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Inhibition of Human Breast Cancer Colony Formation by Anticalmodulin Agents: Trifluoperazine, W-7, and W-13

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Summary. *The effects of anticalmodulin agents, namely trifluoperazine (TFP) and two naphthalene sulfonamide derivatives (W-7 and W-13), were tested on the growth of a human breast cancer cell line (MDA-MB-231) using a soft agar clonogenic assay. The results of this in vitro study reveal that TFP, W-7, and W-13 had the ability to inhibit the colony formation from this cell line. The inhibitory effect was greater when the cancer cells were exposed to these agents continuously than when the cells were exposed to the drugs for 1 h. The IC_{50} values for TFP, W-7, and W-13 in continuous exposure were about 18, 30, and 38 μM, respectively, whereas the corresponding values for 1-h exposure were 50, 53, and 70 μM, respectively. These findings suggest that anticalmodulin agents can inhibit the growth of human cancer cells at relatively low concentrations in vitro. Whether effective antitumor concentrations of these drugs can be achieved in vivo remains a subject for further study.*

Introduction

It is well documented that calcium and/or calmodulin play significant roles in DNA synthesis and cell proliferation [10, 30]. During the pre-replication stage of a cell cycle, there is a critical period when intracellular ionic calcium and calmodulin levels are increased just before DNA synthesis takes place [6, 14, 17, 23, 30]. It has been reported that the calmodulin levels are significantly elevated in exponentially growing transformed cells [5, 15, 26]. In addition, the levels of calcium and/or calmodulin are increased in hepatomas [9, 12, 18, 27, 28] and in human mammary tumor tissue [24]. Thus, prolonged abnormally high levels of calcium and/or calmodulin may lead to a bypass of this critical step during cell proliferation. This biochemical abnormality may be linked to the relatively autonomous cell growth associated with cancer [10, 30].

The recent finding that increased calmodulin levels in Morris hepatomas are positively correlated with tumor growth rates [28] has raised the possibility that anticalmodulin drugs can inhibit the growth of tumor cells. To test this in the present study, we investigated the effects of some calmodulin-inhibitory agents on the growth of the human breast cancer cell line, MDA-MB-231 [3, 4] in soft agar, using the clonogenic assay of Hamburger and Salmon [11, 22]. The agents tested include the antipsychotic agent trifluoperazine (Stelazine, Terfluzine) [16, 29] and the naphthalene sulfonamides W-7 and W-13 [6, 13].

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Materials and Methods

Drugs. Trifluoperazine hydrochloride (TFP) was obtained from Smith Kline and French Ltd., Mississauga, Ontario, Canada. The compounds: W-7, [*N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide, hydrochloride], and W-13 [*N*-(4-aminobutyl)-5-chloro-2-naphthalene sulfonamide, hydrochoride] were purchased from Caabco, Inc., Houston, Texas, USA. These compounds were dissolved in a small amount of ethanol (e.g., 1 mg in 25 μl) and adjusted to appropriate concentrations with culture medium or saline. The highest concentration of ethanol used in these experiments (0.05% v/v) had no significant effect on colony formation or cell culture growth. All drugs were freshly prepared and were protected from light. Other chemicals were of the highest purity available commercially.

Tumor Cells. The human breast carcinoma cell line (MDA-MB-231) was originally grown from a pleural effusion removed from a patient at the M. D. Anderson Cancer Center, Houston, USA, in 1973 [3, 4]. This cell line was obtained by EG & G Mason Research Institute (57 Union Street, Worcester, MA 01608, USA) and cryopreserved. Dr E. M. Jensen of this Institute kindly sent this cell line to us and it has been maintained in our laboratory since January, 1982. The cells were grown in Corning 75 cm^2 tissue culture flasks which contained 15 ml enriched CMRL-1066; this medium was prepared by adding to 100 ml CMRL-1066 (Gibco Laboratories, Grand Island, NY, USA) the following: 15 ml horse serum (Flow Laboratories, McLean, VA, USA), 2 ml insulin (100 U/ml) (Connaught Laboratories, Willowdale, Ontario, Canada), 2 ml L-glutamine (200 m*M*), 1 ml vitamin C (30 m*M*), and 1 ml penicillin-streptomycin solution (10,000 U/ml) (Gibco Laboratories). The cells were incubated at 37° C in a humid air/carbon dioxide (5%) mixture for 5–8 days. The cells were removed from culture flasks by digesting briefly with trypsin (0.125%) in Hank's balanced salt solution. After two fold dilution with enriched CMRL-1066 medium, the cell suspensions were centrifuged at 580 *g* fof 5 min and pellets were resuspended in an appropriate amount of fresh culture medium. These cell suspensions were then passed through 25-gauge needles several times to make single-cell

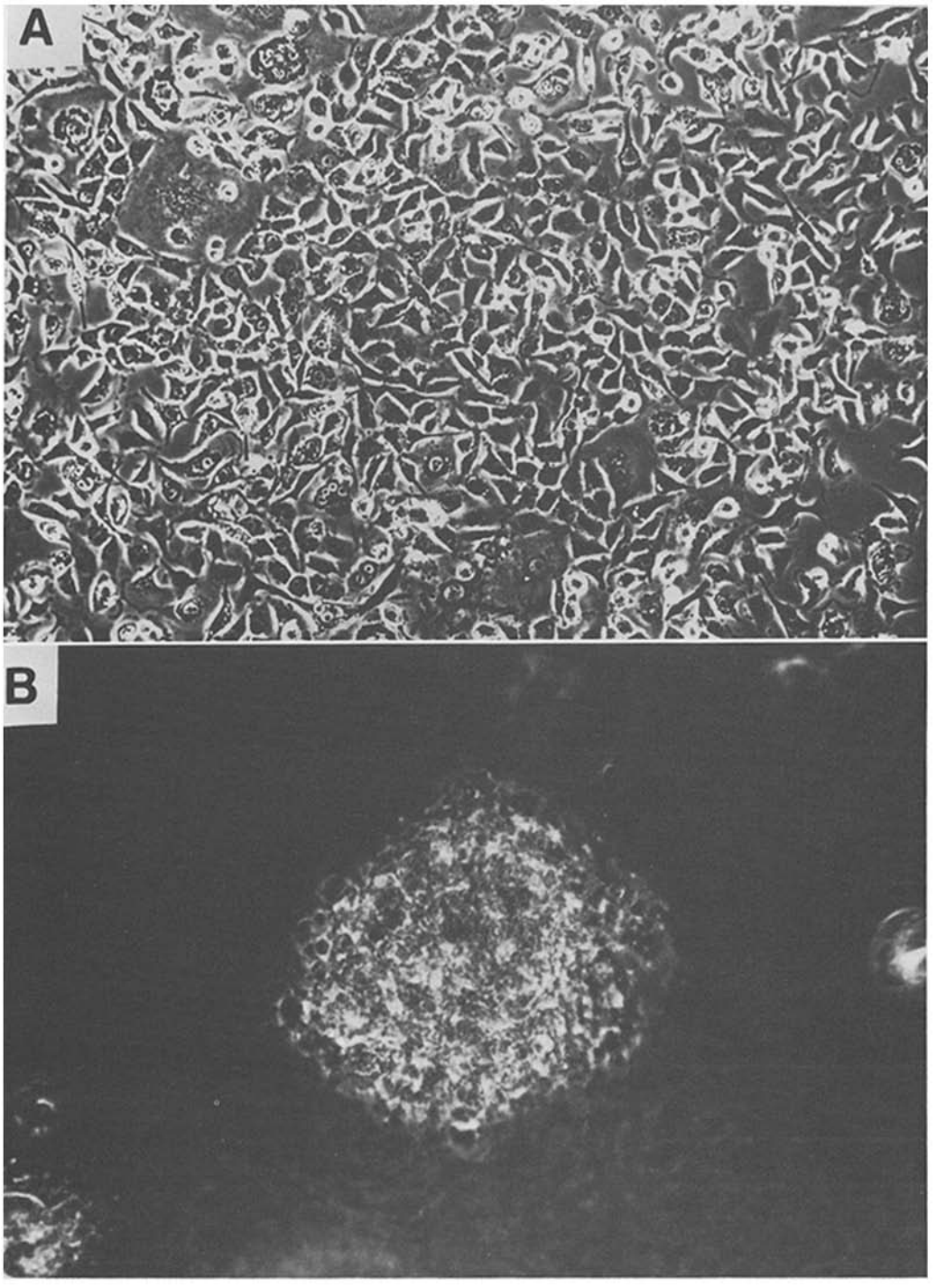

Fig. 1. A Human breast cancer cells (MDA-MB-231) in a confluent culture (100×). **B** A typical human breast cancer colony growing in soft agar on day 10 (400×)

suspensions for subcultures or clonogenic assay. The number of cells per milliliter was counted using a hemacytometer and cell viability was determined by trypan blue dye exclusion (0.1% trypan blue in saline). The viability of the culture cells used in this study was greater than 90%.

Soft Agar Cultures. The culture system for clonic breast cancer cells is a double-layer agar system similar to the reported initially by Hamburger and Salmon [11], with the exception that conditioned medium was not added. All cultures were performed in 35-mm petri dishes (Miles Laboratories, Naperville, ILL., USA). The underlayer consisted of 1.0 ml McCoy's 5-A medium (Gibco Laboratories), 10% fetal calf serum (Gibco Laboratories), other nutrients [i.e., 5% horse serum, 1% sodium pyruvate (2.2%), 1% L-glutamine (200 m*M*), 0.2% L-serine (21 mg/ml), and 1% penicillin-streptomycin solution (10,000 U/ml)] [11], and 0.5% agar (Difco Laboratories, Detroit, Mich., USA). The upper layer, to which the tumor cells were added, consisted of 1.0 ml CMRL-1066 medium with 15% horse serum (Gibco Laboratories), other nutrients

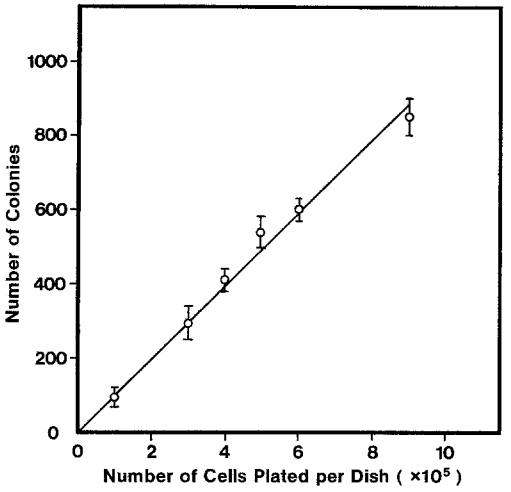

Fig. 2. Linear relationship between colony formation and the number of nucleated breast cancer cells plated. *Points* represent the means of three dishes; the *bars* represent SE. These are the results of a typical experiment

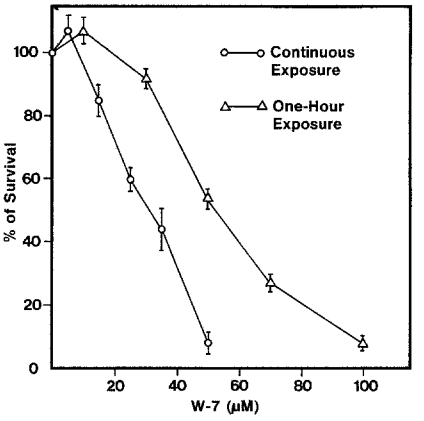

Fig. 4. Effect of W-7 on survival of breast tumor colony-forming cells following 1-h exposure (−△−) and continuous exposure (−○−) to the drug. Results are expressed as the mean (± SE) survivals as percentages of the values in untreated controls

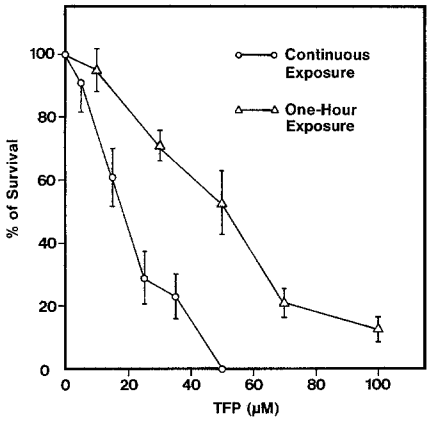

Fig. 3. Effect of trifluoperazine (TFP) on survival of breast tumor colony-forming cells following 1-h exposure (−△−) and continuous exposure (−○−) to the drug. Results are expressed as the mean (± SE) survivals as percentages of the values in untreated controls

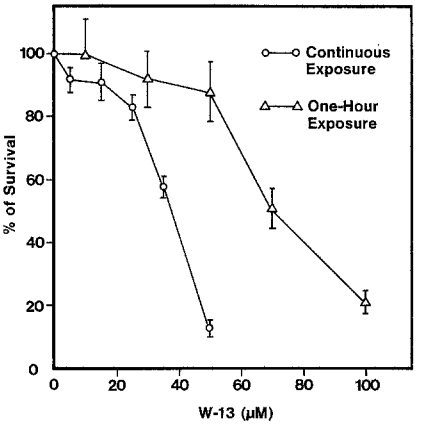

Fig. 5. Effect of W-13 on survival of breast tumor colony-forming cells following 1-h exposure (−△−) and continuous exposure (−○−) to the drug. Results are expressed as the mean (± SE) survivals as percentages of the values in untreated controls

[i.e., 2% insulin (100 U/ml), 2% L-glutamine (200 m*M*), 1% vitamin C (30 m*M*), and 1% penicillin-streptomycin solution (10,000 U/ml)] [11], and 0.3% agar. Cultures were incubated at 37° C in humidified 5% CO_2/air mixture.

Drug Sensitivity Studies

a) One-Hour Exposure. From 3 to 8 $\times 10^5$ breast cancer cells in 1.5 ml McCoy's 5-A medium (plus 10% fetal calf serum) were incubated for 1 h at 37° C in the presence of varying concentrations of drugs. The cells were then washed twice with drug-free medium and the cell concentration adjusted to 1−3 $\times$ 10^6 cells/ml. To 0.5 ml of the cells were added 2.2 ml of enriched CMRL-1066 and 0.3 ml of molten 3% agar. After mixing, the cells were plated in triplicate and incubated as described above.

b) Continuous Exposure. The cell concentrations were adjusted to 1−3 $\times$ 10^6 cells/ml in McCoy's 5-A medium with 10% fetal calf serum. To 0.5 ml of the cell suspension was added 1.9 ml of enriched CMRL-1066, 0.3 ml of the drugs at various concentrations, and 0.3 ml of 3% agar. The cells were plated and incubated as outlined above. For continuous exposure to a drug, the final drug concentration in the upper layer (1 ml) after equilibration with the bottom feeder layer (1 ml) was estimated to be one-half of that originally introduced in the upper layer; this equilibrated concentration of drug was, therefore, used in the *Results* section.

Examination of Cultures. Cultures were examined using an inverted microscope (Nikon Diaphot, Nikon Canada Inc., Mississauga, Ontario). Aggregates of 30 cells or more (with cell size of 70−150 μm) were counted as colonies. Colony counting was performed after 7−14 days of incubation. For drug-treated plates, the results are expressed as the mean percent survival (± SE) of colonies compared with that of untreated controls.

Results

Growth of Breast Cancer Cells

a) In Liquid Medium. The cell line (MDA-MB-231) has been established as a pure epithelial cell line [3, 4]. This line grows rapidly (i.e., doubling time is about 16 h) and, at a dilution of 1 : 5 or higher has to be transferred every 5–8 days. The cells remain granular and either form a loose network of round or short, spindle-shaped cells or pile up as heavy clumps of round cells (Fig. 1A). This cell line has a near-triploid chromosome number (ranging from 65 to 69) and contains several characteristic markers [4]. These findings have been confirmed by Prof. H. C. Wang (Department of Anatomy, College of Medicine, University of Saskatchewan) since this cell line was established in our laboratory.

b) In Soft Agar. When cells are plated in soft agar some of the cells proliferate as colonies. Some 7–14 days after plating, colonies that arise from stem cells can be enumerated. A photomicrograph of a typical breast carcinoma colony at 10 days is shown in Fig. 1B. The colony appears as a group of cells that aggregate, particularly near the center, and with time (e.g., after 10–12 days) this central core may become darkened, possibly due to the development of necrosis. Preliminary experiments indicate a linear relationship between number of cells plated (from 1 to 9 × 10^5 cells/plate) and colony formation (Fig. 2). The plating efficiency was calculated by dividing the number of colonies formed by the number of nucleated cells plated. From the data in Fig. 2 this value is calculated to be about 0.1%.

Drug Sensitivity Testing

The effects of anticalmodulin agents (e.g., TFP, W-7, and W-13) on colony formation of breast cancer cells are shown in representative dose-response curves illustrated in Figs. 3–5. The IC_{50} values (i.e., concentration that causes 50% inhibition of colony formation of untreated controls) of TFP, W-7, and W-13 were calculated to be 18, 30, and 38 μM, respectively, with continuous drug exposure; whereas the corresponding values were 50, 53, and 70 μM, respectively with 1-h exposure. Although all drugs had the ability to inhibit colony formation, it appears that continuous exposure is more effective than 1-h exposure for the three agents studied. The cell-inhibitory potency of these drugs (based on IC_{50} values) was as follows: TFP > W-7 > W-13 in both continuous exposure and 1-h exposure experiments.

Discussion

The findings that anticalmodulin agents such as TFP, W-7, and W-13 have the ability to inhibit colony formation from human breast cancer cells suggests that these agents might be useful in cancer chemotherapy in vivo. The concentrations of these agents (IC_{50}) required to inhibit colony formation in continuous exposure are similar to those values reported in other in vitro systems, where these agents are assumed to interact with calmodulin [6, 13, 21]. Therefore, it is likely that the inhibitory effect of these agents on the colony formation is through the anticalmodulin mechanism.

Accumulating evidence suggests that calcium and calmodulin play an important role in regulating cell proliferation, being essential for the early DNA synthesis (i.e., late G_1 or early S phase) of the cell cycle [14, 17]. Although cell proliferation is a complex phenomenon, presumably involving interactions between several endogenous components in addition to calcium and calmodulin (e.g., cyclic nucleotides, peptide and steroid hormones, peptide growth factors, polyamines, prostaglandins, etc.), it is becoming evident that calcium and calmodulin likely play a pivotal role in coordinating these interactions.

Previous studies have revealed that trifluoperazine or chlorpromazine inhibits DNA synthesis in bone marrow granulocytes [19] or in T51B rat liver cells [2], which results in prolonged generation time and delayed cell division. It is also known that chlorpromazine and other phenothiazines have the ability to inhibit the growth of mouse B-16 melanoma [25] and mouse sarcoma 37 [1], but not of Ehrlich ascites carcinoma [7], or mouse mammary adenocarcinoma [8]. The mechanism of action of these phenothiazine derivatives on these susceptible tumors has not yet been ascertained. However, the present studies suggest that the ability of phenothiazine derivatives to inhibit tumor growth likely depends not only upon the dose but also upon the levels of calmodulin (and calcium) in the tumor cells.

Whether the antitumor concentrations of anticalmodulin agents that are effective in vitro will be achievable in vivo in man remains to be determined. Trifluoperazine, a phenothiazine derivative used clinically in treating psychiatric disorders such as schizophrenia, is usually given in doses of 20–60 mg/day PO. Unfortunately, there is at present no known published information on the pharmacokinetics of trifluoperazine in man. The naphthalene sulfonamides (W-7 and W-13) were recently developed by Hidaka and co-workers and have been tested for their anticalmodulin effects in various biological systems [6, 13, 14]. As with trifluoperazine, there are no known published pharmacokinetic data in man with either W-7 or W-13. Whether these agents have antitumor effects without causing severe adverse effects must still be investigated.

Our results show that the colony growth suppression observed with 1-h exposure was less effective than with continuous incubation of these agents. This may be due to the cell cycle-dependent growth-inhibitory nature of these drugs, although other factors such as drug permeability through cell membranes or intracellular metabolism may also contribute to the enhanced effect with prolonged exposure; alternatively, this effect may be simply due to an increased C × T with continuous exposure. Since most neoplastic cells have generally more rapid growth rates than normal cells [20], anticalmodulin agents may selectively inhibit tumor growth with minimal adverse effects on normal cells.

The plating efficiency of this cell line is rather low (0.1%). This may be at least partly due to the method used to obtain a single-cell suspension, namely, passing the cells through 25-gauge needles several times.

Finally, these studies indicate that the soft agar clonogenic assay shows promise as a system for testing the potential efficacy of cell growth regulators (or their inhibitors) as anticancer agents.

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