Effect of Thiazinotrienomycin B, an Ansamycin Antibiotic, on the Function of Epidermal Growth Factor Receptor in Human Stomach Tumor Cells

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Thiazinotrienomycin B (TT-B), an ansamycin isolated from fermentation broths of *Streptomyces* sp. MJ672-m3, inhibited the growth *in vitro* of human stomach tumor SC-6 cells over 10 times more strongly than the growth of other human tumor cells, such as HeLa (cervix), T24 (bladder) and LX-1(lung). The extent of growth inhibition by TT-B of SC-6, but not of LX-1 nor T24, was lowered in a competitive manner by raising serum concentrations in the culture medium. TT-B inhibited the cell cycle progression of SC-6 at an early stage of the progression from G0/G1 to S. The inhibition was again competitive with serum concentrations in the culture medium. No direct inhibition of DNA synthesis was observed at the concentration range which caused the cell cycle arrest. TT-B and anti-epidermal growth factor receptor (anti-EGFR) were antagonistic to each other in inhibiting the cell cycle progression of SC-6 from G0/G1 to S, suggesting that the two compounds share the same target, EGFR. The kinase activity of EGFR was little inhibited by TT-B in a cell-free system.

Many human tumors express high levels of growth factors and growth factor receptors[1~3]. Blocking the function of growth factor receptors seems to be useful for the treatment of cancer. In cancers of stomach and some other origins, gene amplification and over-expression of epidermal growth factor receptor (EGFR) have been reported[4,5]. Thiazinotrienomycin B (TT-B)[6] inhibited the growth of human stomach tumor SC-6 cells over 10 times more strongly than the growth of human tumor cells derived from other organs, suggesting that TT-B interfered with the signal transduction issued from EGFR. The present studies on the mode of action of TT-B support this hypothesis.

Materials and Methods

Cell Lines

Human bladder carcinoma T24 having a G12V mutation in H-ras gene and human epidermoid carcinoma A431 were provided by the Japanese Cancer Research Resources Bank, Tokyo. Human cervical carcinoma HeLa was purchased from Dainippon Seiyaku Co., Osaka. Human stomach carcinoma SC-6 which showed LOH in p53 gene (Hiratsuka, personal communication), human stomach carcinoma MKN28 and human lung carcinoma LX-1 were provided by the Institute for Chemotherapy, Shizuoka.

Cell Culture in Liquid Medium

The culture media used for the cell lines were as follows: Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS) for T24 and HeLa; RPMI 1640 medium supplemented with 10% fetal calf serum (RPMI·FCS) for SC-6, MKN28 and LX-1; and Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (DMEM·FCS) for A431. Cells were grown in these media in 5% CO_2-containing humidified air at 37°C. To assay the growth inhibition by drugs, the cells were seeded at $1\sim4\times10^4$ cells/1 ml medium/2 cm^2 well of Coster 24-well tissue culture clusters (day 0). Drugs were added to the wells on day 1, and incubation was continued until day 3. Cell growth was quantified by cell counting or by a colorimetric MTT assay as reported previously[7].

Progression of Cell Cycle from G0/G1 to S

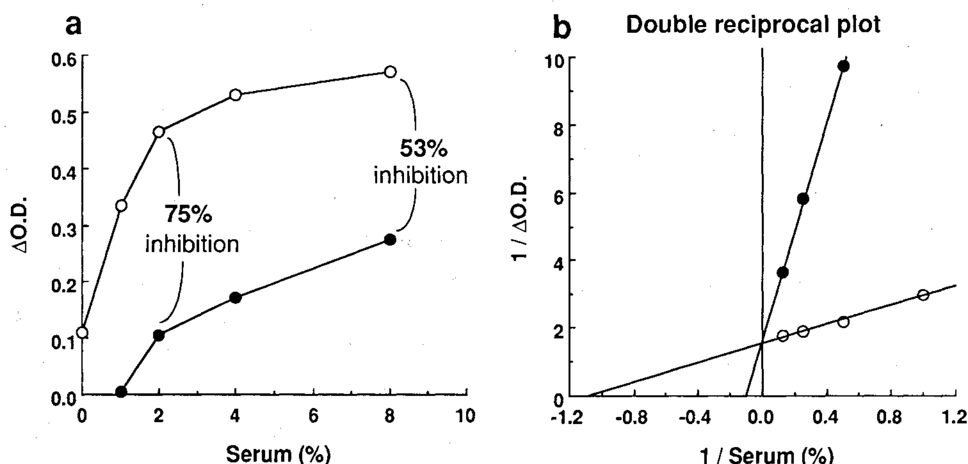
SC-6 cells were seeded at 2×10^4 cells/1 ml RPMI·FCS/2 cm^2 well of Coster 24-well tissue culture clusters and were incubated for 20 hours. After the medium was removed, the cells were washed with 1 ml of FCS-free RPMI and were incubated in FCS-free RPMI further for 72 hours. For progression of the cell cycle from G0/G1 to S, FCS (final conc.: 5%) was added to the incubation medium and cells were further incubated. After 20 hours DNA synthesis was measured by incorporation of [^{3}H]-thymidine into the acid-insoluble fraction of the cells at 37°C for 30 minutes as reported previously[8].

Activation of EGFR in A431 Cells

A431 cells were seeded at 2×10^5 cells/2 ml DMEM·FCS/8 cm^2 dish and incubated for 20 hours. The medium was changed for 1 ml of DMEM containing 2% FCS and a desired amount of TT-B. After 20 minutes, 200 ng/ml EGF was added to the medium and the cells were incubated for one hour at 37°C. After the medium was removed, the cells were washed with 1 ml of PBS three times and lysed in 100 μl of 2×SDS Sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 2% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, TEFCO, Japan). The lysate was heated at 95°C for 10 minutes and centrifuged for 30 seconds at 15,000 rpm. The supernatants were analyzed by SDS-polyacrylamide gel electrophoresis using 6% gels for 90 minutes at 20 mA in 25 mM Tris·192 mM glycine·0.1% SDS. Proteins were transferred to polyvinylidene difluoride membrane for 90 minutes at 100 mA in 25 mM Tris·20 mM glycine·20% methanol. The membrane was blocked overnight at 4°C in 8.1 mM $NaHPO_4\cdot12H_2O$·1.5 mM KH_2PO_4·137 mM NaCl·2.7 mM KCl·0.1% Tween 20·5% nonfat dry milk (Tween·PBS·milk) and incubated with mouse monoclonal anti-EGFR activated form (Transduction Laboratories, USA) in Tween·PBS·milk overnight at 4°C. After washing, the membrane was incubated with anti-mouse Ab for 1 hour at a room temperature. Activated EGFR was detected using VECTASTAIN ABC-AP kit (VECTOR LABORATORIES, INC, USA) with BCIP/NBT.

Fig. 1. Effect of TT-B on growth of SC-6 cells in the medium containing various concentrations of serum.

Cells were incubated for 48 hours in the presence (●) or absence (○) of 2 ng/ml TT-B in the medium containing various concentrations of serum.



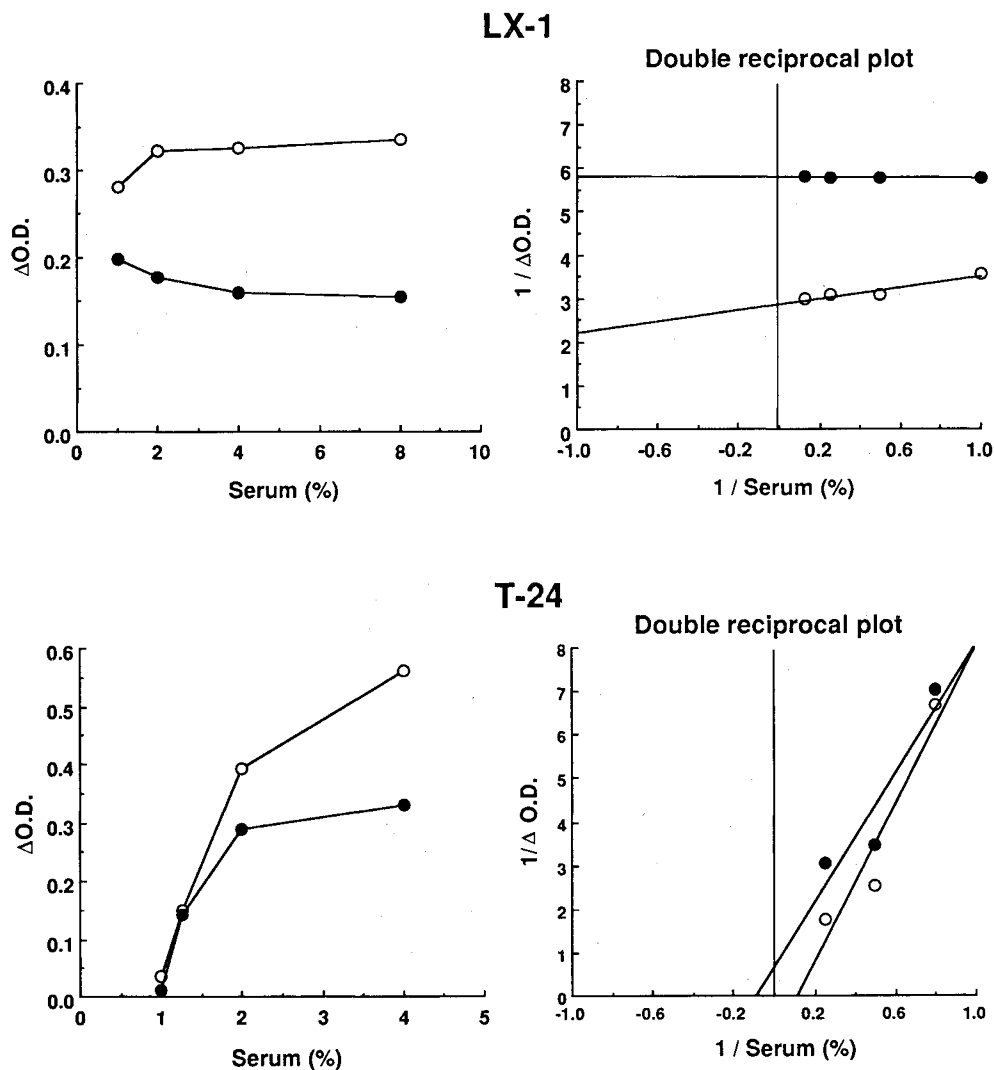
Results and Discussion

Cell Growth Inhibition in Culture Media Containing Vaious Concentrations of Serum

When cells were incubated in a medium containing 10% serum, TT-B inhibited the growth of human stomach carcinoma SC-6 cells over 10 times more strongly than it inhibited the growth of human carcinoma cells of HeLa (cervix), T24 (bladder) and LX-1(lung); IC_{50}'s were 1.6 ng/ml (SC-6), 16 ng/ml (HeLa), 17 ng/ml (T24) and 33 ng/ml (LX-1). The basic idea of our screening for antitumor compounds is to find candidates that have unique mechanisms of action. Since many known antitumor compounds are inhibitors of DNA synthesis, we first tested if TT-B had this effect as its major activity. A preliminary experiment showed that TT-B did not inhibit the incorporation of [^{3}H]thymidine into the acid-insoluble fraction of SC-6 cells at concentrations that inhibited the cell growth (data not shown), we therefore thought that the high sensitivity of SC-6 cells to TT-B should be due to a TT-B-sensitive process, somewhat specific to the cells. Since signal transduction pathways for cell growth vary from one cell type to another, we focused our attention to test the inhibition of signal transduction. Inhibitors of the growth factor-dependent signal transduction pathways inhibit the cell growth more strongly in a serum-free medium than in a serum-containing medium[9]. We therefore determined the extent of cell growth inhibition by TT-B

Fig. 2. Effect of TT-B on growth of LX-1 and T24 cells in the medium containing various concentrations of serum.

LX-1 and T24 cells were incubated for 48 hours in the presence (●) or absence (○) of 30 ng/ml TT-B and 18 ng/ml TT-B, respectively.



in media containing various concentrations of serum and found that the extent of growth inhibition by TT-B of SC-6 cells was lowered by increasing the serum concentrations in the medium (Fig.1a). The results were analyzed by the double reciprocal plot in which the reciprocal of growth velocity (Y-axis) was plotted *versus* the reciprocal concentrations of serum (X-axis). The extraporated lines of the absence and the presence of TT-B met on the Y axis (Fig. 1b), suggesting that TT-B competed with growth factors in the serum for the signal transduction pathways. Another human stomach carcinoma cell line (MKN-28) also showed a similar competitive relationship between growth inhibition by TT-B and serum concentrations in the culture medium (data not shown). No such competitive relationship was observed with LX-1 (lung) and T24 (bladder); growth inhibition of these cells by TT-B became rather stronger when serum concentration was raised (Fig. 2). Most stomach carcinoma cells may share a process needed to grow, which is vulnerable to TT-B.

Inhibition of Cell Cycle Progression in G1 Phase

As some growth factors promote G1 phase cell cycle progression, we investigated the effect of TT-B on the progression of SC-6 promoted by serum. Cells were synchronized at G0/G1 phase by serum starvation for three days and were allowed to progress to S phase by addition of serum. DNA synthesis was measured for the detection of S phase using [^{3}H]thymidine 20 hours after serum addition. TT-B inhibited G1 phase cell cycle progression of SC-6 cells, and here again the inhibition was competitive with serum concentrations (Fig. 3). No such competitive relationship was observed in the cell cycle inhibition by cycloheximide or colchicine (data not shown). We investigated further to determine which period of the progression was inhibited by TT-B. After addition of serum to the serum-starved cells, *i.e.*, the cells synchronized at G0/G1 phase (the time of serum addition is referred to as 0 hour), the cells were exposed to TT-B during the period from 0 to 4, from 4 to 8, from 8 to16 or from 16 to 20 hour. TT-B inhibited cell cycle

Fig. 3. Effect of TT-B on cell cycle progress from G0/G1 to S in SC-6 cells.

Syncronized cells at G0/G1 phase were incubated for 20 hours in RPMI·5% FCS with (●) or without (○) 3 ng/ml TT-B.

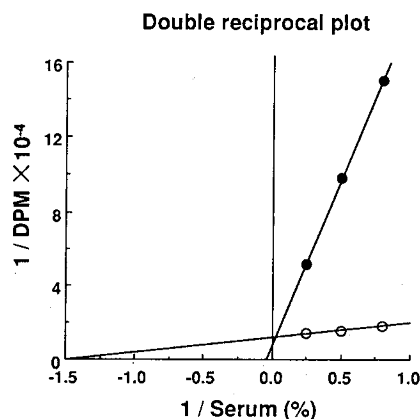
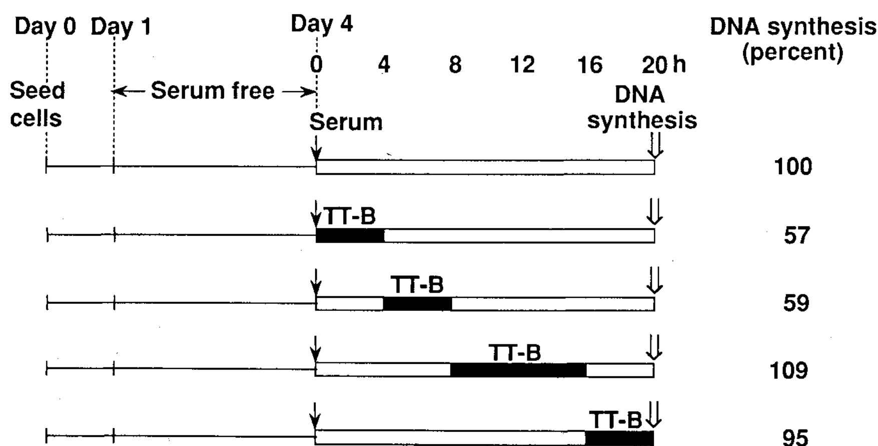


Fig. 4. Effect of TT-B on cell cycle progress from G0/G1 to S in SC-6 cells.



Syncronized cells at G0/G1 phase on Day 4 were exposed to 3 ng/ml TT-B during the periods which are shown by black bars.

progression only when it was present during the first 8 hours in the G1 phase (Fig. 4). In other words, addition of TT-B after 8 hours was too late to stop the cell cycle reaching S phase.

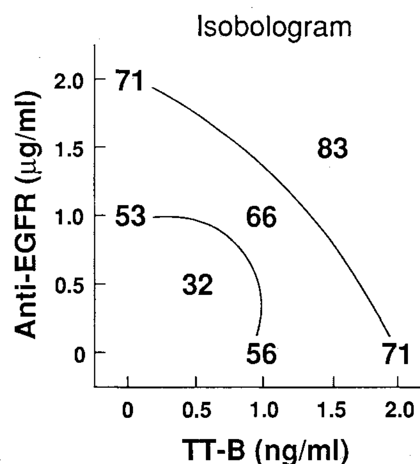
Antagonism between TT-B and Anti-EGFR in Inhibiting Cell Cycle Progress from G0/G1 to S Phase

As TT-B competed with the serum in inhibiting cell cycle progress, we investigated to determine what growth factor(s) in the serum would promote the cell cycle progress. Candidate growth factors for stomach cells were added to the serum-starved SC-6 cells and DNA synthesis was measured after 20 hours described as above. TGF-α and, less strongly, EGF promoted the cell cycle progress but neither did HB-EGF or insulin (data not shown). As TGF-α binds to EGFR, we suspected the target of TT-B was EGFR of SC-6 cells. If it is true, we presumed that TT-B and anti-EGFR antibody should inhibit the cell cycle progress antagonistically by competing for the same target. TT-B and anti-EGFR antibody were added to the serum-starved SC-6 cells and DNA synthesis was measured 20 hours later as above. Antagonism between TT-B and anti-EGFR antibody was obvious as shown in the convex-shaped isobologram (Fig. 5).

Inhibiton of TT-B on Activation of EGFR

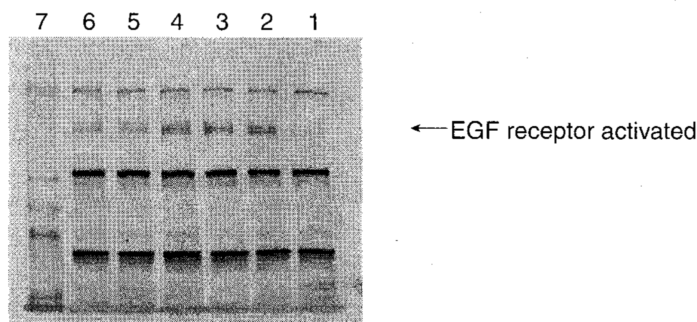
EGFR is overexpressed in human epidermoid carcinoma A431 and is activated (phosphorylated) in response to EGF[10]. We therefore thought that A431 could be a good system to demonstrate the proposed inhibitory effect of TT-B on the receptor. Cells were first exposed TT-B for 20 minutes, then EGF was added, and after one hour the cells were harvested and lysed. Activated (phosphorylated) forms of EGFR was detected in the lysate by western blotting (Fig. 6). TT-B inhibited the activation of EGFR, although higher concentrations of TT-B were needed for the inhibition. We attempted to determine the effect of TT-B on activation of the receptor in SC-6 cells, but the receptor itself was hardly

Fig. 5. Effect of TT-B and anti-EGFR in combination on cell cycle progress from G0/G1 to S in SC-6 cells.



Syncronized cells at G0/G1 phase were incubated with various concentrations of TT-B and anti-EGFR in RPMI·5% FCS. After 20 hours DNA synthesis was measured. Numbers in this figure show relative inhibition of cell cycle progress.

Fig. 6. Effect of TT-B on EGFR activation in A431 cell.



Cells (lanes from 3 to 6) were incubated for 20 minutes with TT-B; 0.01 μg/ml (lane 3), 0.1 μg/ml (lane 4), 1 μg/ml (lane 5) and 10 μg/ml (lane 6), and were further incubated for one hour with (lanes from 2 to 6) or without (lane 1) 200 ng/ml EGF.
Lane 7 shows molecular weight markers.

detectable, possibly because the number of the receptor in SC-6 cells may be far less than that in A431.

As the kinase activity of EGFR was little inhibited by TT-B in a cell-free system at a concentration as high as 10 μg/ml (data not shown), the results presented so far indicate that TT-B binds to EGFR and inhibits its function to mediate the cell growth signal.

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