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Transferrin Dependence of Ga $(NO_3)_3$ Inhibition of Growth in Human-Derived Small Cell Lung Cancer Cells

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Abstract The effect of a combination of anti-transferrin receptor (TFR) antibody, 42/6, and $Ga(NO_3)_3$ on cell growth was examined in small cell lung cancer (SCLC) cell lines: classic, NCI-H209, NCI-H345, NCI-H510; and variant, NCI-H82 and NCI-N417. The role of TFR and transferrin (TF) in $Ga(NO_3)_3$ cellular uptake was also tested. Exogenous TF did not enhance the cytotoxicity of Ga. At > 3 μg/mL, $Ga(NO_3)_3$ inhibited growth in all cell lines in TF-supplemented or deficient media. At < 3 μg/mL, Ga stimulated growth for all cells but this effect was eliminated by TF or 42/6. Classic SCLC lines required 3–4-fold less exogenous gallium than variant lines to reduce cell number by 50%. The mean Ga uptake (ng/10^6 cells) in H345 and H209 cell lines was 4–5-fold compared to H82 and N417 uptake ($P < 0.001$). 42/6 reduced exogenous TF-stimulated growth. Antibody plus $Ga(NO_3)_3$ caused a slight further cell number decline in all cell lines in TF-supplemented or deficient media. These results suggest that the addition of 42/6 antibody treatment would not increase the effectiveness of $Ga(NO_3)_3$ in patients. Both exogenous and endogenous TF and TFR play an important role in Ga uptake in these cells.

Key words: Ga nitrate, transferrin, transferrin receptors, small cell lung cancer

Iron deprivation is a valuable approach to enhance tumor cell killing because this ion is required for cell growth and multiplication [1]. Iron is an essential cofactor in the proteins involved in the electron transport chain which provides energy for the cell and in ribonucleotide reductase (RRase), the rate limiting enzyme for the synthesis of deoxyribonucleotides from ribonucleotides. Since this enzyme turns over rapidly, the need for iron is particularly high when the demands for deoxyribonucleotides are unusually high in S-phase [2]. Transferrin (TF), the iron transport protein, normally transports the iron into the cell by receptor-mediated endocytosis [3,4]. Because the blood concentration of Fe-TF is sufficient to saturate these receptors, the transferrin receptors (TFR) must be upregulated in order to increase the iron uptake into the cell. Intracellular iron appears to regulate TFR expression. Rapidly proliferating hematopoietic and other neoplasms, which express increased numbers of surface TFR, are therefore candidates for iron deprivation treatment [4]. TF is a consistent requirement for the growth of lung cancer cells in vitro as reviewed by Oie in this supplement [5]. Neutralization of such growth factor circuits represents an attractive strategy both for cancer treatment and chemoprevention [6].

Previous strategies have targeted various aspects of this iron accumulation pathway. Gallium (Ga), a trivalent metal with chemical properties similar to iron, has the ability to bind TF and this complex can be incorporated into cells

Abbreviations used: BSA, bovine serum albumin; FCS, fetal calf serum; Fe, iron; Ga, Gallium; IS, insulin and selenite; NSB, non-specific binding; PBS, phosphate buffered saline; R_5, RPMI-1640 containing 2 mM glutamine, 1 mM HEPES plus phenol Red, 1% pen/strep and 5% heat-inactivated (0.5 h at 56°C) fetal calf serum; RRase, ribonucleotide reductase; SCLC, small cell lung cancer; TF, transferrin; TFR, transferrin receptors; TIS, transferrin, insulin and selenite.

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via the TFR [7,8]. Incubation with $Ga(NO_3)_3$ causes cell growth inhibition of hemopoietic tumor cells [9]. Although the exact mechanism of this inhibition is unknown, the cells up-regulate their TFR as if starved for iron and Ga directly interferes with RRase activity [10–12]. These facts imply that Ga affects the later steps of iron accumulation pathway; the intracellular Fe concentration [1]. How Ga enters tumor cells in vivo is somewhat controversial. Data from patient studies [13,14] and a variety of model systems, mainly using trace concentrations (pM) of ^{67}Ga, suggest that TFR are rate-limiting [8]. $Ga(NO_3)_3$, at pharmacological concentrations (μM), has been effective in patients with malignant lymphoma [15,16], a tumor known to express a high concentration of TFR [17]. Alternatively, at μM concentrations both Ga and Fe can be incorporated in tumor cells by a TF-independent pathway under iron-rich conditions [4,9,18,19]. While Fe requires TF to be transported in the blood, Ga can form a soluble non-TF bound complex, $[Ga(OH)_4]^-$ [8]. When $Ga(NO_3)_3$ is used therapeutically, there is a high concentration of non-TF bound Ga and both TF-dependent and -independent pathways may play a role in cellular incorporation [20]. Recent data in an in vivo model suggest that TFR may not be rate-limiting even for trace ^{67}Ga uptake [21].

Fe incorporation into the tumor cell by TF may also be hindered at the initial steps by monoclonal antibodies that are directed against TFR. These antibodies that prevent binding of the Fe-TF to TFR appear to down regulate the total number of TFR by cross-linking the TFR to the cell surface and increasing TFR degradation and/or impairing TFR internalization [22–24]. Anti-TFR antibodies are most effective in inhibiting hematopoietic tumor cell growth in vitro and prolonging the life of animals bearing this type of tumor [1].

In an effort to improve the in vivo effectiveness of gallium, we have investigated the relationship of TF and TFR number on the uptake and growth inhibition in vitro of this ion in a number of SCLC cell lines. $Ga(NO_3)_3$ has been modestly effective in patients with SCLC [15]. Small cell lung cancer (SCLC) cells possess TFR and we and others have reported that a TF-like molecule can mediate autocrine proliferation of certain SCLC lines in vitro [25,26]. In addition, the combination of $Ga(NO_3)_3$ with another antagonist of iron accumulation, an anti-TFR monoclonal antibody, 42/6 was tested. We hypothesized that synergy might be achieved with this combination because each has an opposite effect on TFR number. $Ga(NO_3)_3$ deprives the cell of iron, causing an increase in TFR [10] and the 42/6 antibody inhibits Fe uptake and causes a decrease in TFR [27]. In HL-60 cells, this combination of $Ga(NO_3)_3$ and 42/6 has a supra-additive effect on cell killing but not in KB cells [24].

METHODS

The classic SCLC cell lines, NCI-H209, NCI-H345, and the variant SCLC cell lines [28], NCI-H82 and NCI-N417, were used in all experiments. NCI-H209 grows in suspension as a tight spheroid and can be only reliably separated to a single cell suspension with vigorous agitation, which decreases viability. For that reason in the growth inhibition studies, the classic SCLC cell line H510 was also studied. Cells were grown in 175 cm^2 growth flasks as previously reported [25]. All cell lines were free of mycoplasma contamination. Cells were maintained in RPMI-1640 supplemented with 5% fetal calf serum (FCS), penicillin and streptomycin [pen/strep] (Gibco, Grand Island, NY), in the presence of a 5% CO_2 atmosphere at 37°C.

TF Receptor Studies

The number of surface TFR and affinity constant, K_a, were determined in four SCLC cell lines; H209, H345, N417, and H82. The cells were removed from the flasks and washed twice (5 min at 4,000 RPM in a Beckman GPR centrifuge at 4°C; Beckman, Fullerton, CA) with phosphate buffered saline containing 1% (w/v) bovine serum albumin (PBS/BSA), pH 7.4. The cell viability was determined using a trypan blue assay and the number determined using a hemacytometer. All cells were ~90% viable except H209 cells which were 65–70% viable. The cells were removed from the flasks, washed twice and re-suspended in PBS/BSA without N_3, and kept on ice. The cell concentration was adjusted to 5×10^6 cells/mL and a 100 μL aliquot was placed in small (~250 μL), coated (1% BSA overnight, then aspirate BSA) Sarstedt tubes. Six samples were prepared in triplicate plus one control containing 100 μL of PBS/BSA buffer used to determine the non-specific binding (NSB). Then 25 μL of Dupont NEN (Boston, MA) ^{125}I-diferric TF (specific activity is 0.78 μCi/μg, ~1,300 CPM/ng and 82.6 μCi/mL) or

^{125}I-apoTF labeled in-house (specific activity 14 μCi/μg) was added to each sample to yield ~2,000 CPM/sample. The concentration of TF was varied by adding 50 μL of different diferric or apo TF stock solutions in PBS/BSA buffer. The stock concentration was adjusted to yield a final 10,000, 1,000, 100, 50, 25, and 7.6 ng/mL. In the control sample, 50 μL of buffer was added. The samples were incubated at 4°C for 4 and 24 h. The non-cell bound TF activity was removed by centrifuging the samples for 5 min at 4,000 RPM in a Beckman GPR centrifuge at 4°C. The supernatant was aspirated and then 200 μL of cold PBS/BSA buffer was added. The centrifugation was repeated and the supernatant decanted. The ^{125}I activity was measured and the data corrected for NSB. The 4 h incubation gave unreliable results while the data from the 24 h was much more consistent. This indicated that at least 24 h were required to achieve equilibrium and this data is presented. The data was plotted as bound/free vs. bound nM and analyzed by the method of Scatchard using a linear least squares regression [29]. Estimated values and standard errors of K_a and number of sites per cell are presented.

Growth Inhibition Studies

To determine the effect of $Ga(NO_3)_3$ and TF on cell growth, a semi-automated colorimetric assay was used. In this assay, the numbers of cells are directly correlated to the ability of viable tumor cells to reduce a tetrazolium compound which was monitored at 540 nm [25]. Three classic cell lines, H209, H345, H510, and two variants, N417 and H82, were studied. Cells were grown as described previously. The cells were re-suspended in RPMI-1640 supplemented with 5 μg/mL insulin, and 5×10^8 M selenium, and then seeded in a 96-well plate at a density of $10–20 \times 10^3$/well. To determine if cell growth was more sensitive to the effects of $Ga(NO_3)_3$ in the presence of TF, the growth media was also supplemented with apo TF (Sigma Chemical Co, St. Louis, MO) at 10 μg/mL or as a control, buffer. $Ga(NO_3)_3$ (National Cancer Institute, Bethesda, MD) in buffer was added to the wells in a range of concentrations from 0.003–25 μg/mL and buffer alone was added as a control. The plates were incubated for 5 days and assayed for cell number. Mean optical density ± standard deviation of at least 6 data points is reported in the figures. All experiments were repeated on at least three separate occasions.

Effect of Combining $Ga(NO_3)_3$ and an Anti-TF Antibody

To assess the potential for additive effects with a combination of 42/6 antibody and $Ga(NO_3)_3$ on growth inhibition, we first examined the ability of the anti-TF receptor antibody, 42/6 (Oncogene Science, Inc., Manhasset, NY), to directly inhibit cell growth. This antibody was added in a range of 0.01–10 μg/mL to the cells grown in 96 well plates. TEPC-15 (Organon Teknika-Cappel Inc., Malvern, PA), an isotypic antibody, was added to control wells. The plates were incubated and assayed for cell number as described above. Next, an aliquot of 42/6 antibody solution was added to the wells to achieve 10 μg/mL and the $Ga(NO_3)_3$ concentration was varied as described above. The plates were incubated and assayed for cell number as described above.

Ga Uptake Studies

Four cell lines studied above, H345, H510, N417, and H82, were used also in these studies. Cells were removed from the flasks and washed twice with PBS/BSA buffer and re-suspended in RPMI-1640 containing: 2 mM glutamine, 1 mM HEPES plus phenol Red, 1% pen/strep, and 5% heat-inactivated (0.5 h at 56°C) fetal calf serum (R_5). Aliquots of cells were placed in sterile, uncoated 12 × 75 mm polypropylene tubes to yield four duplicate samples with $2–7 \times 10^6$ cells/mL. The total reaction volume was 1 mL except for H345 which was 600 μL. Two additional duplicate samples were prepared with R_5, to serve as a control for NSB and with PBS, for total ^{67}Ga counts. Radiopharmaceutical grade ^{67}Ga citrate (Dupont Pharma, Billerica, MA), 170 μCi, was diluted into 5 mL of 0.25 μg/μL $Ga(NO_3)_3$ in water and an aliquot of this stock added to each of the 6 samples. This yielded a final $Ga(NO_3)_3$ concentration of 30 μg/mL for H209, H82, and N417 cells and 30 or 50 μg/mL for H345 cells. These concentrations were used to ensure that cell death occurred within a reasonable time frame in all cell lines. To determine the influence of TF on $Ga(NO_3)_3$ uptake in these cell lines, stock solutions of ApoTF were prepared in PBS and aliquots of the stocks or PBS were added to the samples to yield 0, 0.1, 1, and 10 μg/mL, final TF concentration. The samples were then placed in a test tube rack on a shaker in an incubator at 5% CO_2 and 37°C.

To assay for the amount of cell-bound Ga, 200 μL was removed from each tube, placed in 1.5 mL polypropylene Eppendorf tubes and 1 mL of cold PBS/BSA buffer was added. The samples were centrifuged 5 min at 3,000 RPM at 4°C, the supernatant was removed by aspiration, 1 mL of cold PBS/BSA buffer was added, and the sample vortexed. This washing process was repeated twice. Then the tips of the tubes were cut off and placed in 12 × 75 mm tubes for measuring the ^{67}Ga activity. The cell-bound Ga, cell viability and number were assayed at times indicated in the figures. To compare the uptake by the different cell lines, the values for time points where the uptake plateaus, 41 to 86 h for all lines expect H82, were included in the statistical analysis. For H82 time points 17 and 41 h post Ga addition were used.

Statistical Analysis

The Student's t-test was used to test the differences between means of the data and $P > 0.05$ was used to indicate significance.

RESULTS

TF Receptor Studies

Previous work [25] suggested that both classic and variant cell lines possess TF receptors. To more fully characterize the role of TF in cell growth, the K_a for TF binding and number of receptors/cell for each cell line were determined. The estimates for these parameters are shown in Table I. Even though the K_a vary almost 8-fold these differences are not significant among the cell lines. The classic cells had more receptors than the variants and the difference between H209 and the variant cell lines was significant ($P < 0.05$). However, the difference for H345 cells was not significant. The number of TF-sites/cell and K_a for H209 cells is within the range for other tumor cells with high density and high affinity receptors, like the human erythroleukemia cell line, K562 [8 and references therein]. In contrast to the K562 cells, the K_a values for apo and holoTF binding to H209 cells were not different, which is somewhat unusual.

Growth Inhibition Studies

To determine if TF would have any effect on $Ga(NO_3)_3$ inhibition of SCLC cell line growth, cells were incubated in the presence and absence of 10 μg/mL of TF (Fig. 1). This concentration is sufficient to saturate all the TF binding sites

TABLE I. Best Estimates of K_a and Receptors/Cell for SCLC Classic and Variant Cell Lines

Cell line	K_a 1/M × 10^8	Sites/cell
H209-Apo	1.1 ± 0.4[a]	190,000 ± 22,000[a]
H209-Holo	1.6 ± 0.4	250,000 ± 5,100
H345	2.4 ± 0.7	87,000 ± 2,300
H82	8.6 ± 1.9	62,000 ± 4,700
N417	4.4 ± 0.3	66,000 ± 570

[a]Least squares estimate ± standard error.

(Table I). Whether or not TF was present, $Ga(NO_3)_3$ inhibited growth in all cell lines (Fig. 1). The presence of TF did have a small effect, mainly eliminating the $Ga(NO_3)_3$-induced growth-stimulating in H209 and variant lines. The initial growth-stimulating effect of $Ga(NO_3)_3$ occurred for all cells when TF was not present in the incubation media (Fig. 1A and C). With TF present, growth of the H209 line was not consistently stimulated (Fig. 1B). Gallium clearly stimulated growth for H345 and H510 lines at low concentrations (Fig. 1B) and had no stimulatory influence on the H82 and N417 lines (Fig. 1D).

These studies did show, however, that the classic cells were more sensitive to the external gallium concentration than the variants. That is, for a 50% reduction of viable cells, ~3.1 μg/mL of $Ga(NO_3)_3$ was required for H345 cells and ~6.3 μg/mL for H510 (Fig. 1A and B). In contrast, for the variants, H82 and N417, 9–11 and 16–20 μg/mL of gallium, respectively, was required to achieve a similar percentage decline in cell growth (Fig. 1C and D). These values also demonstrate that H345 cell growth was inhibited at lower concentrations by gallium than H510, and H82 cell growth was inhibited at lower concentrations than N417.

Ga Uptake Studies

To compare the effect of TF-dependent vs. TF-independent processes on $Ga(NO_3)_3$ incorporation, SCLC cell lines were incubated with a wide range of TF concentrations (Figs. 2 and 3). Exogenous TF did not appear for most cell lines to significantly enhance the uptake of $Ga(NO_3)_3$ under these conditions. There was a modest TF-effect in the H345 cell line but only when Ga was incubated with the cells for a short time. In a short-term (under 4.5 h) experiment. the uptake at 0.5 h peaked at 1 μg/mL TF ($P < 0.05$) compared to the lower TF concentration values

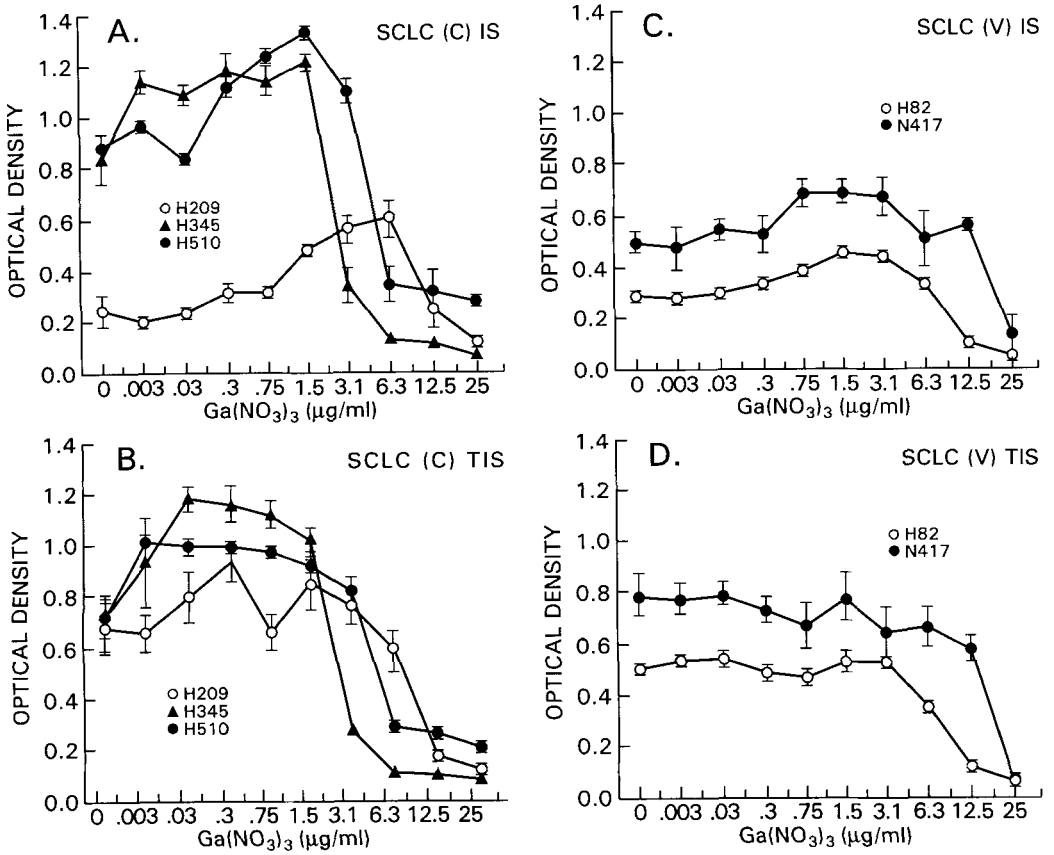

Fig. 1. Effect of $Ga(NO_3)_3$ concentration on in vitro growth of SCLC cell lines in the absence of TF (IS) and presence of 10 μg/mL TF (TIS). **A, B:** Classic SCLC cell lines. **C, D:** Variant SCLC cell lines. Cells were seeded in 96-well plates at 1–2 10^5 cells/mL as outlined in Methods and incubated with various concentrations of $Ga(NO_3)_3$. Cell growth was determined after 5 days by the MTT assay. Data points represent mean optical density at 540 nm ± SD of 6 values.

(0.0 and 0.1 μg/mL) (data not shown). At 4.5 h, there was no longer any significant TF concentration dependence. In the longer term experiment (up to 1 week incubation with $Ga(NO_3)_3$), at 1 h the Ga binding to the H345 cells again showed a typical TF-dependent with a maximum at 0.16 μg/mL (Fig. 2A). While this uptake value was not significant compared to the absence of TF, it was significantly higher, $P < 0.02$, than the uptake at higher TF concentrations (receptor saturation with excess apoTF). This 1 h uptake value was consistent with the value at 0.5 h in the earlier experiment. Both Ga uptake values at these TF concentrations were ~ 2-fold greater than in the absence of TF and a decrease in the cell concentration (from 5 to 3×10^6 cells/mL) would likely shift the maximum binding to lower TF concentration (0.16 μg/mL). At 17 h and beyond there was no significant influence of TF concentration on the H345 cell line. Also, the rate of uptake slowed and incorporation remained generally constant. While it appeared that the 41 h, 1.6 μg/mL uptake value increased more than 2-fold, this was not significantly different than the other TF values. Viability precipitously declined at 41 h and after that was only modestly reduced. In H209 cells, the uptake was similar to the H345 line but there was no significant effect of TF, even at the early time point. Ga incorporation leveled off at 41 h and the large decline in viability occurred at 86 h (Fig. 2B). At further time points when most cells were not viable, Ga concentration declined instead of remaining constant.

The uptake pattern for the N417 cells is similar to the other 2 lines; linear uptake occurred from 1 to 41 h and then it flattened until 86 h (Fig. 3A). Most of the reduction in cell viability occurred within this time frame. While the addition of TF to the incubation media did not appear to significantly effect Ga uptake, the

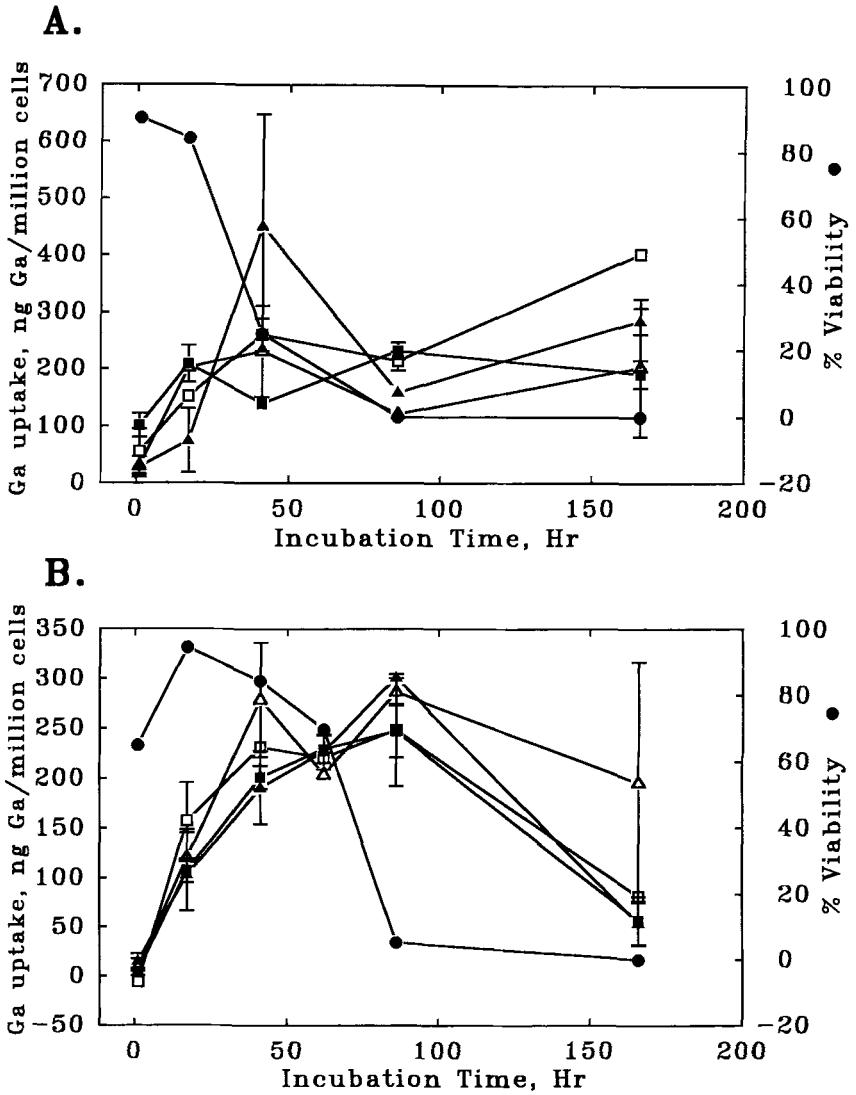

Fig. 2. Effect of time and TF concentration on uptake of $Ga(NO_3)_3$ in (A) H345 and (B) H209 cells. H345 cells were incubated with 50 μg/mL of $^{67}Ga(NO_3)_3$ in the presence of 0 (□), 0.16 (■), 1.6 (▲), and 16 (△) μg/mL of apoTF. H209 cells were incubated with 30 μg/mL of $^{67}Ga(NO_3)_3$ in the presence of 0 (□), 0.1 (■), 1 (▲), and 10 (△) μg/mL of apoTF. At times indicated the amount of ^{67}Ga bound to the cells and the viability (●) of the cells was determined as described in Methods. Data points represent means of duplicate samples.

uptake values in the presence of TF were generally lower when the cells were viable. In this cell line, further incubation when the cells were mostly non-viable yielded a modest decline in bound Ga. In Figure 3B, a similar uptake pattern was observed for the H82 cells. The uptake peaked a little earlier (between 17 and 41 h) and then leveled off when the incubation was continued to 86 h. Viability substantially diminished up to 62 h and then declined minimally. Uptake did increase after 86 h but only when the cells were no longer viable. For viable cells, TF seemed to neither help nor hinder Ga uptake.

The most striking difference among the different cell lines was the absolute Ga incorporation. The Ga uptake/10^6 cells for viable cells fell into two groups: the classic lines, H345 and H209, and the variants, H82 and N417. If the values from time points where uptake plateaus were combined, the classic cells had a 4–5-fold highly significant greater mean uptake compared to either variant cell lines whether or not TF was present (Table II). The different classic lines had no difference between the mean uptake in the presence or absence TF. However, for the variant cells, N417 uptake was significantly

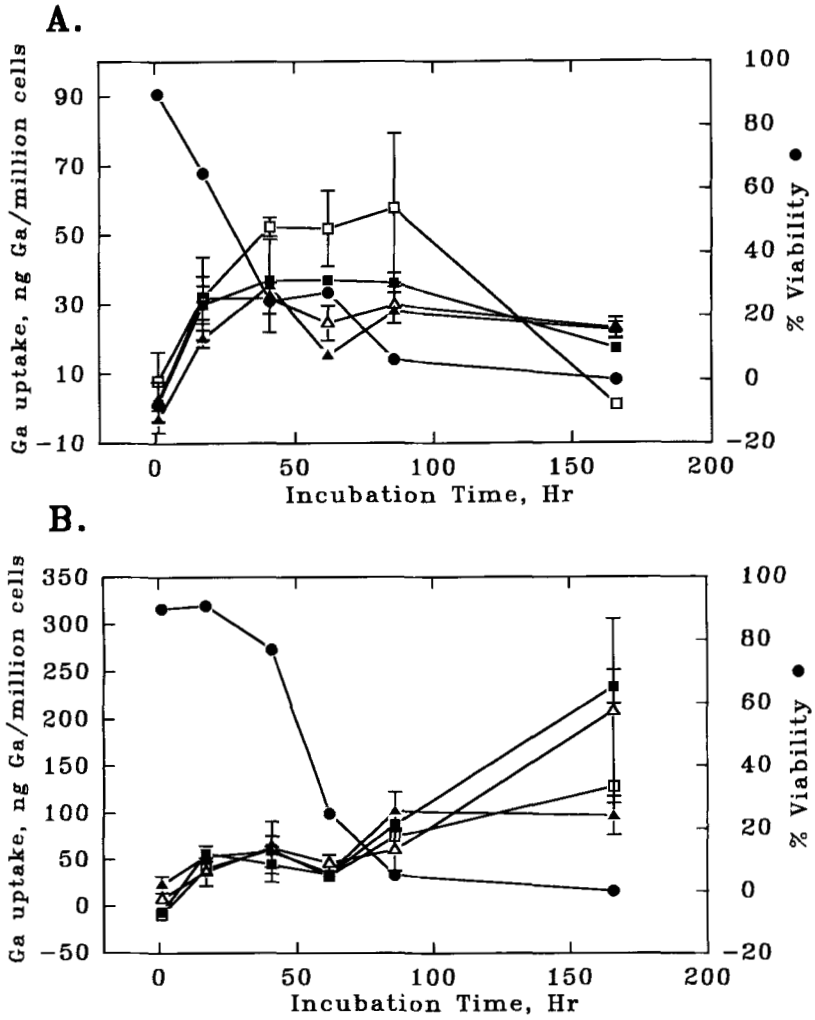

Fig. 3. Effect of time and TF concentration on uptake of $Ga(NO_3)_3$ in (**A**) N417 and (**B**) H82 cells. N417 and H82 cells were incubated with 30 μg/mL of $^{67}Ga(NO_3)_3$ in the presence of 0 (□), 0.1 (■), 1 (▲), and 10 (△) μg/mL of apoTF. For other experimental details see Figure 2 legend.

($P < 0.001$) lower than the H82 value but only in the presence of TF.

Effect of $Ga(NO_3)_3$ and Anti-TF Antibody on Cell Growth

The influence of the anti-TF receptor antibody, 42/6, at 10 μg/mL, was initially tested by itself in both TIS and IS media. This concentration is in large excess of what is needed to prevent TF binding to its receptor [22]. In the control (IS) media, this antibody clearly reduced the cell number for H345, H510, and N417 lines but the growth of the cell line, H82, was only moderately inhibited (Fig. 4A and B). An effect could not be detected for H209 cells because they grew poorly. In contrast, 42/6 reduced exogenous TF-stimulated growth in all cell lines (Fig. 4B and D). There was a 10-fold difference in the effective concentration. The growth of N417 cells was significantly reduced at 0.1 μg/mL of antibody while the other cell lines required a higher value (1 μg/mL) to achieve a significant reduction. Experiments with an isotypic control antibody showed no growth inhibition (data not shown).

Since the 42/6 antibody was an effective antiproliferative agent, the antibody was next examined in combination with $Ga(NO_3)_3$. Figure 5 shows that the addition of 10 μg/mL of 42/6 antibody to $Ga(NO_3)_3$ caused only a modest additional decline in cell number in all cell lines. This modest additive effect on cell growth inhibition is clearly observed at the highest $Ga(NO_3)_3$ concentration, 12.5 μg/mL in all lines. How-

TABLE II. Comparison of Combined Mean Cellular $^{67}Ga(NO_3)_3$ Uptake for SCLC Classic and Variant Cell Lines

Cell type	Transferrin concentration [μg/mL (number of data points)]			
	0	0.1	1	10
NCI-H345	237 ± 23[a] (4[b])	188 ± 38 (6[c])	299 ± 47 (6)	185 ± 120 (6)
H209	233 ± 12 (6)	226 ± 19 (6)	240 ± 46 (6)	256 ± 37 (6)
N417	54 ± 3* (6)	37 ± 0.4* (6)	26 ± 8* (6)	29 ± 3* (6)
H82	51 ± 10* (4[d])	51 ± 6* (4[d])	56 ± 3** (4[d])	50 ± 13* (4[d])

[a]Mean ± SD, ng Ga/10^6 total cells.
[b]Includes time points 41, 86 h.
[c]All others include time points 41, 62, 86 h.
[d]Includes time points 17, 41 h.
*$P < 0.001$, compared to H345 or H209 values at identical TF concentrations.
**$P < 0.005$, compared to H345 or H209 values at identical TF concentrations.

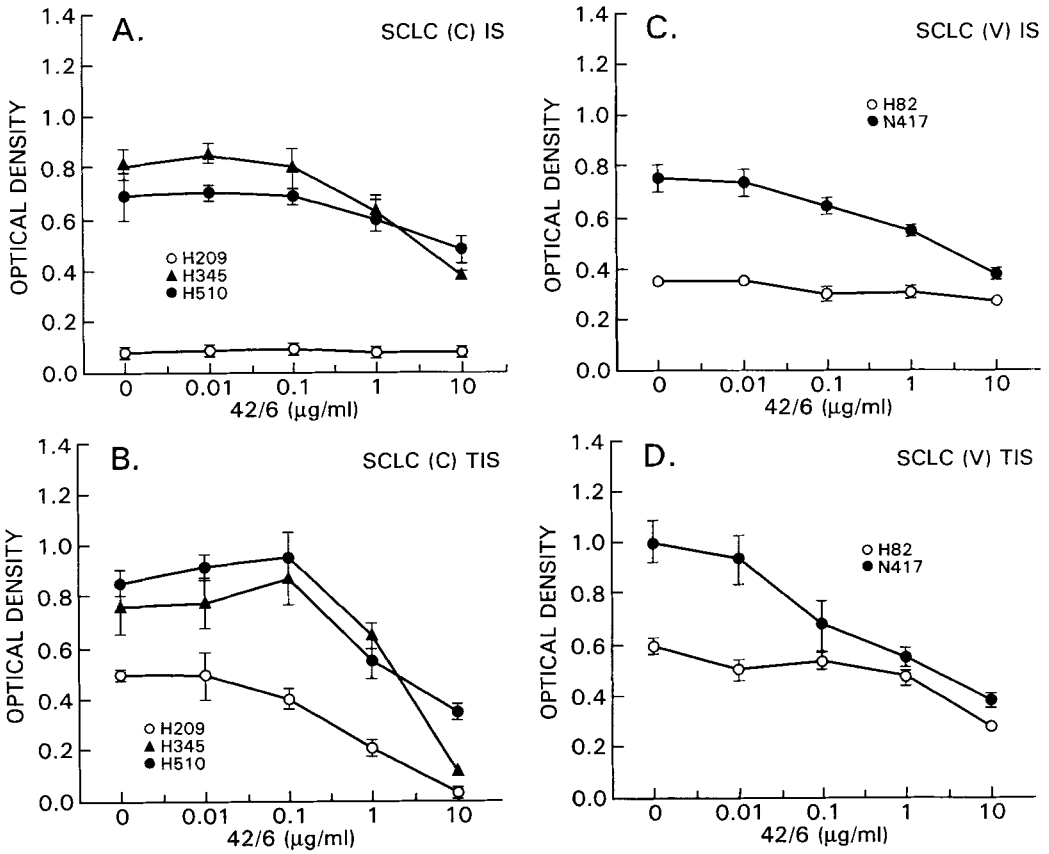

Fig. 4. The effect of anti-TF receptor antibody, 42/6, concentration on the in vitro growth of SCLC cell lines in the absence of TF (IS) and presence of 10 μg/mL TF (TIS). **A, B:** Classic SCLC cell lines. **C, D:** Variant SCLC cell lines. Cells were seeded in 96-well plates at 1–2 10^5 cells/mL as outlined in Methods and incubated with various concentrations of the 42/6 antibody. For other experimental details see Figure 1 legend.

ever, several cell lines were responsive to as low as 3.1 μg/mL (Fig. 5C,D,H, and J). The effect of TF in the media produced no great differences like those that were easily apparent with either Ga or 42/6 antibody alone. The addition of 42/6 did eliminate or reduce the growth stimulation that was observed at lower Ga concentrations (Fig. 5A,D–G, and I). In the absence of TF, there was no longer any significant augmentation for lines H209, H510, and H82. In the N417 line, a small amount of growth increase remained. In the presence of TF, at these Ga concentrations, H510 was the only cell line to show initial growth enhancement and this was eliminated with the

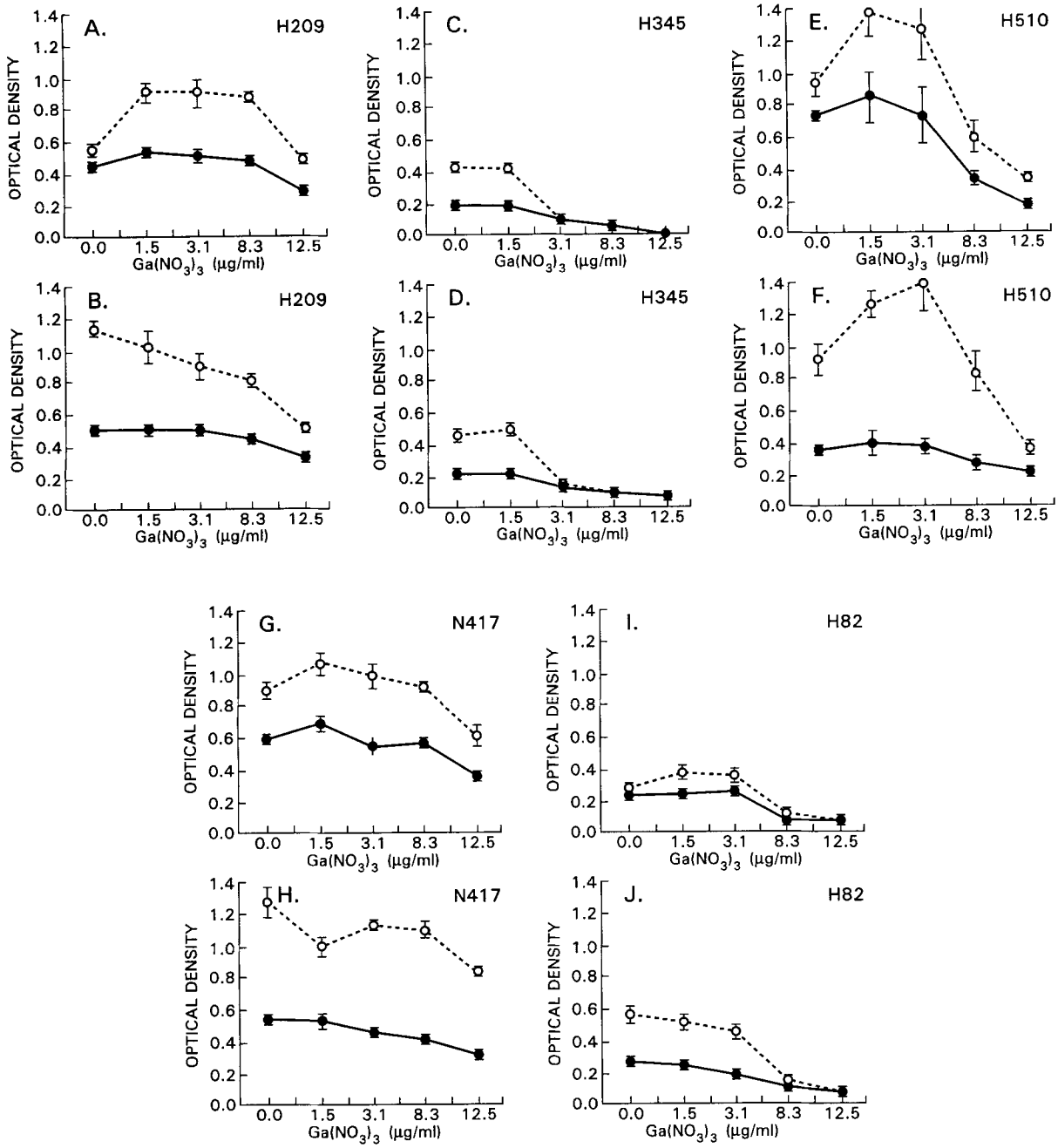

Fig. 5. The effect of anti-TF receptor antibody, 42/6, on in vitro growth of classic SCLC cell lines (A–F) and variant SCLC cell lines (G–J) in combination with various $Ga(NO_3)_3$ concentrations. Antibody concentration was 10 μg/mL (●) and no antibody was added in the controls (○). Experiments were also performed in the absence of TF (A,C,E,G,I) and presence of 10 μg/mL TF (B,D,F,H,J). For other experimental details see Figure 1 legend.

42/6 antibody. H345 cells did not show stimulation as was observed in the earlier set of experiments (compare Figs. 1A,B and 5C,D).

DISCUSSION

In an attempt to enhance the clinical efficacy of $Ga(NO_3)_3$ [15,16,20], the anti-TFR antibody, 42/6, was tested to determine if it would enhance the growth inhibitory ability of $Ga(NO_3)_3$ on SCLC cell lines. In addition, we examined the influence of TF and TFR on the growth inhibition and cellular incorporation of $Ga(NO_3)_3$ in these cell lines. We hypothesized that a "double hit" on the iron accumulation system might

increase the effectiveness of these two agents. Unfortunately, in our experiments with the SCLC cells, the combination of $Ga(NO_3)_3$ and anti-TF receptor antibody had only a slight additive effect (Fig. 5). Each agent by itself was effective. This lack of synergy could be that both Ga and 42/6 are directed at the same intracellular target. The main target, at least for gallium, appears to be RRase [10–12]. RRase is in constant need of Fe for activity [2]. Chitambar and co-workers have shown in two different cell lines that Ga can directly influence the activity of RRase [10,12]. It is possible that Ga can bind to the iron binding site of RRase [30,31]. The noncatalytic Ga^{3+} could compete with and replace the Fe^{3+} which would inactivate the enzyme. The 42/6 antibody would affect the RRase activity by blocking iron entry into the cell and reducing the intracellular metal ion concentration. This antibody interferes with Fe accumulation by cross-linking the TFR and preventing internalization of the Fe*TF-TFR complex [27]. It is likely that there is a critical level of intracellular iron to keep RRase operating. If the iron concentration is reduced below this level, the enzyme is inactivated. In the SCLC cells, each agent could inactivate RRase but an additional agent would have no further effect. An alternative explanation for this lack of synergy is that one agent could interfere with the other. The 42/6 could simply limit intracellular Ga concentration by reducing the entry of Ga into the cell. Since the main access of gallium to the tumor cell is thought to be as the Ga*TF complex via the TFR [30], the antibody would just reduce the effectiveness of gallium while its effect of reducing intracellular Fe would unimpaired by Ga. The elimination of the gallium-induced growth spurt by the 42/6 is consistent with this explanation.

In the uptake experiments, there appeared no clear influence of TF on $Ga(NO_3)_3$ incorporation. Evidence from a variety of sources suggests that the Ga*TF complex at trace concentrations (pM) is the rate-limiting means by which Ga enters the malignant cell [30]. In contrast, when cell lines are incubated with μM concentrations of $Ga(NO_3)_3$ in the absence of TF, non-TF metal ion transporters are synthesized to facilitate TF-independent entry [9,19]. The typical TF-dependent stimulation where Ga incorporation is increased at low exogenous TF levels and declines at higher levels, was not observed in all the cell lines tested for the long-term experiments. We also did not detect the typical TF-independent Ga process where uptake declines directly with TF concentration [8]. Cell viability was essential to Ga uptake (Figs. 2 and 3). A TF-dependent effect was observed only at early time points for the H345 cells but by 4.5 h any stimulatory effect was over. Since H345 cells can synthesize endogenous TF, a certain lag time would be expected before the endogenous TF would overcome any effect of the exogenous TF added to the media. While this might not be expected at normal synthesis rates, endogenous TF might be more effective in stimulating Ga uptake because of the proximity of release and effective local concentrations. The other cell line, N417, would be expected to act similarly. For lines H209 and H82, if these also produce endogenous TF when intracellular iron is unusually low, we would expect these also to be uninfluenced by exogenous TF. Alternatively but less likely, both TF-independent and dependent accumulation processes may be operational in these cells. In low TF environments, especially in the present of high concentration of Ga, Ga could be taken up in a TF-independent manner. As TF concentration would be increased, the TF-dependent process becomes primary. We may not have observed the TF-independent process in the growth experiments because a relatively low concentrations of Ga was used. The increase in transporter number is related to external Ga concentration [19].

The receptor data suggests there is only a weak correlation with receptor number and Ga incorporation in these cell lines. In normal hemoproliferative cells, it is thought that upregulation of receptors is essential to increase Fe uptake [3]. However, there is data from a number of in vitro studies with malignant cells that suggest receptor number is not the only factor governing Fe uptake. Shterman et al. [32] showed that there was a 30% increase in receptor number on breast cancer cells vs. epithelial cells while the actual cellular uptake of Fe was ~30% lower. In a similar study, the TFR number in transformed cells was twice that of normal cells while Fe uptake was only increased a modest 20% and the difference was attributed to less efficient cycling of the TFR [33]. Most recently, Collawn et al. [34] have demonstrated some of the molecular details for cycling efficiency. There appears to be present on the TFR, a tetrapeptide amino acid sequence, YTRF, within the 61-residue amino-terminal cytoplas-

mic tail which promotes efficient endocytic cycling. Amino acid substitution in this motif varied the rate of Fe delivered to the cells more than an order of magnitude.

Lastly, while $Ga(NO_3)_3$ has shown some clinical effectiveness in patients with SCLC [15], based on our data there would not appear to be a benefit of the combination of anti-TFR receptor antibody and $Ga(NO_3)_3$ as a treatment for advanced SCLC. Alternative strategies that targets both iron uptake and other aspects of TF's role in cellular metabolism may be more effective in neutralizing growth-factor dependent cancer cell growth.

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