ample, in precursor uptake, transmitter synthesis, transmitter degradation) or, alternatively, to restore the balance between two or more age-altered neurotransmitter systems before a consistently positive effect on memory is obtained. Nevertheless, these results indicate that (i) reliable changes in performance on this memory task do occur under physostigmine, (ii) certain age-related differences in these effects also exist, and (iii) some aged monkeys perform significantly better under a number of short-term doses. Although these results do not provide strong support for the use of physostigmine as a reliable therapeutic agent for geriatric cognition, they do provide additional circumstantial evidence for an important cholinergic role in age-related memory impairments. More important, they provide an objective rationale for believing appropriate pharmacological manipulation of the cholinergic system (perhaps in conjunction with other neurotransmitter systems) may eventually be developed to alleviate some of the cognitive declines associated with advanced age.

RAYMOND T. BARTUS*
*Department of Pharmacology, Warner-Lambert/Parke-Davis Research Laboratories, Ann Arbor, Michigan 48106,* and *Medical Research Division, American Cyanamid Company, Pearl River, New York 48106*

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10. During each test session, each monkey was tested under equally distributed, 0-second (nonmemory) control intervals, as well as longer (memory-dependent) retention intervals. The duration of the longer retention intervals was adjusted so that all monkeys responded accurately between 50 and 70 percent of the time on each nondrug session. In this way, age-related differences in performance were controlled, providing an assessment of physostigmine unconfounded by differences in performance. The duration of the retention intervals for the four young monkeys (in order of appearance in Fig. 2) were 150, 68, 75, and 150 seconds. Similarly, the retention intervals for the eight aged monkeys were 40, 23, 45, 15, 23, 30, 23, and 4 seconds. These large differences in duration reflect the large difference in ability of the two age groups on this task after several months of training.
11. Individual analyses of variance revealed significantly greater variability between age groups on the three lower doses (0.0013 to 0.005 mg/kg; $F(7, 3) = 9.6$, 52.3, and 10.4, respectively). Age-related differences in variability were not observed on the three higher doses.
12. No impairment was seen at any dose in either age group on the 0-second control condition. These selective effects on the longer, memory-dependent intervals are often interpreted as evidence for disruption in centrally mediated mnemonic processes [H. J. Fletcher, in *Behavior of Nonhuman Primates*, A. M. Schrier, H. F. Harlow, F. Stollnitz, Eds. (Academic Press, New York, 1965), vol. 1, pp. 129-165].
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14. Still other alternatives include (i) Changes in the absorption and metabolism of drugs with age [J. R. Gillette, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 1901 (1979)], coupled with the relatively short half-life of physostigmine (*5*) could result in age-related differences in concentrations in the blood. These differences could result, in turn, in differences in the effective dose of physostigmine during the 30-minute test session. (ii) The two age groups might be differentially sensitive to adverse side effects of physostigmine, since no peripheral cholinergic blocking agents were administered to either group. Careful monitoring of each monkey revealed no overt effects or changes in response rates except at the highest dose. At this dose some monkeys in both age groups appeared somewhat lethargic, but three other aged monkeys actually improved. Thus, it is unlikely that differences in peripheral side effects could account for these results. The data do indicate that careful individual titration of doses of physostigmine might be necessary to enhance the chances of finding significant positive effects in aged subjects.
15. Data taken from R. T. Bartus *et al.* (*2*).
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* Present address: American Cyanamid Company, Pearl River, N.Y. 48106.

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## Elimination of Metabolic Cooperation in Chinese Hamster Cells by a Tumor Promoter

Abstract. *Wild-type Chinese hamster V79 cells (6-thioguanine-sensitive) reduce the recovery of 6-thioguanine-resistant cells when they are cultured together at high densities, through a form of intercellular communication (metabolic cooperation). Cooperation is inhibited by 12-O-tetradecanoyl phorbol-13-acetate, rescuing the 6-thioguanine-resistant cells. These results may be useful in the study of an aspect of the mechanism of tumor promotion and in assaying for promoters.*

Metabolic cooperation is a form of intercellular communication in which the mutant phenotype of enzyme-deficient cells is corrected by normal cells or by different mutant cells. Two types of metabolic cooperation have been observed: one requires cell-to-cell contact, the other does not. A typical example of the former is the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) system described by Subak-Sharpe *et al.* (*1*). Subsequent investigations have indicated that metabolic cooperation also occurs with the products and functions of the enzymes coded by genes for adenine phosphoribosyltransferase (*2*), thymidine kinase (*2*), $Na^+$, $K^+$-activated adenosinetriphosphatase (*3*), β-adrenergic receptor (*4*), and plasminogen activator (*5*). The second type of metabolic co-

Table 1. The effects of phorbol ester analogs on the recovery of the 6-TG$^r$ cells. The percentage of recovery for each treatment group was obtained by averaging the results for 21 plates, each of which contained $8 \times 10^5$ 6-TG$^s$ cells and 100 6-TG$^r$ cells. The concentration of phorbol, TPA, and all phorbol ester analogs was 1 ng/ml. Statistical significance was determined according to a modification of the Student-Newman-Keuls test. Recovery of cells treated with TPA, phorbol-12,13-didecanoate, and phorbol-12,13-dibutyrate was superior to that of the control group at the $P < .01$ level of confidence; for cells treated with 4-*O*-methylphorbol-12-myristate-13-acetate, recovery was superior to that of the control group at $P < .05$. For each treatment group there were six control plates, in which 6-TG$^r$ cells were cultured alone. The phorbol drugs had no significant effect on their attachment and growth. Tumor-promoting potential was rated on a scale of 0 to 4, with 0 representing no significant activity and 4 the greatest activity.

| Phorbol analog | Tumor-promoting activity in vivo | Percentage recovery ± standard error |
|---|---|---|
| Control (ethanol) | 0 | 26.7 ± 1.3 |
| Phorbol | 0 | 27.5 ± 1.3 |
| 4-α-Phorbol-12,13-didecanoate | 0 | 31.6 ± 1.5 |
| Phorbol-12,13-diacetate | 1 | 32.8 ± 1.1 |
| 4-*O*-Methylphorbol-12-myristate-13-acetate | 1 | 33.5 ± 1.5 |
| Phorbol-12,13-dibutyrate | 2 | 51.0 ± 1.9 |
| Phorbol-12,13-didecanoate | 3 | 91.5 ± 2.2 |
| Phorbol-12-myristate-13-acetate | 4 | 100 ± 2.3 |


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operation is exemplified by the different syndromes of mucopolysaccharidosis (*6, 7*). Here cell-to-cell contact is not required, since cooperation appears to be mediated by means of a diffusible product. Metabolic cooperation has been shown to be influenced by such factors as different chemical analogs (*8*), cell lines (*9*), and membrane modifications (*10*). Cell-to-cell communication, thought to be involved in metabolic cooperation, has also been implicated in a variety of biological processes, including immune response (*11*) and growth control (*12*).

We report here a series of experiments that demonstrate the elimination of metabolic cooperation between 6-thioguanine-resistant (6-TG$^r$) and 6-thioguanine-sensitive (6-TG$^s$) Chinese hamster V79 cells by the potent tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA).

Since the original demonstration by Berenblum (*13*) of two-stage (initiation and promotion) carcinogenesis in mouse skin, a number of more recent studies have corroborated the two-stage conceptualization of tumorigenesis. Initiation seems to be the result of an irreversible cellular event that is induced by physical or chemical changes, whereas promotion appears to be a reversible process (up to a point) that depends on repeated treatment of the initiated cell by agents which are weakly carcinogenic or noncarcinogenic by themselves. Many different chemical compounds, given to animals after initiation with chemical or physical carcinogens, have been implicated as tumor promoters in several organ systems. The list includes chemicals as structurally and functionally dissimilar as butylated hydroxytoluene (*14*), phenobarbital (*15*), thyroid-stimulating hormone (*16*), bile acids (*17*), Tween 80 (*18*), alkanes (*19*), and cholesterol (*20*). Evidence favors the hypothesis that tumor initiation is a mutagenic event and promotion an epigenetic change (*21*).

In an attempt to delineate the biochemical mechanism of tumor promotion, TPA, the most potent of all known tumor promoters, has been examined quite extensively. When TPA is administered to cells in culture, a large number of responses are elicited. Among the most striking are an increase in the synthesis of DNA and RNA, stimulation of ornithine decarboxylase activity, an increase in the uptake of 2-deoxyglucose, an increase in prostaglandin synthesis, altered cellular morphology, and an increase in malignant transformations, and in the recovery of specific mutant somatic cells (*22*). Clearly, TPA is capable of inducing major cellular changes whose significance we do not yet fully understand.

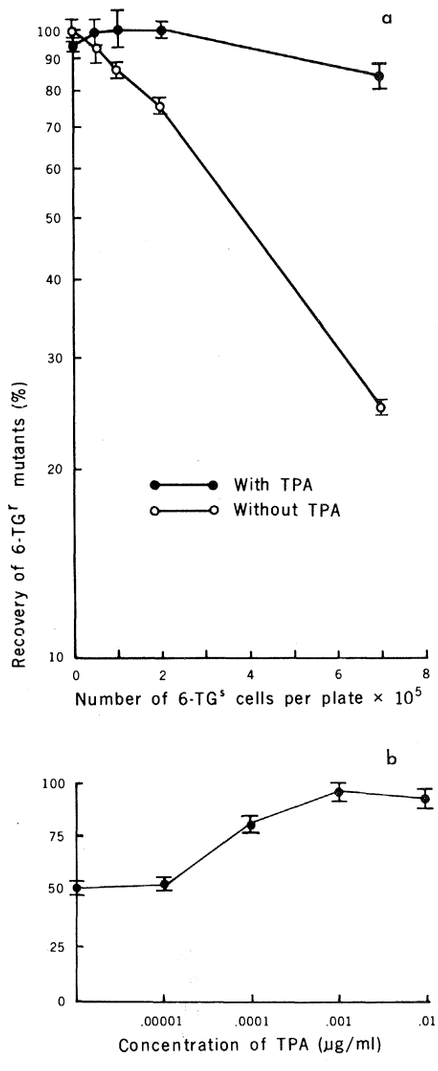


Fig. 1. (a) Effect of cell density on the recovery of 6-TG$^r$ cells cultured with and without TPA. Wild-type 6-TG$^s$ cells and a mixture of approximately 100 x-ray-induced 6-TG$^r$ colonies were grown in modified Eagle's medium (Earle's balanced salt solution with a 50 percent increase of essential amino acids and vitamins) supplemented with a 100 percent increase of nonessential amino acids, 1 m*M* sodium pyruvate, and a 5 percent increase of fetal calf serum. In a humidified air atmosphere (5 percent $CO_2$) at 37°C, the two cell lines had a generation time of approximately 12 hours. Both cell lines were cultured simultaneously, allowed to attach themselves to the 9-cm-diameter plates (Falcon), and then were treated with TPA (1 μg/ml) and 6-thioguanine (10 μg/ml). The TPA was removed about 4 days after the cells were first cultured and replaced by a medium containing only 6-thioguanine. The colonies were fixed, stained with Giemsa, and scored for recovery about 3 days later. Percentage of recovery was determined as the average of the recovery in the ten plates in each treatment group. (b) Effect of concentration of TPA on the recovery of 6-TG$^r$ cells. The culture conditions were identical to those in (a). In each plate, 100 6-TG$^r$ cells were cultured with $8 \times 10^5$ 6-TG$^s$ cells. For each treatment group there were four control plates, in which 100 6-TG$^r$ cells were cultured alone. None of the TPA concentrations had any significant effect on the efficiency with which cells in each group attached themselves to the plates and grew. Percentage of recovery was determined as the average of the recovery in the ten plates in each treatment group.

In an attempt to examine the biological effects of TPA on cellular membranes and the intercellular transport of small molecules, we cultured a small number of 6-TG$^r$ V79 cells in the presence of various numbers of 6-TG$^s$ cells; in each case, the number used was sufficient to reduce the recovery of the mutant cells (Fig. 1a). With no treatment, the recovery of the 6-TG$^r$ cells diminishes precipitously when the number of wild-type cells increases. In the presence of $7 \times 10^5$ wild-type cells it is possible to recover as colonies approximately 25 percent of the 6-TG$^r$ cells originally cultured. However, if the same experiment is conducted with the addition of TPA, the recovery of 6-TG$^r$ cells is not significantly reduced; it is still possible to recover approximately 85 of the 100 6-TG$^r$ cells originally cultured. In this series of experiments, TPA was present during the first 4 days of growth, at the end of which virtually all of the 6-TG$^s$ wild-type cells had been killed. The TPA was then removed, and cultivation of the colonies in selective medium was continued for 4 to 5 days. Control experiments have clearly indicated that TPA does not enhance the efficiency with which 6-TG$^r$ cells attach to the plate and grow when they are cultured alone (data not given). Therefore, we feel that TPA somehow blocks metabolic cooperation, thereby allowing mutant 6-TG$^r$ cells to proliferate in the medium.

In an attempt to determine whether the modification of the recovery of 6-TG$^r$ cells by TPA was dose-responsive, we performed the following experiment. Using $8 \times 10^5$ 6-TG$^s$ cells and 100 6-TG$^r$ cells per plate, we measured the recovery of the resistant cells after exposure to TPA (0.01 to 10 ng/ml). A dose-responsive relationship was clearly demonstrated when TPA (1 ng/ml) was sufficient to allow the recovery of almost 100 percent of the 6-TG$^r$ cells (Fig. 1b).

Table 1 gives the results of an experiment to determine whether this system is capable of discriminating between tumor promoters of various degrees of potency in vivo. In addition to TPA and phorbol (the parent alcohol of TPA), we examined five commercially synthesized, structural analogs of TPA. Excellent correlation was observed between the abili-

ty of each drug to increase the recovery of the 6-TG$^r$ mutants and its ability to promote tumors in vivo. Both phorbol and 4-$\alpha$-phorbol-12,13-didecanoate (neither of which promotes the growth of skin tumors) gave essentially negative results.

These results, we believe, are applicable for the detection of tumor promoters in Chinese hamster cells in vitro. At present, phenobarbital, anthralin, butylated hydroxytoluene, DDT, and saccharin have blocked metabolic cooperation in the V79 system (*23*). We are now testing suspected promoters of tumors in humans by using Lesch-Nyhan fibroblasts (HGPRT$^-$) cocultivated with normal diploid fibroblasts (HGPRT$^+$).

It would appear that the mechanism through which TPA increases the recovery of 6-TG$^r$ cells in the V79 system is related to a reduction of metabolic cooperation. This could occur through alteration of membrane structure or function or both, which would reduce the amount of cell-to-cell communication. This explanation is consistent with a variety of experimental and theoretical reports. Borek and Sachs (*24*) showed that nontransformed hamster or rat cells can inhibit cell replication of transformed cells. Sivak and Van Duuren (*25*) noted that croton oil, a potent promoter of skin tumors in mice, enhances the recovery of virus-transformed cells that are cocultivated with many nontransformed 3T3 mouse cells. Krieg *et al.* (*26*) showed that TPA is an effective inhibitor of a G1 chalone in mouse skin. A mathematical model was postulated by Bell (*27*) to explain how cancer cells escape regulation by diffusible mitotic inhibitors. He postulated a locally reduced mitotic inhibitor to account for a single transformed cell proliferating until it reaches a "critical mass." Bertram (*28*) and Lloyd *et al.* (*29*) showed that untransformed 10T$^1/_2$ cells can, by cocultivation, suppress the expression of transformed C3H 10T$^1/_2$ cells. Recently, Bertram (*30*) showed that the mechanism by which nontransformed 10T$^1/_2$ cells inhibit the growth of cocultivated malignant cells seems to involve the modulation of cyclic nucleotides in mediating intracellular communication between normal and transformed cells.

Two additional observations should be considered: (i) transformed human cells exhibit reduced contact-mediated communication when compared to normal cells (*9*) and (ii) malignant cells in mice melanomas do not form tumors when mixed with cells of a nonmalignant clone (*5*). Since tumorigenicity has been shown to be a recessive trait (*31*) in all but virally transformed cells, it is likely that tumorigenic cells can be converted into normal cells when regulatory substances from normal cells are transferred by metabolic cooperation. Therefore, we speculate that a possible mechanism for tumor promotion could be the elimination of metabolic cooperation, allowing cells in the initation phase of carcinogenesis to express their transformed phenotype.

L. P. YOTTI, C. C. CHANG, J. E. TROSKO
*Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, East Lansing 48824*

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## Noncycling Tumor Cells Are Sensitive Targets for the Antiproliferative Activity of Human Interferon

Abstract. *Resting Burkitt's lymphoma cells (Daudi) in culture are more sensitive targets for the antiproliferative activity of purified human fibroblast interferon than cells that are rapidly multiplying. Thus, interferon may be of significant clinical value in neoplasms involving stem cells and, after chemotherapy, in suppressing the re-emergence of tumors.*

Interferons (IF's), produced by different cell types, are glycoproteins with antiproliferative as well as antiviral activities. They are being tested as therapeutic agents in a variety of malignant tumors and viral diseases (*1*). Although IF's affect a wide range of tumor cell types, little is known about the events in the mitotic cycle that are targets of IF action. Better insight into the action of IF's could result in greater suppression of tumor cell proliferation and possibly effective combinations with available chemotherapeutic drugs. This would be very useful for clinical trials, which are now limited by both the expense and the amount of IF's available.

Lymphoblastoid cell lines, established from Burkitt's lymphoma, have been used to study the IF effect on cell division and the virus growth cycle (*1, 2*). Simultaneous measurements of IF effects on expression of virus genes (Epstein-Barr virus) and cell growth show that the kinetics of appearance of the antiproliferative and antiviral effects are parallel, illustrating the close functional similarities between them. Moreover, these two properties of IF's are always coordinately influenced, as shown by the gradient of IF sensitivity observed among certain lymphoid tumor lines (*2*). Both biological effects reside in the same glycoprotein (*1, 3*).

The antiviral effect of IF's is enhanced in noncycling human cells (*4*). In this report we demonstrate that the antiproliferative activity of purified human fibroblast IF (HFIF) on Daudi lymphoblastoid cells is most pronounced in resting or nondividing cells. Our experiments imply that intracellular events, in conjunction with the extracellular IF concentration, are critical determinants of the magnitude of antiproliferative response. They suggest a strategy for the


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