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# Inhibition of the $G_1$/S transition in A65 cells by H-7, a protein kinase C inhibitor

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**Abstract**—The effects of protein kinase inhibitors on the proliferation of A65 murine leukemia cells were studied. The proliferation of phorbol ester-dependent A65 cells was inhibited by *N*-(2-methylpiperazyl)-5-isoquinolinesulfonamide (H-7), a protein kinase C inhibitor, at a significantly lower concentration than the phorbol ester-independent variant, while both cell types had the same sensitivity to *N*-[2-[*N*-[3-(4-chlorophenyl)-1-methyl-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide, a selective inhibitor of protein kinase A, and staurosporine, a non-selective inhibitor of protein kinases. When the effect of H-7 on the cell cycle was analysed by flow-cytometry, the agent at concentrations that completely inhibited the cell proliferation significantly increased the proportion in the $G_0/G_1$ phase of both cell types but decreased that in the S phase, without much change in the $G_2$/M phase. These results suggest that H-7 blocks the $G_1$/S transition by inhibiting protein kinase C, whether the proliferation is dependent on phorbol ester or not.

The A65T cell line was isolated from symbiotic AKRL-65 mouse leukemia cells by Kaneshima *et al.* [1]. Cell growth is dependent on close contact with thymic epithelial reticular cells, but tumor promoters such as 12-*O*-tetradecanylphorbol-13-acetate (TPA*) can induce growth *in vitro* without thymic epithelial cells being present [2]. The TPA-independent revertant A65IND cell line was obtained from A65T cells. Tumor promoters can act like diacylglycerol and activate protein kinase C. However, it is not known if protein kinase C is involved in the proliferation of A65 cells.

In this study, we examined the effects of inhibitors of protein kinase on the proliferation of leukemia cells and suggest that protein kinase C is involved in the transition from the $G_1$ to the S phase of the cell cycle, whether the proliferation is dependent on TPA or not.

* Abbreviations: TPA, 12-*O*-tetradecanylphorbol-13-acetate; H-7, *N*-(2-methylpiperazyl)-5-isoquinolinesulfonamide; H-87, *N*-[2-[*N*-[3-(4-chlorophenyl)-1-methyl-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide.

## *Materials and Methods*

*Cells.* A65T and A65IND cells were kindly provided by H. Hiai and Y. Nishizuka of the Aichi Cancer Center Research Institute (Nagoya, Japan). The medium was a high glucose version of Dulbecco's modified Eagle's minimum essential medium (Gibco, Grand Island, NY, U.S.A.) with added pyruvate, vitamins, amino acids and 10% unheated fetal calf serum [2]. A65T cells were cultured in the same medium containing 10 ng/mL TPA. A65IND cells were cultured in the absence of TPA, unless stated otherwise.

*Flow cytometry.* The DNA content of the cells was measured by flow cytometry. Cells treated with a protein kinase inhibitor for designated periods were stained with propidium iodide by the method of Taylor [3]. The samples were analysed with an EPICS 753 flow cytometer (Coulter Electronics Inc., Hialeah, FL, U.S.A.). The cell cycle pattern was analysed by the MDADS II program (Coulter).

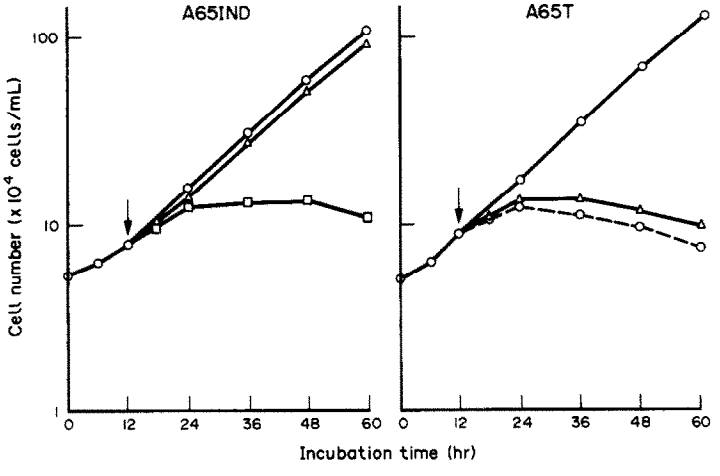


Fig. 1. Growth curves of A65IND and A65T cells and the effect of H-7. A65IND cells were cultured with 10 μM H-7 (△), with 50 μM H-7 (□) or without (○), in the absence of TPA. A65T cells were cultured with 10 μM H-7 (△) or without (○) in the presence (solid line) or absence (dotted line) of 10 ng/mL TPA. Arrows indicate the time of addition of H-7 or removal of TPA.

Table 1. Inhibition of growth of A65IND and A65T cells

| Agent | $IC_{50}$ ($\mu$M) A65IND −TPA | A65IND +TPA | A65T +TPA |
|---|---|---|---|
| H-7 | 30.4 ± 1.1 | 24.4 ± 3.4 | 4.0 ± 0.2* |
| H-87 | 3.5 ± 0.5 | 3.0 ± 0.3 | 2.8 ± 0.3 |
| Staurosporine | 0.0016 ± 0.0003 | — | 0.0014 ± 0.0005 |
| Vinblastine | 0.00041 ± 0.00006 | — | 0.00046 ± 0.00010 |

Cells were cultured with varying concentrations of inhibitor in the absence or presence of 10 ng/mL TPA for 48 hr, and the 50% growth-inhibitory concentration ($IC_{50}$) of the agent was calculated.

Data are the means ± SEM of three experiments performed in triplicate.

* Significantly different from A65IND, $P < 0.01$.

Table 2. Effects of H-7 on the proportion of A65 cells at different stages of the cell cycle

| Cell line | Period of treatment with H-7 (hr) | Percentage of cells $G_0/G_1$ | S | $G_2/M$ |
|---|---|---|---|---|
| A65IND | 0 | 41.3 ± 1.9 | 32.0 ± 1.0 | 26.3 ± 0.9 |
| | 12 | 55.7 ± 5.0* | 15.7 ± 3.0* | 28.7 ± 3.2 |
| A65T | 0 | 42.3 ± 2.4 | 43.0 ± 3.0 | 14.7 ± 0.7 |
| | 12 | 58.0 ± 4.7* | 26.7 ± 3.7* | 15.7 ± 0.9 |

A65IND and A65T cells were cultured with 50 and 10 $\mu$M H-7, respectively, for 12 hr. The proportions in the different stages were analysed as described in Materials and Methods.

Data are the means ± SEM of three experiments.

* Significantly different from the 0 hr control, $P < 0.05$.

*Chemicals.* Vinblastine (Shionogi and Co., Osaka, Japan), TPA, ribonuclease A (Sigma Chemical Co., St Louis, MO, U.S.A.), staurosporine (Kyowa Hakko Kogyo Co., Tokyo, Japan) and propidium iodide (Calbiochem, La Jolla, CA, U.S.A.) were purchased. *N*-(2-Methylpiperazyl)-5-isoquinolinesulfonamide (H-7) and *N*-[2-[*N*-[3-(4-chlorophenyl)-1-methyl-2-propenyl]amine]ethyl]-5-isoquinolinesulfonamide (H-87) were synthesized in our laboratory [4, 5].

*Results and Discussion*

A65T cells in the presence of 10 ng/mL TPA and A65IND cells in the medium alone, proliferated exponentially with a doubling time of 11.7 and 12.5 hr, respectively (Fig. 1). Previously, Tanaka *et al.* [6] reported that A65T cells have about one-third of the activity of protein kinase C as A65IND cells even when cultured with TPA, and that both cell types contained only the type III isozyme. However, it is not known whether the proliferation of TPA-dependent A65T cells is maintained by down-regulation or by stimulation of protein kinase C by TPA. We therefore examined the effect of inhibitors of protein kinases on the proliferation of A65 variants. Table 1 summarizes the 50% growth inhibition concentrations of H-7, an inhibitor of protein kinase C [4], H-87, a selective inhibitor of protein kinase A [5], staurosporine, a non-selective protein kinase inhibitor [7, 8] and vinblastine, a mitotic inhibitor antitumor drug, for both cell lines. These data indicate that the TPA-dependent cells were much more sensitive to H-7 than the independent cells regardless of whether they were cultured with or without TPA. Both cells had the same sensitivities to H-87, staurosporine and vinblastine. In this experiment, cell viability determined by a Trypan blue dye exclusion method was decreased more by higher concentrations of H-87, staurosporine and vinblastine, but not by H-7. Figure 1 also shows that 10 $\mu$M H-7 stopped the proliferation of A65T cells and that 50 $\mu$M was needed for complete inhibition of A65IND cells. Removal of TPA from the culture of A65T cells decreased not only cell proliferation but viability, as reported previously [2]. These results suggest that H-7 affects the progress of the cell cycle and slows or suppresses cell proliferation. Cell cycle analysis was done in asynchronous cells. When A65T and A65IND cells were cultured with 10 or 50 $\mu$M H-7, respectively, for 12 hr, the proportion of cells in the $G_0/G_1$ phase increased significantly but the proportion of cells in the S phase, especially the early S phase, was decreased without much change in the proportion of cells in the $G_2/M$ phase (Table 2 and Fig. 2). H-87 and staurosporine had no effect on the cycle phases, except for increasing cell death and debris (data not shown).

Abe *et al.* [9] reported that low concentrations of staurosporine blocked the early $G_1$ phase and that high concentrations blocked the $G_2$ phase in rat 3Y1 fibroblasts. They speculated that the block of the $G_2$ phase caused by staurosporine may result from inhibition of $p34^{cdc2}$ kinase but did not speculate on the $G_1$ block. In A65 cells, staurosporine was only cytotoxic; it had no strong effect on the cell cycle. This may be due to multiple and

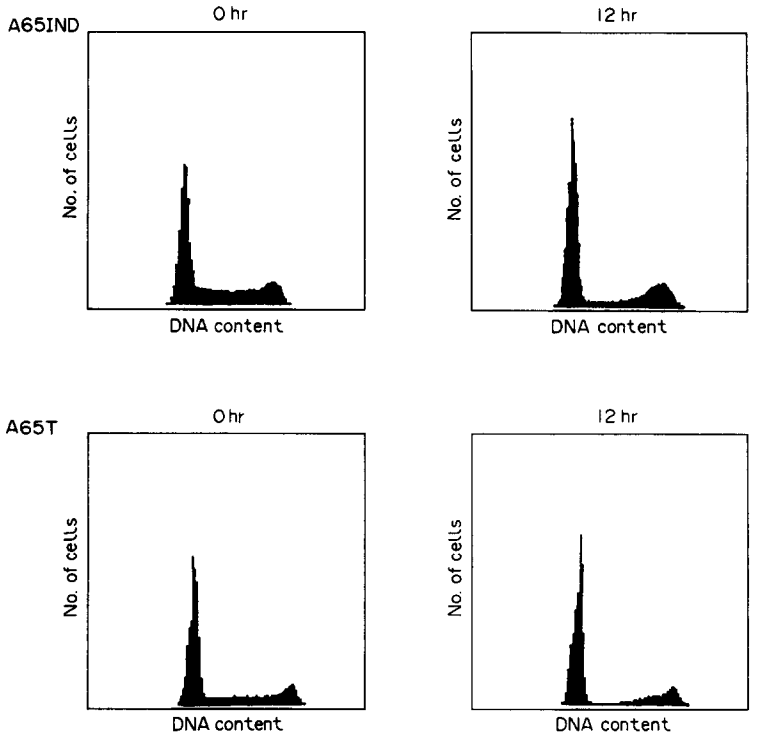


Fig. 2. Typical DNA histogram of asynchronous A65IND and A65T cells before and after culture with 50 and 10 μM H-7, respectively, for 12 hr.

simultaneous inhibition of different cell functions by the potent and unselective protein kinase inhibitor. On the other hand, the stimulation of protein kinase C increases the expression of the proto-oncogenes c-*fos* and c-*myc*, which are closely associated with progress through the $G_1$ phase [10, 11]. This may mean that the $G_1$ block caused by H-7 in A65 cells seen in this study and by staurosporine in rat 3Y1 fibroblasts [9] results from inhibition of protein kinase C.

Our findings indicate that H-7 inhibits proliferation by the blocking of movement from the $G_0/G_1$ phase to the S phase in A65T and A65IND cells whether TPA-dependent or not, and suggest that type III protein kinase C has a role in the $G_1$/S transition in leukemic cells.

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