Pergamon

European Journal of Cancer Vol. 32A, No. 2, pp. 342–345, 1996

Printed in Great Britain
0959–8049/96 $15.00+0.00

0959-8049(95)00536-6

Original Paper

Effect of Tyrphostin Combined with a Substance P Related Antagonist on Small Cell Lung Cancer Cell Growth *In Vitro*

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The protein tyrosine kinase inhibitor [(3,4,5,-trihydroxyphenyl)-methylene]-propanedinitrile (tyrphostin) was originally designed to inhibit polypeptide growth factor receptor signalling, but was also found to inhibit neuropeptide stimulated tyrosine phosphorylation and mitogenesis in Swiss 3T3 cells [*J Biol Chem* 1993, 268, 9548–9554]. Here, we demonstrate that tyrphostin inhibits *in vitro* colony growth of the H-345 and H-69 small cell lung cancer (SCLC) cell lines stimulated by the neuropeptides, bombesin and bradykinin, respectively. This effect was dose-dependent and, at tyrphostin concentrations above 5 μM, both background and neuropeptide stimulated colony formation were reduced. In liquid culture, tyrphostin inhibited the growth of the H-345 and H-69 SCLC cell lines with an IC_{50} of 7 μM. Time course experiments in liquid culture revealed that tyrphostin delayed the rate of entry of both SCLC cell lines into rapid phase growth and reduced the number of cells reaching a plateau phase of growth compared with control cells. Furthermore, tyrphostin concentrations at or above 50 μM reduced the number of cells present over time compared with untreated cells. When combined with a substance P (SP) analogue, which inhibits the action of multiple neuropeptides and SCLC cell growth, both in semisolid media and liquid culture, tyrphostin additively inhibited the growth of the H-345 and H-69 SCLC cell lines in liquid culture.

Key words: SCLC, tyrphostin, substance P
Eur J Cancer, Vol. 32A, No. 2, pp. 342–345, 1996

INTRODUCTION

THE PAUCITY of effective treatments for small cell lung cancer (SCLC) has generated a crucial need to develop new therapies for this disease. An understanding of the growth factors involved in SCLC proliferation and their signalling pathways may be helpful in this respect. There is increasing evidence that multiple neuropeptides, including bombesin and bradykinin, stimulate SCLC proliferation via autocrine/paracrine growth circuits [1–3]. This knowledge has already led to the development of a substance P (SP) analogue [Arg^6, $DTrp^{7,9}$, $MePhe^8$] SP(6–11) (AntG), which inhibits the action of many different neuropeptides and blocks the growth of SCLC cells in liquid culture, semisolid media and as xenografts *in vivo* [4–7]. This broad spectrum neuropeptide antagonist probably acts at the receptor level by inhibiting ligand binding [8]. Investigation of the downstream signalling events from neuropeptide receptors has revealed multiple pathways leading to the induction of the mitogenic response [9, 10]. The development of inhibitors to specific components of these pathways could provide potential new strategies for cancer therapy [11, 12].

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Revised 10 Aug. 1995; accepted 23 Aug. 1995.

The protein tyrosine kinase inhibitor [(3,4,5,-trihydroxyphenyl)-methylene]-propanedinitrile (tyrphostin), blocks bombesin stimulated tyrosine phosphorylation and mitogenesis in Swiss 3T3 cells [13], a useful model system for elucidating signal transduction pathways for both neuropeptide and polypeptide growth factors [10]. Tyrphostin has also been shown to inhibit tyrosine phosphorylation and mitogenesis induced by other neuropeptides in Swiss 3T3 cells [14] as well as a spectrum of polypeptide growth factors [15]. Interestingly, we have recently shown that the polypeptide growth factors, stem cell growth factor and hepatocyte growth factor, stimulate colony formation in several SCLC cell lines [16]. It is therefore conceivable that tyrphostin may not only inhibit the growth promoting activity of neuropeptides such as bombesin, but also polypeptides including stem cell and hepatocyte growth factors in SCLC cell lines. Here we show that SCLC growth is inhibited by tyrphostin in a concentration-dependent manner. Furthermore, combining tyrphostin with the SP analogue broad spectrum neuropeptide antagonist AntG produced additive growth inhibitory effects.

MATERIALS AND METHODS

Cell culture

SCLC cell lines H-69 and H-345 were generously donated by Dr A. Gazdar (Bethesda, U.S.A.) and purchased from the

American Type Culture Collection. Stocks were maintained in RPMI 1640 supplemented with 10% (v:v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 10% CO_2/90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA which consists of RPMI 1640 supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM oestradiol, 30 nM selenium and 0.25% bovine serum albumin.

Colony assay

SCLC cells, 3–5 days after passage, were washed and resuspended in HITESA. Cells were then disaggregated into an essentially single cell suspension by two passes through a 19 g needle and then through a 20 μm Nylon gauze. Cell number was determined using a Coulter Counter, and 10^4 viable cells were mixed with HITESA containing 0.3% agarose and agonist at the concentrations indicated, and layered over a solid base of 0.5% agarose in HITESA with agonist at the same concentration in 33 mm plastic dishes. The cultures were incubated in humidified 10% CO_2/90% air at 37°C for 21 days and then stained with the vital stain nitroblue tetrazolium. Colonies of > 120 μm diameter (16 cells) were counted using a microscope.

Liquid culture assay

SCLC cells, 3–5 days after passage, were washed and resuspended in HITESA. Cells were then aliquoted in 24-well Falcon plates at a density of 5×10^4 cells in 1 ml HITESA in the presence or absence of antagonists/inhibitors. After 8–10 days, when control cells were in log phase growth, or at various times, cell number was determined from a minimum of 3 wells per condition using a Coulter Counter, after cell clumps were disaggregated by passing the cell suspension five times through a 19 and subsequently 21 gauge needle.

Materials

Tyrphostin 25 was purchased from Calbiochem Corporation (La Jolla, California, U.S.A.). AntG was from Bachem Fine Chemicals. Bombesin and bradykinin were obtained from Sigma Chemical Co (St. Louis, Missouri, U.S.A.). Agarose was from SeaKem (Rockland, Maine, U.S.A.). All other reagents were of the highest grade available.

RESULTS

Tyrphostin inhibits the growth of SCLC cells in vitro

It has previously been shown that bombesin induces colony formation in the H-345 SCLC cell line above basal levels in a dose dependent manner with a maximum effect at 10 nM [1, 5]. Figure 1a shows that 5 μM tyrphostin completely suppressed colony formation in response to 30 nM bombesin. The inhibition was selective as 5 μM tyrphostin had no effect on the number of background colonies formed in the absence of added neuropeptide. This result is in agreement with previous data demonstrating that similar concentrations of tyrphostin can inhibit bombesin stimulated DNA synthesis in Swiss 3T3 cells [13]. Raising the tyrphostin concentration above 5 μM resulted in an increasing inhibition of both background and bombesin stimulated colony formation. To ensure this effect was not confined to one SCLC cell line and one neuropeptide, we tested whether tyrphostin could inhibit the bradykinin stimulated colony growth of the H-69 SCLC cell line [5, 17]. As shown in Figure 1b, tyrphostin inhibited both basal and bradykinin stimulated colony formation in the H-69 SCLC cell line similarly to that seen with bombesin in the H-345 SCLC cell line. These data demonstrate that tyrphostin can inhibit neuropeptide stimulated growth in semisolid medium of the H-345 and H-69 SCLC cell lines.

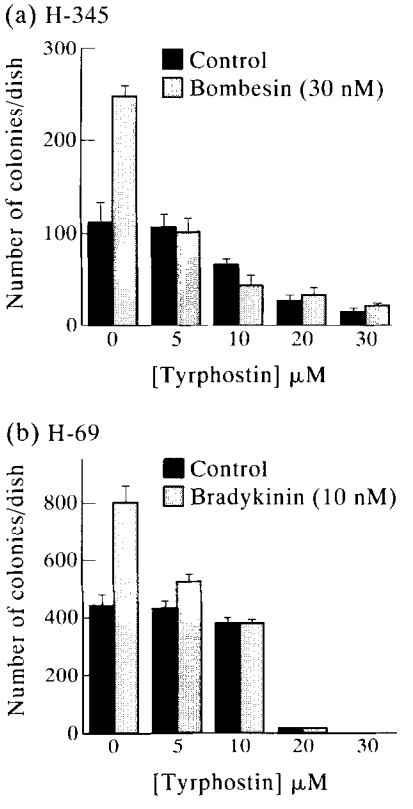

Figure 1. Effect of tyrphostin on neuropeptide stimulated colony formation in (a) H-345 and (b) H-69 SCLC cell lines. Each point represents the mean number of colonies formed on five separate dishes ± S.E. In all cases, a representative of at least two independent experiments is shown. Where no error bar is visible, it lies within the symbol.

Figure 2 demonstrates that tyrphostin also inhibits growth of the H-345 and H-69 SCLC lines in serum-free (HITESA) liquid medium dose dependently with an IC_{50} of 7 μM. Thus, tyrphostin inhibits growth of the H-345 and H-69 SCLC cell lines to a similar degree in liquid culture and colony assays.

Cells grown in HITESA in the absence of inhibitors have previously been shown to grow slowly for the first 2–3 days (lag period), before entering an exponential increasing rate of growth (log phase), and then stop at 12–15 days (plateau period), presumably when the medium has been depleted of essential constituents to support growth [4]. Figure 3 demonstrates that increasing concentrations of tyrphostin, up to 20 μM, progressively prolongs the lag period and reduces the total number of cells which reach the plateau phase in both SCLC cell lines. However, at these concentrations, there is little effect of tyrphostin on the slope of the log phase indicating that those cells entering log phase growth proliferate as rapidly as the control cells. Interestingly, this pattern of growth curve inhibition by tyrphostin (i.e. a prolonged lag period with a reduced number of cells reaching a plateau but with a similar log phase growth rate compared with control cells) has previously been demonstrated with SP analogue

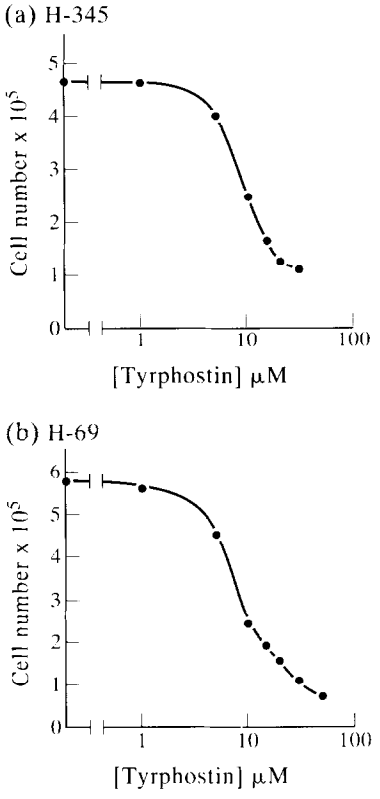

Figure 2. Effect of tyrphostin on liquid culture growth of (a) H-345 and (b) H-69 SCLC cell lines. Each point represents the mean of three determinations. In all cases, a representative of at least three independent experiments is shown. The S.E. bars lie within the symbols.

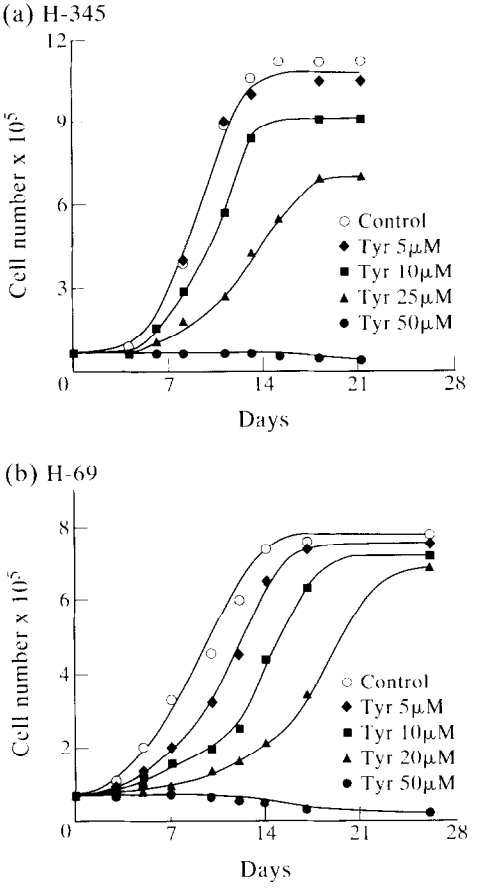

Figure 3. Time course of tyrphostin effect on liquid culture growth of (a) H-345 and (b) H-69 SCLC cell lines. Each point represents the mean of three determinations. In all cases, a representative of at least two independent experiments is shown. The S.E. bars mostly lie within the symbols and are not shown for clarity.

broad spectrum neuropeptide antagonists in SCLC cell lines [4].

Strikingly, concentrations of tyrphostin at or above 50 μM not only completely inhibited growth but reduced the numbers of H-345 and H-69 cells from the original day 0 values.

Combined effects of tyrphostin and the SP broad spectrum neuropeptide antagonist AntG on SCLC growth in liquid culture

Figure 4 demonstrates that in both SCLC cell lines, the combination of AntG with tyrphostin significantly prolonged the lag phase period compared with the use of either agent alone or with control cells not exposed to the inhibitors. Little change was seen in the slope of the log phase of growth in keeping with results using AntG or tyrphostin alone. Strikingly, the number of cells reaching plateau phase in both cell lines treated with combined AntG and tyrphostin was additively reduced compared with either agent alone or control cells.

DISCUSSION

The development of novel therapies for SCLC is urgently required. Since this tumour has been shown to proliferate in response to multiple neuropeptides in an autocrine/paracrine manner, one approach has been the use of broad spectrum neuropeptide receptor antagonists [4–6]. However, as more is learnt about the downstream signalling events, leading from the activated neuropeptide receptor to the nucleus where DNA synthesis occurs, new targets are becoming apparent for SCLC therapy. In addition, the recent finding that polypeptide growth factors, such as stem cell factor, can induce colony formation in SCLC cell lines [16] indicates that inhibitors of polypeptide growth factor receptor signalling may be of potential therapeutic interest.

We recently showed in Swiss 3T3 cells that tyrosine phosphorylation is important in bombesin mediated mitogenesis in Swiss 3T3 cells using the tyrosine kinase inhibitor tyrphostin [13]. The results presented here show that tyrphostin not only inhibits neuropeptide stimulated colony growth of two SCLC cell lines but also reduces background colony formation dose dependently. Furthermore, we found that tyrphostin inhibits colony formation induced by the polypeptide growth factor stem cell factor (unpublished results). The inhibitory effect of tyrphostin on the growth of the SCLC cell lines was also examined in liquid culture, which is a less stringent assay than the semisolid media colony assay. Tyrphostin was similarly potent in inhibiting the growth of the H-345 and H-69 SCLC cell lines in liquid and semisolid media. The inhibitory effect of tyrphostin on SCLC growth could reflect a delay in entry of the cells into their proliferative phase. However, time course studies revealed that tyrphostin not only prolonged the lag phase before the cells started to proliferate but also reduced the total numbers of cells reaching the plateau stage of the growth curve. Interestingly, concentrations of tyrphostin at or above 50 μM actually reduced cell counts with time implying

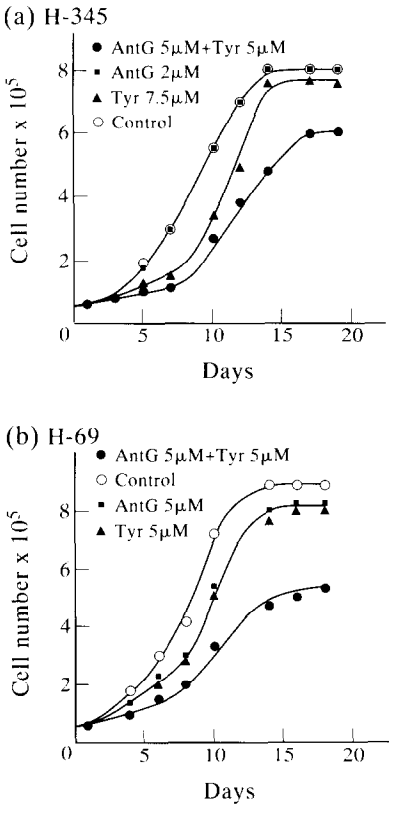

Figure 4. Combined effects of suboptimal inhibitory concentrations of tyrphostin and AntG on (a) H-345 and (b) H-69 SCLC cell growth. Each point represents the mean of three determinations. In all cases, a representative of at least three independent experiments is shown. The S.E. bars mostly lie within the symbols and are not shown for clarity.

that this agent may be inducing cell death in both SCLC cell lines.

AntG and tyrphostin inhibit neuropeptide induced mitogenesis in Swiss 3T3 cells via different mechanisms; AntG competes for ligand binding [8], while tyrphostin blocks the downstream induction of tyrosine phosphorylation [13]. In addition, tyrphostin is known to inhibit tyrosine phosphorylation and mitogenic signalling induced by many polypeptide growth factor receptors [15]. It is plausible that these agents may behave in a similar manner in SCLC lines, which are now known to be stimulated not just by neuropeptide growth factors but also by the polypeptide growth factors, stem cell factor and hepatocyte growth factor [16]. We therefore investigated the combined inhibitory effect of AntG and tyrphostin on SCLC growth. The results demonstrate that when AntG and tyrphostin were combined, each at suboptimal inhibitory concentrations, there was marked prolongation of the entry of cells into log phase growth. Interestingly, the total number of cells achieving plateau phase was additively reduced in the presence of both inhibitors in both SCLC cell lines examined. These data indicate that AntG and tyrphostin act additively to block SCLC growth.

Tyrphostin has been used by others *in vivo* without toxicity even at very high concentrations [18, 19]. In contrast, AntG has a narrow therapeutic index in animal experiments designed to test its efficacy at inhibiting SCLC xenograft growth [6]. It is therefore conceivable that the combined use of tyrphostin with AntG may permit reduced doses of SP analogue antagonist to be administered, thereby reducing toxicity without loss of effect.

In conclusion, the data presented here demonstrate for the first time that tyrphostin inhibits SCLC growth *in vitro*. Furthermore, the combination of suboptimal inhibitory concentrations of tyrphostin and the broad spectrum neuropeptide antagonist AntG additively inhibit SCLC proliferation *in vitro*.

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