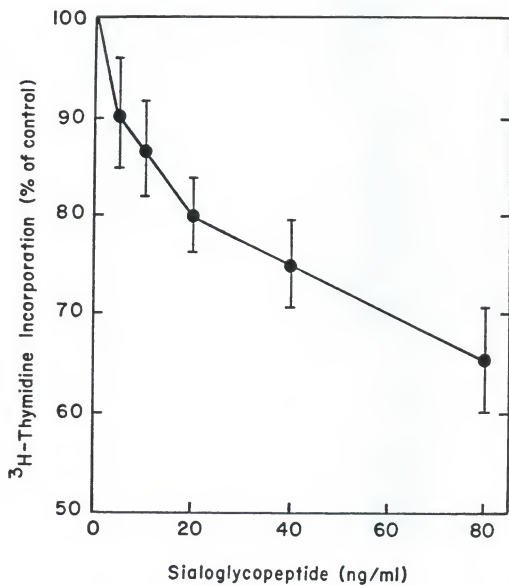


Fig. 2. Reversal of the inhibitory effect of the sialoglycopeptide. Cells were treated as in figure 1 except following 20 h incubation with 40 ng/ml of the sialoglycopeptide, a set of cultures received fresh medium indicated by the arrow. The other cultures were not disturbed until assayed for DNA synthesis. The broken line denotes the DNA synthesis in control cultures. The open circles indicate the cultures fed with fresh medium following 20 h incubation with the sialoglycopeptide. The filled circles are the cultures treated with the sialoglycopeptide throughout the experiments. The data represent average and range of triplicate determinations.

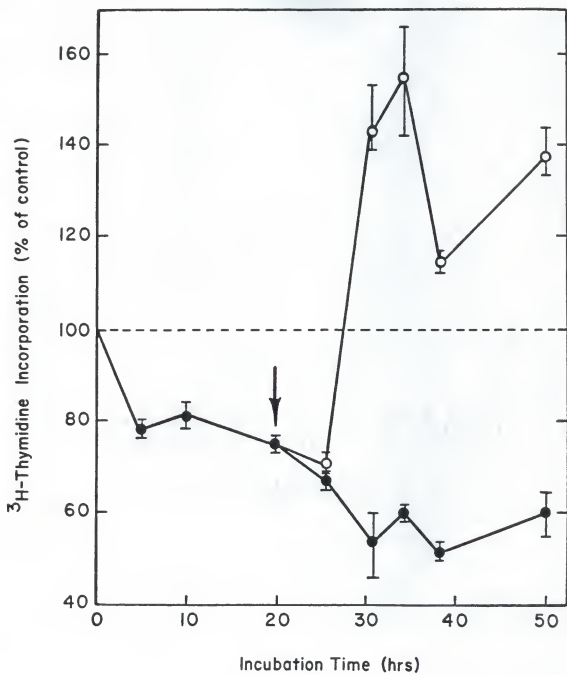
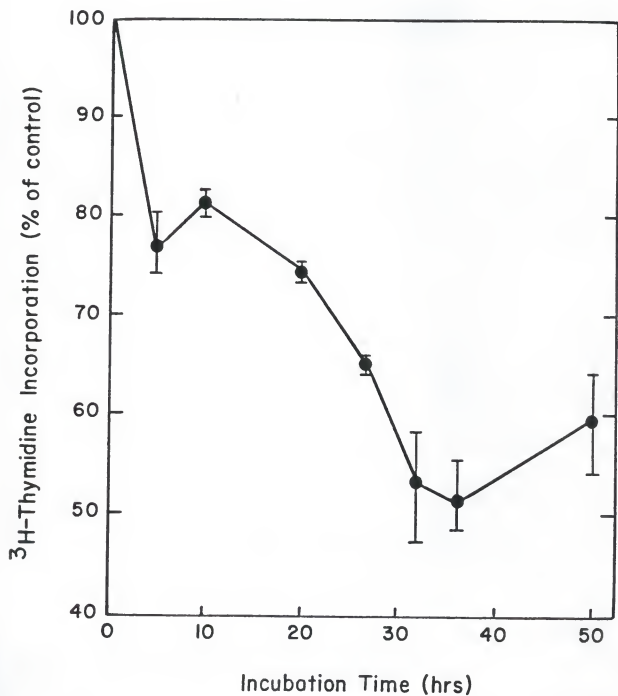




Fig. 3. Kinetics of inhibition of DNA synthesis. As in figure 2, growing 3T3 cells were incubated with 40 ng/ml of the sialoglycopeptide for a total period of 50 h. DNA synthesis was determined as indicated beginning in 5 h in triplicate cultures. The remaining cultures were not disturbed until determination of DNA synthesis. The data were graphed as percent of DNA synthesis in control cultures and represented averages and ranges of triplicate determinations.



III. A UNIQUE SIALOGLYCOPEPTIDE  
GROWTH REGULATOR THAT  
INHIBITS MITOGENIC ACTIVITY OF  
12-O-TETRADECANOYLPHORBOL-13-  
ACETATE (TPA)

## SUMMARY

The ability of a naturally occurring cell surface sialoglycopeptide growth inhibitor to antagonize the induction of DNA synthesis by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) was studied with mouse 3T3 cells. The bovine sialoglycopeptide was shown to be a potent antagonist of TPA-stimulated DNA synthesis in confluent 3T3 cell cultures. Kinetic studies demonstrated that inhibition of TPA-induced DNA synthesis required the addition of sialoglycopeptide within 15 min of TPA treatment. Addition of the sialoglycopeptide 30 min or longer after the cells were exposed to TPA did not block stimulation of DNA synthesis by TPA. The inhibition of TPA action was shown not to be restricted to DNA synthesis in 3T3 cells since the sialoglycopeptide also inhibited TPA-induced ornithine decarboxylase (ODC, L-ornithine carboxylase, EC 4.1.1.17) activation in suspensions of mouse epidermal as well as 3T3 cells.

## INTRODUCTION

TPA is a potent tumor promoter and experiments with the phorbol ester in the mouse skin system have provided the strongest evidence for two-stage carcinogenesis (26). This experimental model has been used extensively to study the mechanisms of tumor promotion and led to the use of TPA in studies of cell transformation in culture (9, 11). The addition of TPA to cell cultures has been shown to have numerous effects on cell proliferation, virus replication and cell morphology (7, 8, 11, 28). Although the mechanism of TPA action is not fully understood, protein kinase C appears to be the cell surface receptor for the phorbol ester (1). The process of signal transduction between TPA-induced phosphorylation of cellular proteins and the induction of DNA synthesis involves cascade reactions that remain to be studied.

We have described a cell surface sialoglycopeptide from bovine cerebral cortex cells which was shown to inhibit both protein synthesis and DNA synthesis in nontransformed cells (25). Many transformed cells are relatively resistant to the inhibitory activity. The inhibitor did not have to be internalized to exert the inhibitory activity (23) and the inhibition is nontoxic and reversible (Chou *et al.*, in preparation). In addition, the sialoglycopeptide did not alter the uptake of radioactive precursors (25). This sialoglycopeptide is very likely a candidate for studying the antagonism of some TPA-induced effects. The ability of a growth

inhibitor to block responses of the cells to tumor promoter would be important since only a few agents have been shown to inhibit TPA activities in vivo and in vitro. These agents are, in general, free radical scavengers, such as D-alpha-tocopherol or glutathione level raising agents that enhance the activity of the glutathione peroxidase detoxifying system (18, 20, 21, 26).

## MATERIALS AND METHODS

### Chemicals

12-0-tetradecanoylphorbol-13-acetate (TPA) and L-ornithine-HCl were purchased from Sigma Chemical Co. (St. Louis, MO). DL-[1-<sup>14</sup>C]-ornithine-HCl (specific activity: 57.3 mCi/mmol) was obtained from New England Nuclear (Boston, MA) and [methyl-<sup>3</sup>H]thymidine (specific activity: 0.5 Ci/mmol) from ICN Radiochemical (Irvine, CA).

### Isolation and Purification of Sialoglycopeptide

The sialoglycopeptide was isolated from bovine cerebral cortex cell surface and purified as described previously (25). Briefly, the sialoglycopeptide was released from cortical cells by mild pronase treatment, precipitated with ethanol, extracted by chloroform/methanol (2/1, v/v) and dialyzed prior to lyophilization. The sialoglycopeptide was purified by diethylaminoethyl ion exchange chromatography, wheat germ agglutinin chromatography and size exclusion high pressure liquid chromatography.

### Cell Culture

Swiss 3T3 cells were grown in culture in a humidified incubator with 5% CO<sub>2</sub>/95% air atmosphere. The TPA nonresponsive TNR-2 and TNR-9 cell lines were kindly provided by Dr. H. Herschman, University of California,

CA. Cells were plated in 24-well plates from Costar (Cambridge, MA) and allowed to grow to confluent prior to experiments with TPA. TPA was dissolved in dimethylsulfoxide (DMSO) and diluted in Dulbecco's Modified Eagle's Medium (DMEM) containing 2.5% calf serum and filter-sterilized. The final volume added to 3T3 cells was 0.2 ml containing 0.1% DMSO (v/v) and the control cultures were incubated with 0.1% DMSO only.

#### Preparation of Mouse Epidermal Cells

Female CF-1 mice from Sasco Inc. (Omaha, NE) at age 7-9 weeks were used; suspensions of isolated mouse epidermal cells were prepared by trypsin digestion as described elsewhere (19). Approximately  $2 \times 10^6$  viable cells were suspended in 0.8 ml of serum-free modified Eagle's medium containing 25mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.0).

#### Measurement of DNA synthesis

DNA synthesis was measured as described elsewhere (25). Briefly, the medium was replaced with 0.2 ml of fresh DMEM that contained 2.5% calf serum and 2.0  $\mu$ Ci of [ $^3$ H]thymidine and incubated for 1 h. Cells were washed three times with HKM buffer (10 mM HEPES, 120 mM KCl, 5mM  $MgCl_2$ ; pH 7.1) and cells were disrupted with 0.5 ml of 0.2 N NaOH. DNA was precipitated with 0.5 ml of 20% trichloroacetic acid (TCA) at 1,000g for 10 min. The precipitates were washed two times with 10% TCA and centrifuged. The final precipitates were suspended in 0.5 N NaOH. Fractions were mixed with scintillation fluid



and radioactivity was determined.

#### Measurement of ODC

Mouse epidermal cells were incubated with 62 ng/ml of TPA in serum-free medium as described above for 5 h. Cells were frozen and thawed three times and ODC activity was determined in 0.2 ml aliquots of the clear supernatants as described before (19). Assays were carried out in triplicate and values were corrected by use of no enzyme or boiled enzyme blanks. The results were expressed as nmoles of  $\text{CO}_2$  released in 60 min per  $2 \times 10^6$  cells. 3T3 cells were manipulated in a similar manner.

## RESULTS

In order to measure the magnitude of TPA-induced DNA synthesis in confluent 3T3 cells, various concentrations of TPA were incubated with the cells for 24 h. DNA synthesis was then measured. DNA synthesis was stimulated with the maximal stimulation of 7-8 fold in the range of 50 ng/ml to 100 ng/ml of TPA in agreement with results reported by others (4, 6). The possibility of antagonism between the sialoglycopeptide and TPA was then tested. 3T3 cells were incubated with 50 ng/ml of TPA in combination with various concentrations of the sialoglycopeptide for 18 h and DNA synthesis was determined. The presence of 40 ng/ml of the sialoglycopeptide in the incubation medium reduced DNA synthesis by 40% and 80 ng/ml by 90% as compared to TPA-induced DNA synthesis (Figure 1).

The kinetics of inhibition of the TPA effect by the sialoglycopeptide was studied. 3T3 cells were treated with the sialoglycopeptide at various time points following incubation with TPA. Addition of 80 ng/ml of the sialoglycopeptide and 50 ng/ml of TPA simultaneously to the cells blocked TPA-induced DNA synthesis by 87% (Figure 2). Only 70% reduction of DNA synthesis was observed with 15 min delay of the sialoglycopeptide treatment following exposure to TPA. Furthermore, addition of the sialoglycopeptide to the cells 30, 60, or 150 min after exposure to TPA had no effect on the mitogenic activity of TPA (Figure 2). These data indicated the antagonism between TPA and the

sialoglycopeptide occurred in the early steps of signal transduction.

The possibility of shared cell surface receptors between TPA and the sialoglycopeptide was examined. Radio-labeled sialoglycopeptides were incubated with 3T3 cells in the presence of increasing concentrations of TPA. The level of binding was not affected by increasing concentrations of TPA as high as 300-fold in excess (in moles) over the sialoglycopeptide (data not shown). Clearly the two ligands did not share the same receptors, implying that the antagonism occurred in the process of signal transduction. The same conclusion was drawn in the experiments with TPA nonresponsive cell lines TNR-2 and TNR-9. These two cell lines did not initiate mitogenic response after binding to TPA (4) while both cell lines responded to the sialoglycopeptide in inhibition of protein synthesis to a similar extent as measured in the parental 3T3 cells (data not shown).

In most cells, elevated levels of polyamines were observed in response to a growth stimulus (12, 13). In order to measure ODC activity, we incubated the mouse epidermal cells with 62 ng/ml of TPA for 5 h, conditions which resulted in the maximal stimulation of ODC activity (19). The TPA-induced ODC activity was sensitive to the sialoglycopeptide and as little as 60 ng/ml of the sialoglycopeptide reduced the enzyme activity by 50% (Figure 3). In contrast to the inhibition of TPA-induced DNA synthesis, no further decrease in the ODC activity was observed with increasing concentrations of the sialoglycopeptide. Similar results were obtained when ODC activity was measured in 3T3 cells (Table I).

Incubation of 40 ng/ml of the sialoglycopeptide inhibited TPA-induced ODC activity by 40% similar to the inhibition observed in the epidermal cells (Figure 3). However, the addition of the sialoglycopeptide 1 h after exposure of 3T3 cells to TPA only reduced the activity by 20% (Table I).

## DISCUSSION

The results of this paper show that the bovine sialoglycopeptide is a potent inhibitor of TPA action. The sialoglycopeptide inhibited both TPA-induced DNA synthesis and ODC activity (Figures 1 and 3) and the kinetics of antagonism showed that the counteraction was rapid (Figure 2). This conclusion was supported by the results of ODC activity (Table I). The data strongly suggested that the sialoglycopeptide interfered with TPA action at early stage of TPA-induced signal transduction. ODC activity in epidermal and 3T3 cells was inhibited by approximately 40% with 60 ng/ml of the sialoglycopeptide. Unlike the counteraction of DNA synthesis, however, increasing concentrations of the sialoglycopeptide did not result in further inhibition of ODC activity. The fraction of ODC activity that remained refractory to the action of the sialoglycopeptide might reflect the de novo synthesis of the enzyme which was probably not subjected to TPA induction.

TPA synergized with a number of serum factors in stimulating DNA synthesis (10). Thus, it is possible that the sialoglycopeptide in effect blocks the activity of other serum factors. We rule out this possibility. For instance, measurement of ODC activity was carried out in the absence of serum in the epidermal system and that inhibition of TPA-induced ODC activity by the sialoglycopeptide was direct. In addition, the sialoglycopeptide inhibited bombesin-induced DNA

synthesis in the absence of serum (Sharifi et al., in preparation).

The kinetic studies suggested that the sialoglycopeptide intervened at the TPA-induced early steps of signal transduction. It has been shown that protein kinase C is the receptors for TPA and that TPA activates protein kinase C, which in turn leads to phosphorylation of other proteins. In addition, TPA stimulated ion flux transport systems that include  $\text{Na}^+/\text{H}^+$  antiport and  $\text{Na}^+/\text{K}^+$  pump (14,29). It is likely that the sialoglycopeptide interferes with either the activity of protein kinase C, the activation of ion transport systems or both.

The findings in this paper show that a cell surface growth inhibitor can antagonize the mitogenic activity of a phorbol ester tumor promoter. The data suggested that the counteraction of TPA-induced mitogenesis did not involve a direct competition between TPA and the sialoglycopeptide for cell surface receptors and the interaction occurred at the post-receptor level. These observations are consistent with the proposition that cell growth is regulated by growth inhibitors as well as growth factors (27) and that cell transformation could result from an imbalance in growth regulating signals mediated at cell surface.

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Table I. The Sialoglycopeptide Inhibits  
TPA-Induced ODC Activity in 3T3 Cells

Treatment	ODC Activity	ODC Activity
	(nmoles CO <sub>2</sub> /2x10 <sup>6</sup> cells)	(% inhibition)
Control	0.42 ± 0.02	-
TPA	1.31 ± 0.18	0
TPA plus inhibitor (0 time)	0.95 ± 0.13	40
TPA plus inhibitor (1 h delay)	1.11 ± 0.06	20

3T3 cells were incubated with 62 ng/ml of TPA alone or TPA plus 40 ng/ml of the sialoglycopeptide. The sialoglycopeptide was added to TPA treated cells either simultaneously (0 time) or 1 h later and ODC activity was determined. The data represent the average and range for triplicate determinations.

Fig. 1. Antagonism of TPA-induced DNA synthesis by the bovine sialoglycopeptide. Confluent 3T3 cells were treated simultaneously with 50 ng/ml of TPA and increasing concentrations of the sialoglycopeptide for 18 h and thymidine incorporation was measured. The basal level of [ $^3$ H] thymidine incorporation by confluent 3T3 cells (in the absence of TPA) is indicated by the open circle on the ordinate and the broken line. The results represent the mean and the range for each concentration carried out in duplicate.

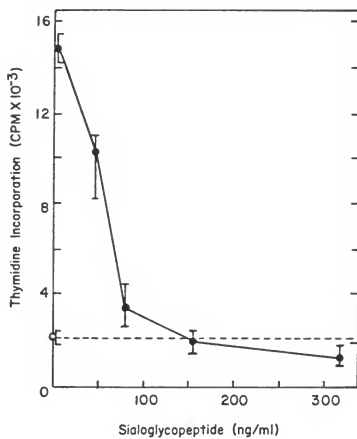


Fig. 2. Kinetics of inhibition of TPA-induced DNA synthesis by the sialoglycopeptide. 3T3 cells treated with TPA (50 ng/ml) alone (open bars) or TPA plus the sialoglycopeptide (80 ng/ml) (shaded bars), the sialoglycopeptide was added simultaneously (panel A) or 15 min (panel B) or 30 min (panel C) or 60 min (panel D) or 150 min (panel E) after TPA and incubated for 20 h. Thymidine incorporation was then measured. The data represent the average and range for triplicate determinations.

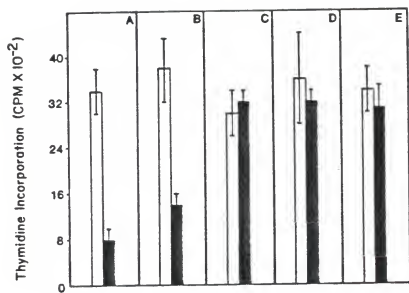
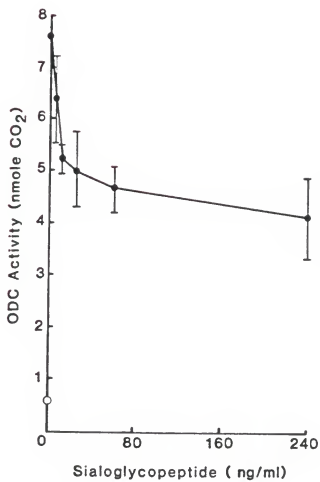


Fig. 3. Inhibition of TPA-induced ODC activity by the sialoglycopeptide. Mouse epidermal cells were incubated with 62 ng/ml of TPA and increasing concentrations of the bovine sialoglycopeptide for 5 h and ODC activity was determined. The results represent the mean and range of triplicate from two independent experiments. The basal level of ODC activity (in the absence of TPA) in mouse epidermal cells was denoted by open circle.





GROWTH INHIBITION BY SIALOGLYCOPEPTIDES  
FROM THE SURFACE OF BOVINE CEREBRAL CELLS  
AND THEIR INTERACTION WITH  
12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA)

by

Hsin-Hwei Joseph Chou

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## ABSTRACT

Sialoglycopeptides purified from the bovine cerebral cortex cell surface were incubated with various lines of cells in logarithmically growing phase. The purified material exhibited inhibitory activity on DNA synthesis of nontransformed cells, whereas transformed cells were refractory to the molecules. Also 3T3 cells were blocked in cell division. LM 22 cells upon pretreatment with ganglioside  $G_{D1a}$  remained refractory to the inhibitory activity. The nontoxic nature of the molecules was demonstrated upon removal of the material from the cells. The data suggested that these cells were arrested in  $G_1$  phase or  $G_1/S$  boundary of the cell cycle.

The sialoglycopeptide counteracted the TPA-induced DNA synthesis and ornithine decarboxylase activity in 3T3 cells. Studies of the kinetics of antagonism indicated that interactions between these two species occurred in the early steps of signal transduction. The sialoglycopeptide decreased TPA-stimulated ornithine decarboxylase activity in mouse epidermal cells as well.