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Protein binding modulates inhibition of the epidermal growth factor receptor kinase and DNA synthesis by tyrphostins

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Abstract Inhibition of growth factor-stimulated DNA synthesis carried out in defined medium is often compared with inhibition of serum-stimulated DNA synthesis so as to assess the selectivity of growth-factor-receptor tyrosine kinase inhibitors such as tyrphostins. We investigated whether protein binding may influence the interpretation of these experiments. Protein binding of tyrphostins was determined by ultrafiltration, equilibrium dialysis or spectrophotometer, and was quantitated by high-performance liquid chromatography (HPLC). For growth factor-stimulated DNA synthesis, we used the non-small-cell lung cancer cell line L23/P stimulated by transforming growth factor α (TGFα). The epidermal growth factor (EGF)-receptor kinase was assayed by phosphorylation of a peptide substrate or by receptor autophosphorylation. Protein binding of a number of tyrphostins ranged from 64% to 98%. There was a positive correlation ($r = 0.995$) between the degree of protein binding and the hydrophobicity. Inhibition of the EGF-receptor tyrosine kinase activity by the highly protein-bound tyrphostin B56 [*N*-(4-phenylbutyl)-3,4-dihydroxybenzylidene cyanoacetamide] was reduced by bovine serum albumin (BSA), but BSA had less of an effect on inhibition of the EGF-receptor kinase by the weakly protein-bound tyrphostin A47 (RG 50864; 3,4-dihydroxybenzylidene cyanothioacetamide). Tyrphostins B46 [*N*-(3-phenylpropyl)-3,4-dihydroxybenzylidene cyanoacetamide] and B56 (both highly protein-bound) inhibited DNA synthesis of L23/P cells with approximately 3-fold greater potency in 0.5% serum than in 10% serum, but the inhibition of DNA synthesis in 0.5% serum was reduced by the addition of BSA. Tyrphostins B46 and B56 inhibited DNA synthesis stimulated by TGFα in defined medium to a greater extent than DNA synthesis stimulated by serum. However, this apparent selectivity for inhibition of TGFα-stimulated DNA synthesis was lost when the protein concentration in the defined medium was made equivalent to that in the serum-containing medium. By contrast, BSA enhanced the selective inhibition of TGFα-stimulated DNA synthesis by tyrphostin A47. These results demonstrate that protein binding accounts for the apparent selectivity of some highly protein-bound tyrphostins for TGFα-stimulated DNA synthesis of L23/P cells. Therefore, protein binding should be taken into consideration in assessments of the selectivity of tyrphostins.

Key words Lung cancer · Tyrphostins · Protein binding

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Introduction

Current chemotherapy has minimal impact on the survival of patients with non-small-cell lung cancer (NSCLC). A common occurrence in NSCLC is overexpression of growth-factor-receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor [11], c-erbB-2 [8] and insulin-like growth factor-1 receptor [18]. Inhibition of signaling pathways is now recognised as an important new strategy for the therapy of cancer [22]. Thus, receptor tyrosine kinase inhibitors warrant investigation as potential chemotherapeutic agents for NSCLC.

Numerous molecules have been identified that inhibit tyrosine kinases [22]. Since cells express many different, yet structurally conserved, tyrosine kinases, a major challenge of current research is to develop agents that selectively inhibit tyrosine kinases associated with the malignant phenotype. Some of the tyrphostin group of tyrosine kinase inhibitors selectively inhibit the EGF-receptor tyrosine kinase and have minimal activity against a number of other tyrosine

kinases and other protein kinases [14]. These tyrphostins are therefore of interest for the treatment of the histological sub-types of NSCLC in which over-expression of the EGF receptor occurs.

Selective inhibition of a sub-set of tyrosine kinases in vitro must translate into selective inhibition of the malignant cell phenotype in vivo if an agent is to display therapeutic potential. In evaluating the selectivity of inhibitors of signal-transduction pathways it is to common practice to compare the inhibition of growth factor-stimulated DNA synthesis (carried out in low-serum or serum-free medium) with the inhibition of serum-stimulated DNA synthesis [10, 16]. We used this method to compare the efficacy of tyrphostins as selective inhibitors of EGF-receptor-mediated DNA synthesis of NSCLC cells. During the course of this work, we gained evidence suggesting that the apparent selectivity of some tyrphostins is due to differences in the protein concentrations of the culture media and that the selectivity of these agents is reduced when protein binding is taken into consideration. This work has been reported in preliminary form [5].

Mateials and methods

Materials

Tyrphostins (Cookson Chemicals Ltd, Southampton, UK) were dissolved in either dimethyl sulphoxide (DMSO) or a solution of 10% DMSO in methanol and were stored as stock solutions at − 20° C. We adopted the numbering system for tyrphostins used by LC Laboratories [Calbiochem-Novabiochem (UK) Ltd] in which compounds described by Gazit et al. in 1989 [9] are given the prefix *A*, and compounds described in 1991 [10] are given the prefix *B*. Growth factors were obtained from Bachem UK (Saffron Walden, UK) and were stored in aliquots at − 70° C until required. Monoclonal mouse anti-EGF-receptor antibody clone 528 was supplied by Cambridge Bioscience (Cambridge, UK). Tissue-culture media, excluding foetal calf serum (FCS), were obtained from Gibco (Paisley, Scotland). Crystallised and lyophilised bovine serum albumin (BSA), FCS and other chemicals were supplied by Sigma.

Cells

The NSCLC cell line L23/P (large-cell histology) was maintained in RPMI/10% FCS supplemented with antibiotics (100 units penicillin/ml, 100 μg streptomycin/ml).

EGF-receptor characterisation

Cells were grown to about 70%–90% confluence, washed twice in binding buffer (ice-cold RPMI containing 0.1% BSA and 20 m*M* HEPES, pH 7.4) and incubated with [^{125}I]-EGF (0.5 n*M*; Amersham, UK) and various concentrations of cold EGF (in binding buffer) for 2 h at 4° C. The cells were then washed in binding buffer (four times) and solubilised in 1 N NaOH and the solubilisate was counted in a gamma-counter. The binding characteristics of EGF were evaluated using the LIGAND program [20] as modified for the micro-computer by McPherson [19].

DNA synthesis

DNA synthesis was determined by measuring the incorporation of [methyl-^{3}H]-thymidine (0.5 μ Ci/well) into trichloroacetic acid (TCA)-insoluble material over a 6-h period [4]. Cells for proliferation experiments were plated at 2,000 cells/well in microtitre plates in medium containing 10% serum, and then washed with serum-free medium, and fresh medium containing tyrphostin was added. DNA synthesis was determined 24 h later. Cells to be growth-factor-stimulated were plated at 1,000 cells /well in medium containing 10% serum over-night, washed with serum-free medium and cultured in RPMI/0.5% FCS for 6 days. Transforming growth factor α (TGFα, 10 ng/ml) and tyrphostin were then added in protein-free medium (PFM; RPMI containing 10 μg transferrin/ml and 10 n*M* sodium selenite) and DNA synthesis was determined 24 h later.

High-performance liquid chromatography

Tyrphostins were extracted from protein solutions by the addition of 2 vols. of acetonitrile. The mixture was centrifuged (10,000 g, 5 min), and the supernatant was analysed by high-performance liquid chromatography (HPLC). Concentrations of tyrphostins were determined by reverse-phase HPLC (Waters Nova-Pak C18; 4-μm, 8-mm × 10-cm radial packed column). Tyrphostins were eluted using a linear gradient of 10%–60% acetonitrile in 20 m*M* sodium acetate (pH 5) over 8 min followed by a further 2 min with 50% acetonitrile/buffer (flow rate, 2 ml/min). Tyrphostins were detected by UV absorbance at 365 nm and quantified by peak area (Waters Expertease software; version 2.1).

Protein-binding assays

Various techniques were employed to determine protein binding. Ultrafiltration was carried out by two methods [3]. In the first method, mixtures containing tyrphostin (10 μg/ml) and BSA (3.5 mg/ml) in 25 m*M* buffer (pH 7.5) were equilibrated to 22° C and centrifuged in Centricon 30 micro-concentrators (Amicon, Mass., USA; 1,500 *g*, 5 min). The concentration of tyrphostin in the ultra filtrate was determined by HPLC. Protein binding was estimated from values obtained from solutions of tyrphostin in buffer and of tyrphostin in buffer containing 3.5 mg BSA/ml. For the second method, tyrphostin in 25 m*M* HEPES buffer (pH 7.5) containing 7.5% ethanol and 1.67% DMSO was placed in a visking tube that was knotted at both ends, placed in a 50-ml Falcon plastic centrifuge tube and spun (370 g, 4° C) for 15 min. The early ultrafiltrate was discarded, and the sample was respun for 1.5 h. Protein binding was determined from concentrations of tyrphostin in the ultrafiltrate and retentate.

Equilibrium dialysis was carried out in Eppendorf microcentrifuge tubes as described elsewhere [24]. Briefly, 260 μ l of BSA (3.5 mg/ml) in 25 m*M* HEPES buffer (pH 7.6) was placed in the lid of a 2-ml Eppendorf microcentrifuge tube. A visking dialysis membrane was placed over the lid and the bottom of the tube was used to seal the membrane over the lid. Then, 260 μl of tyrphostin (10 μg/ml) in 25 m*M* HEPES buffer (pH 7.6) was introduced into the bottom tube via a hole melted in the side. The Eppendorf tube was sealed with Parafilm and the tubes were left to equilibrate overnight at 22° C with shaking. Parallel experiments were carried out using buffer in the lid to determine when equilibrium was attained. Concentrations on either side of the dialysis membrane were determined using HPLC.

Protein binding was also determined by a spectroscopic method [3]. The maximal absorption difference of tyrphostin [in 25 m*M* HEPES buffer (pH 7.5) containing 7.5% ethanol and 1.67% DMSO] was first determined. Using a range of tyrphostin concentrations the molar extinction coefficient (E1) of tyrphostin could

then be calculated according to Beer's law. Next, an apparent molar extinction coefficient (Eapp) of tyrphostin in the presence of 3.5 mg BSA/ml was determined. Finally, BSA was titrated against 10 μg tyrphostin/ml to determine the maximal absorption of bound ligand allowing calculation of the bound-ligand molar extinction coefficient (E2). The degree of protein binding, *a*, was calculated according to Chignell [3] using the following equation

$$\% \text{ Protein bound} = 1 - \frac{Eapp - E2}{E1 - E2} \times 100.$$

A431 membranes

Membranes were prepared as previously described [15]. Briefly, near-confluent 175-cm^2 flasks of A431 cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (PBS), scraped with a rubber policeman in 10 ml of PBS and centrifuged (800 g, 3 min). The pellet was resuspended in lysis buffer [1 m*M* magnesium chloride, 2 m*M* ethylenediaminetetracetic acid (EDTA), 20 μg soybean trypsin inhibitor/ml, 50 μg phenylmethylsulphonyl fluoride/ml, 5 m*M* TRIS-HCl (pH 7.4); 1 ml/flask]. After 30 min of incubation at 0° C the mixture was homogenised in a Potter homogeniser (10 strokes, 800 rpm, 30 s). A post-nuclear supernatant was obtained by centrifugation (5,000 g, 5 min), from which a membrane pellet was obtained by centrifugation (100,000 g, 60 min). The pellet was resuspended in 10% glycerol 125 m*M* sodium chloride 50 m*M* HEPES (pH 7.6) and stored at − 70° C prior to use.

EGF-receptor tyrosine kinase activity

Tyrosine kinase activity was determined using poly(Glu_6Ala_3Tyr) (polyGAT) as the substrate [15]. Briefly, crude A431 membrane extracts were solubilised in 5 vols. of solubilisation buffer [0.1% (w/v) Triton X-100, 150 m*M* sodium chloride and 10% (v/v) glycerol in 50 m*M* TRIS-MES (pH 7.6)] and pre-incubated with 0.8 μ*M* EGF for 20 min at 4° C and then for 3 min at 22°C. To start the reaction, 15 μl of pre-activated membrane was added to 20 μl of adenosine triphosphate (ATP) cocktail (12.5 μ*M* [γ-^{32}P]-ATP, 5 μCi/ml, 60 m*M* magnesium acetate, 0.625 mg polyGAT/ml; 30 m*M* Tris-MES, pH 7.6; final concentrations) and 5 μl of tyrphostin. When required, 3.5 mg BSA/ml was included in the reaction mixture. After 10 min at 22° C the reaction was terminated by adding 20 μl of stop reagent [0.2 *M* EDTA, 2 m*M* ATP, Phenol red, 50 m*M* TRIS-MES (pH 7.6)]. Then, 50-μl aliquots were spotted onto 2-cm squares of filter paper (Whatman P81), which were washed twice for 10 min in 75 m*M* phosphoric acid and then counted in a beta-counter.

EGF-receptor autophosphorylation

Autophosphorylation was determined as described elsewhere [15]. Briefly, crude A431 membrane extracts were pre-incubated with 3 vols. of 200 n*M* EGF in 125 m*M* sodium chloride/50 m*M* HEPES (pH 7.6) for 10 min at 4° C. To start the reaction, 10 μl of pre-activated membrane was added to 15 μl of ATP cocktail (2 μM [γ-^{32}P]-ATP, 16.6 μCi/ml; 24 m*M* magnesium acetate; 4 m*M* manganese chloride, 2 m*M* sodium vanadate, 50 m*M* HEPES, pH 7.6) and 5 μl of tyrphostin. BSA was added as required. After 30 s at 0° C, the reaction was stopped with 10 μl of 4x stop reagent [400 m*M* dithiothreitol, 16% (w/v) sodium dodecyl sulphate, 40% (v/v) glycerol, 0.08% (w/v) bromophenol blue, 400 m*M* TRIS-HCl (pH 6.8)] and then boiled for 5 min. The samples were electrophoresed in 7.5% acrylamide minigels. Autoradiographs were produced from the gels after they had been fixed for 30 min with 40% methanol 10% acetic acid and dried.

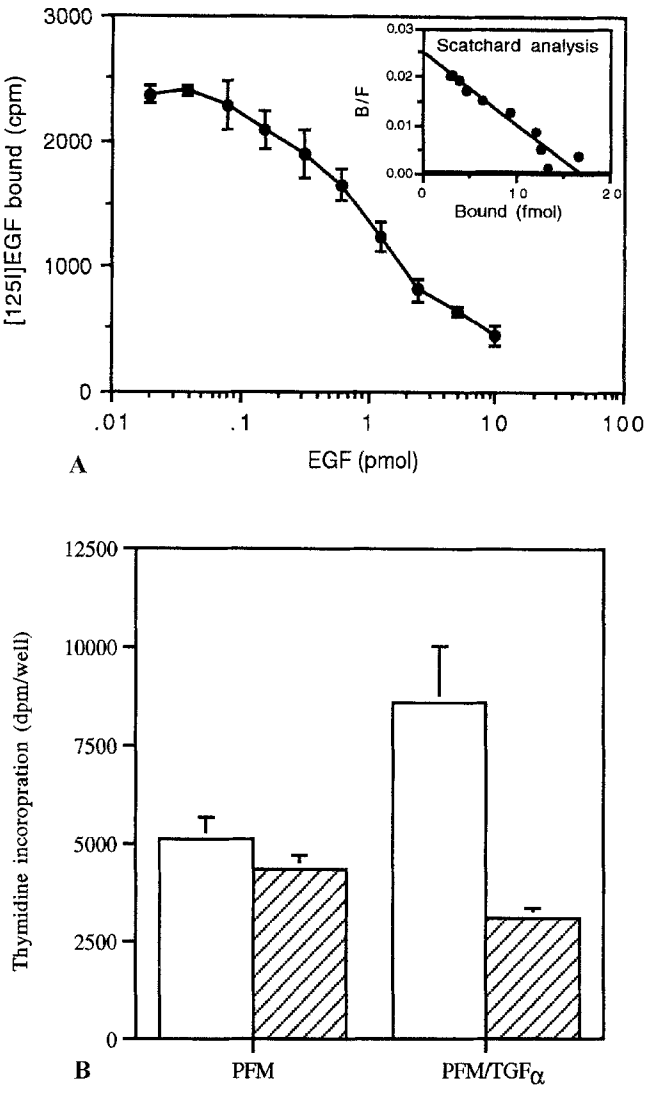


Fig. 1A, B EGF receptor characterisation of L23/P cells. **A** Scatchard analysis of L23/P cells. **B** Stimulation of L23/P cells by TGFα in protein-free medium (PFM) in the absence (*open bars*) or presence of a 20-μg/ml concentration of EGF-receptor antibody clone 528 (*hatched bars*).

Protein determination

Protein concentrations were determined using the Bio-Rad protein assay [2].

Results

EGF-receptor characterisation of L23/P cells

L23/P cells expressed 3.3×10^5 EGF binding sites/cell with a *Kd* of 5.1 n*M* as determined by Scatchard analysis (Fig. 1a). Following a 6-day period in 0.5% serum, TGFα added in PFM stimulated the DNA synthesis of L23/P cells about 2-fold over basal levels (Fig. 1b). EGF-receptor antibody 528 (20 μg/ml) completely

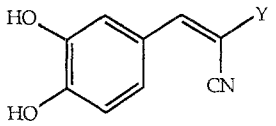


Fig. 2 Structure of tyrphostins. The structures of the Y group of the tyrphostins used in this study are shown in Table 1.

blocked the TGFα stimulation, but the antibody did not inhibit basal DNA synthesis (Fig. 1b). Although the overall rate of DNA synthesis was reduced following a 6-day incubation in 0.5% serum, some cells nonetheless remained in the S phase as determined by flow cytometry (data not shown).

DNA synthesis in 0.5% serum and 10% serum

Tyrphostins B46 and B56 have been reported to induce selective inhibition of EGF-receptor-mediated DNA synthesis relative to serum-stimulated DNA synthesis [10] and were therefore chosen for our initial studies. Tyrphostins B46 and B56 are derivatives of 3,4-dihydroxybenzylidene cyanoacetamide (Fig. 2) and their structures are depicted in Table 1. Tyrphostins B46 and B56 inhibited DNA synthesis of L23/P cells to a greater extent when the cells were grown in 0.5% serum than when they were grown in 10% serum (Fig. 3). This is shown by observations that (a) approximately 3-fold lower concentrations of tyrophostin were required to cause 50% inhibition of DNA synthesis (IC_{50} values) of cells grown in 0.5% serum as compared with cells grown in 10% serum and (b) 20 μ*M* tyrphostin inhibited DNA synthesis by >90% when the cells were grown in 0.5% serum whereas at least 40% of control rates of DNA synthesis were retained when the cells were grown in 10% serum.

The possibility that protein binding is contributing to the reduced inhibition in 10% serum was investigated by supplementing the medium containing 0.5% serum with 3.5 mg BSA/ml. This concentration of BSA is equivalent to the protein concentration in medium containing 10% serum. For both tyrphostins, the presence of 3.5-mg/ml concentration of BSA reduced the inhibition of DNA synthesis in 0.5% serum to values similar to those obtained in 10% serum (Fig. 3).

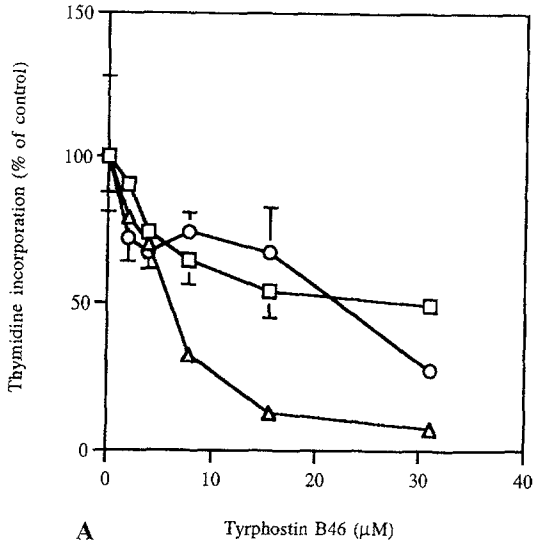
Protein binding of tyrphostins

For protein-binding studies, tyrphostins were quantitated by HPLC. The elution profile of tyrphostins following HPLC is shown in Fig. 4 and retention times are shown in Table 1. Protein binding was determined by more than one method so as to validate the data. Tyrphostins B46 and B56 showed considerable binding to the ultrafiltration membranes. Ultrafiltration of tyrphostin B56 was therefore carried out using a visking tube as the ultrafiltration membrane (see Materials and methods). Tyrphostin B56 was dissolved in 25 m*M* HEPES buffer (pH 7.5) containing ethanol and DMSO in this experiment since this tyrphostin precipitated out in 25 m*M* HEPES buffer (pH 7.5) alone. Protein binding was also determined using a spectroscopic method. The ability to utilise this method is based on our observations that binding of tyrphostin to BSA induces a shift in the absorption spectrum as compared with the spectrum of free tyrphostin. The advantages of this method are (a) the speed of determination, thus minimising potential problems of instability of the tyrphostins, and (b) the absence of membrane binding. There was generally good agreement between the protein binding assessed using these various techniques (Table 1). Protein binding of the tyrohostins ranged from 64% to 98% with tyrphostin B56 being the most highly protein-bound (Table 1).

The tyrphostins examined in the present study are structurally similar apart from the Y group (see Table 1). Increasing the alkyl-chain length of the Y group will increase the hydrophobicity of the tyrphostin and this correlated with an increase in the elution time from the reverse-phase HPLC column. When the elution time from the reverse-phase HPLC

Table 1 Protein binding and HPLC retention times determined for tyrphostins (*UF* ultrafiltration, *Spec* spectrophotometric determination, *ED* equilibrium dialysis)

Tryphostin	Y-group structure	Protein binding (% ± S/E)	Method of determination	HPLC retention time (min)
A47	$C(S)NH_2$	67 ± 2	UF	5.5
		64 ± 4	Spec	
B42	$C(O)NHCH_2C_6H_5$	84 ± 1	UF	8.6
B50	$C(O)NHCH(CH)_3$ $C_6H_5(+)$	82 ± 2	UF	9.2
		87 ± 2	Spec	
		91 ± 1	ED	
B46	$C(O)NH(CH_2)_3C_6H_5$	95 ± 2	Spec	9.9
		97 ± 1	ED	
B56	$C(O)NH(CH_2)_4C_6H_5$	97 ± 2	Spec	10.7
		96 ± 1	UF	
		98 ± 1	ED	



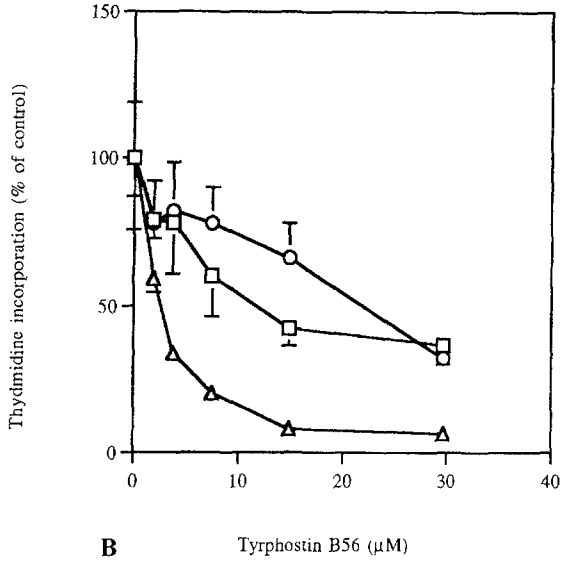


Fig. 3A, B Inhibition of the DNA synthesis of L23/P cells grown in serum by tyrphostins. Cells were plated-down overnight and then incubated in fresh medium containing 10% serum (□) 0.5% serum (△) or 0.5% serum supplemented with 3.5 mg BSA/ml (○) with **A** tyrphostin B46 or **B** tyrphostin B56 for 24 h and DNA synthesis was determined. Data represent mean values ± SD ($n = 3$). Error bars are smaller than the symbols when not shown. Typical control DNA-synthesis values obtained for cells in 10% serum, 0.5% serum and 0.5% serum supplemented with 3.5 mg BSA/ml were 4,516 ± 1,106, 1,946 ± 254 and 3,342 ± 630 cpm/well, respectively

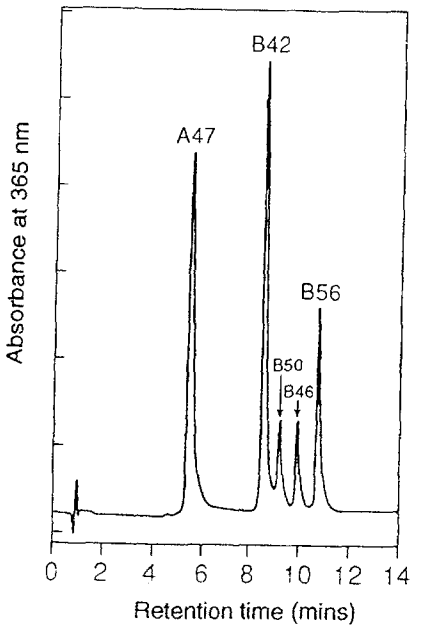


Fig. 4 HPLC trace of tyrphostins

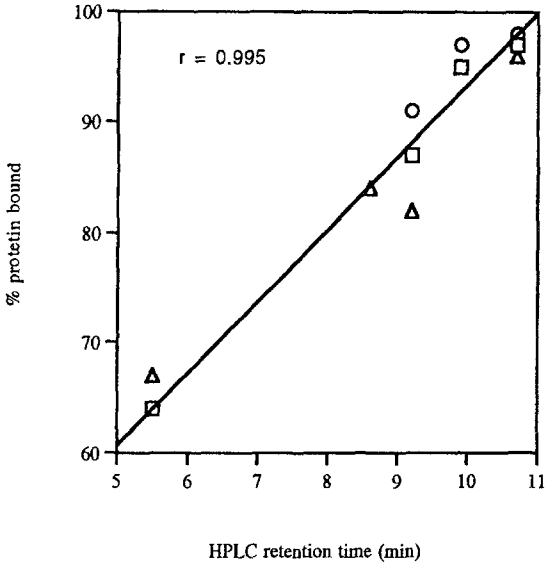
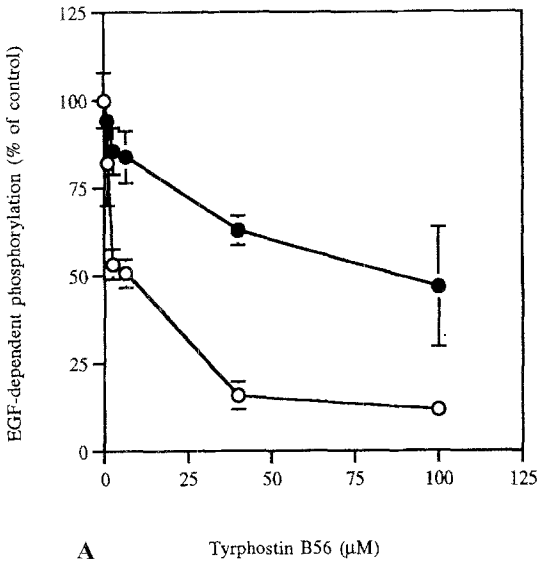


Fig. 5 Correlation between protein binding and hydrophobicity. Protein binding was determined by ultrafiltration (△), equilibrium dialysis (○)and spectroscopy (□)

column is used as an arbitrary measure of hydrophobicity, there is an excellent positive correlation ($R = 0.995$) between protein binding and hydrophobicity (Fig. 5).

Inhibition of EGF-receptor kinase activity

The effect of protein binding on the efficacy of the tyrphostins as tyrosine kinase inhibitors was studied by assaying the phosphorylation of polyGAT by EGF-receptor tyrosine kinase in the presence or absence of 3.5 mg BSA/ml. Tyrphostin B56 (highly protein-bound) was a potent inhibitor of the EGF-receptor kinase in the absence of BSA, and this inhibitory activity was significantly reduced in the presence of BSA (Fig. 6a). IC_{50} values (means ± SD) obtained in three separate



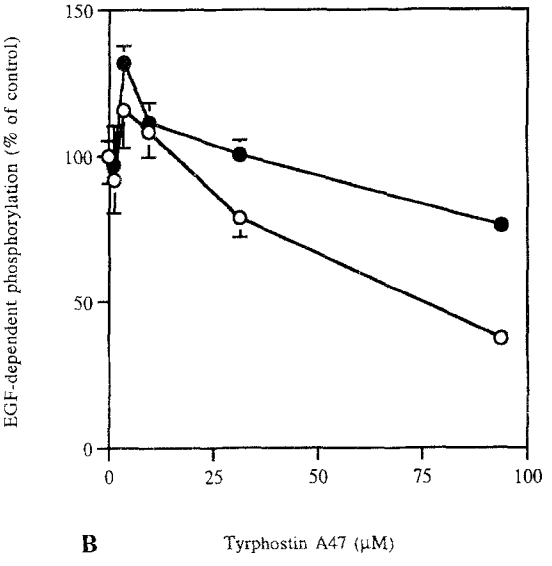


Fig. 6A, B Influence of BSA on the inhibition of polyGAT phosphorylation by tyrphostins. Tyrphostin B56 (**A**) or tyrphostin A47 (**B**) was incubated with EGF-receptor kinase in the absence of BSA (○) or the presence of 3.5 mg BSA/ml (●). Data represent mean values ± SD ($n = 3$). Error bars are smaller than the symbols when not shown.

experiments carried out in the presence and absence of BSA were 74 ± 16 and 12 ± 6 μ*M*, respectively. Tyrphostin A47 (RG 50864, weakly protein-bound) was a much weaker inhibitor of the EGF-receptor kinase and there was less of a reduction in this inhibitory activity in the presence of BSA than was the case for tyrphostin B56 (Fig. 6b). The final protein concentration of the EGF-receptor preparation in these experiments was 30 μg/ml, and this protein concentration did not significantly influence inhibition of the EGF-

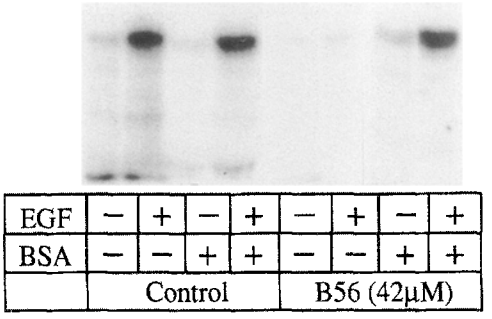


Fig. 7 Influence of BSA on the inhibition of EGF-receptor autophosphorylation by tyrphostin B56. A431 membranes were stimulated for 30 s with 100 ng EGF/ml in the presence or absence of 3.5 mg BSA/ml

receptor kinase by tyrphostin (data not shown). The importance of protein binding as a modulator of the in vitro activity of B56 was also studied by assaying EGF-receptor autophosphorylation. The presence of BSA substantially reduced the ability of B56 to inhibit EGF-receptor auto-phosphorylation (Fig. 7).

TGFα-stimulated DNA synthesis

Tyrphostin B56 inhibited DNA synthesis stimulated by TGFα (in PFM) to a greater extent than DNA synthesis stimulated by serum. For example, 15 μM tyrphostin B56 completely suppressed DNA synthesis stimulated by TGFα in PFM, whereas the cells retained approximately 40% of the DNA synthesis stimulated by serum at this concentration of tyrphostin (Fig. 8a). However, when the cells were stimulated by TGFα in PFM supplemented with 3.5 mg BSA/ml inhibition of DNA synthesis by tyrphostin B56 was reduced to values similar to those obseved for inhibition of serum-stimulated DNA synthesis (Fig. 8a). BSA also reduced the inhibition of TGFα-stimulated DNA synthesis by tyrphostin B46 to values similar to those obtained for serum-stimulated DNA synthesis (Fig. 8b). Overall, tyrphostin B46 was a more potent inhibitor of TGFα-stimulated DNA synthesis than was tyrphostin B56. For tyrphostin A47, there was greater inhibition of TGFα-stimulated DNA synthesis in PFM than inhibition of serum-stimulated DNA synthesis (Fig. 8c). However, in marked contrast to the results obtained with tyrphostins B46 and B56, inhibition of TGFα-stimulated DNA synthesis by tyrphostin A47 was significantly enhanced by the presence of BSA (Fig. 8c). Tyrphostins A47, B46 and B56 inhibited DNA synthesis of L23/P cells incubated in the absence of TGFα with IC_{50} values similar to those observed for inhibition of TGFα-stimulated cells (data not shown).

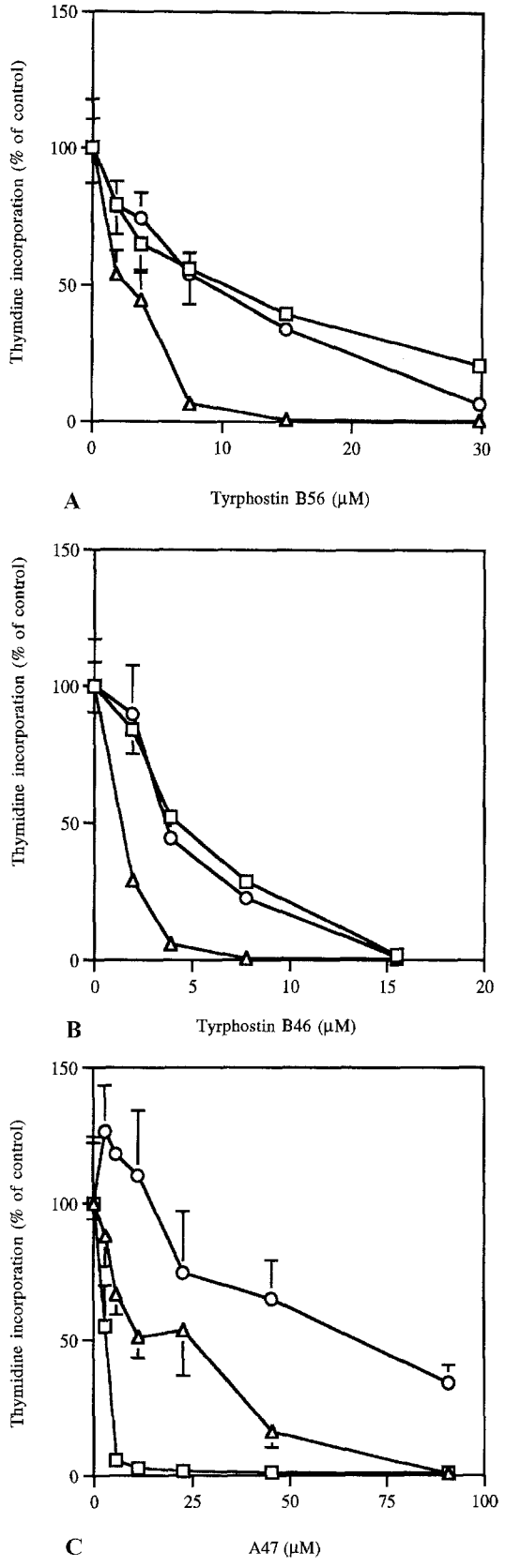


Fig. 8A–C Effect of BSA on the inhibition of TGFα-stimulated DNA synthesis of L23/P cells by tyrphostins. Cells were incubated for 6 days in 0.5% serum as described in Materials and methods and then treated with **A** tyrphostin B56 **B** tyrphostin B46 or **C** tyrphostin A47 in PFM containing TGFα (△), TGFα/BSA (□) or 10% serum (○). DNA synthesis was determined 24 h later. Data represent mean values ± SD ($n = 3$). Typical control values (cpm/well): PFM, 1,300 ± 47; TGFα, 3,205 ± 544; PFM/BSA 2,533 ± 427; TGFα/BSA, 4,771 ± 452; 10% FCS, 5,969 ± 518.

Discussion

This study indicates that the apparently weaker inhibition of serum-stimulated DNA synthesis of L23/P cells by tyrphostins B46 and B56 as compared with the inhibition of TGFα-stimulated DNA synthesis can be explained by protein binding of these tyrphostins in serum-containing medium. These tyrphostins were highly protein-bound. When protein binding is taken into consideration, tyrphostins B46 and B56 are not selective inhibitors of TGFα-stimulated DNA synthesis of L23/P cells. Protein binding reduced the efficacy of tyrphostin B56 in inhibiting autophosphorylation of the EGF receptor in a cell-free system and phosphorylation of a peptide substrate. Reduced inhibition of the EGF-receptor kinase by tyrphostins B46 and B56 due to protein binding may therefore contribute to the lack of selectivity of these tyrphostins for TGFα-stimulated DNA synthesis relative to serum-stimulated DNA synthesis. These findings indicate that protein binding should be taken into consideration when DNA synthesis stimulated by serum is being compared with DNA synthesis stimulated by growth factors in defined medium. In addition, differences in protein concentrations detected in EGF-receptor kinase assays may contribute to discrepancies reported by other workers regarding the relative potencies of tyrphostins [1].

Contrary to the results obtained for tyrphostins B46 and B56, the selectivity for inhibition of TGFα-stimulated DNA synthesis by tyrphostin A47 (RG 50864) was increased rather than decreased in the presence of BSA. A possible explanation is that BSA stabilises tyrphostin A47 in cell-culture medium, since it has been reported that A47 is unstable in tissue culture [17]. In our experiments, A47 was also only a weak inhibitor of the EGF-receptor kinase. BSA caused only a slight decrease in the inhibition of the EGF-receptor kinase, consistent with the weak protein binding of this tyrphostin. Thus, the enhanced inhibition of TGFα-stimulated DNA synthesis in the presence of BSA cannot be explained by a direct effect of BSA in increasing the potency of A47 as an inhibitor of the EGF-receptor kinase. Tyrphostin A47 was originally reported to be a potent inhibitor of EGF-receptor kinase activity in a peptide-phosphorylation assay [25], and Dvir et al. [6] reported an IC_{50} value of 8 μ*M* for inhibition of EGF-receptor autophosphorylation. However, Faaland et al. [7] reported that 100 μ*M* tyrphostin A47 did not inhibit EGF-receptor autophosphorylation. BSA

(0.7 mg/ml) was present in the assay described by Faaland et al. However, our data do not indicate for this tyrphostin that protein binding is a major factor modulating in vitro inhibition of the EGF-receptor kinase.

Inconsistencies between results obtained in EGF-receptor kinase inhibition studies and cell proliferation studies suggest that targets other than the EGF-receptor kinase may contribute to the anti-proliferative activities of some tyrphostins. For example, tyrphostin A47 was reported to accumulate in A431 cells to maximal levels within 1 h after treatment, whereas inhibition of EGF-receptor kinase was evident only 4–24 h later [7]. Similarly, inhibition of EGF-receptor kinase activity in HER14 cells by tyrphostin A47 required 16 h of pre-treatment [17]. Tyrphostin B46 is approximately 7-fold more potent as an inhibitor of EGF-receptor autophosphorylation than is tyrphostin B56 in a cell-free assay, but both tyrphostins have been reported to have comparable activities as inhibitors of EGF-dependent proliferation (IC_{50} values: 2.5 and 3 μM, respectively) [10]. However, our results are consistent with the enzyme-inhibition data since B46 was more potent than B56 as an inhibitor of TGFα-stimulated DNA synthesis of L23/P cells. The comparable inhibition of basal DNA synthesis as compared with the inhibition of TGFα stimulated by tyrphostins A47, B46 and B56 does, however, indicate that these agents may inhibit targets other than the EGF-receptor kinase. The basal DNA synthesis of L23/P cells does not appear to be due to an autocrine loop involving the EGF-receptor, since EGF-receptor antibody 528 blocked TGFα-stimulated DNA synthesis but did not block basal DNA synthesis. An antibody that blocks IGF-1-mediated DNA synthesis (clone αIR3) also had no effect on the basal DNA synthesis of L23/P cells (unpublished observations). A lack of selectivity for receptor kinases by tyrphostin RG-13022 was demonstrated by Reddy et al. [23]. This tyrphostin caused comparable inhibition of DNA synthesis of the breast line T47D stimulated with several different growth factors, and it was suggested that RG-13022 may inhibit a signaling molecule that represents a convergence point for different growth factors. It has also been shown that some tyrphostins inhibit the respiratory chain and other basic metabolic processes [27].

In our studies we addressed the issue of protein binding during the in vitro evaluation of tyrphostins. Since albumin is a major protein in calf serum responsible for protein binding, BSA at a concentration of 3.5 mg/ml was used as a protein equivalence for 10% calf serum. The influence of protein binding on the clinical utility of tyrphostins, however, should be addressed using human serum albumin since this sometimes shows binding properties different from those of BSA [12]. The 10-fold greater albumin concentration in plasma as compared with tissue-culture medium would indicate that some tyrphostins will be substantially protein-bound in man since the fraction of drug bound is related to the protein concentration. Thus, protein binding may be an important issue during the clinical development of certain tyrphostins [13].

Several mechanisms of protein binding have been described, including both ionic and hydrophobic interactions [12]. The positive correlation found between the hydrophobicity of the tyrphostins and the degree of protein binding suggests that hydrophobic interactions are an important component for the binding of tyrphostins to BSA.

Some of the recently developed tyrphostins appear to display more selective action against signal-transduction pathways than do the tyrphostins used in our study. For example, tyrphostin AG879 displayed remarkably selective action on the nerve-growth-factor pathway [21]. In addition, anti-tumour activity against human squamous-cell carcinoma xenografts in mice has been demonstrated for a tyrphostin [26]. Thus, clinical evaluation of some tyrphostins is now warranted. Our study indicates that protein binding should be taken into consideration during the evaluation of tyrphostins for future clinical use.

References

1. Barret JM, Ernould AP, Ferry G, Genton A, Boutin, JA (1993) Integrated system for the screening of the specificity of protein kinase inhibitors. Biochem Pharmacol 46: 439
2. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248
3. Chignell CF (1985) Protein binding. In: Garrett ER and Hirtz JL (eds) Drug fate and metabolism Marcel Dekker, New York Basel, pp 187–228
4. Dealtry GB, Balkwill FR (1987) Cell growth inhibition by interferons and tumour necrosis factor. In: (eds) Clemens MJ, Morris AG, Gearing AJH Lymphokines and interferons. IRL, Oxford, pp. 195–220
5. Dennis IF, Donaldson J Hoffman R (1994) Influence of protein binding on the activity of tyrphostins. Ann Oncol 5 [Suppl 5]: A 126
6. Dvir A, Milner Y, Chomsky O, Gilon C, Gaziy A, Levitzki A. (1991) The inhibition of EGF-dependent proliferation of keratinocytes by tyrphostin tyrosine kinase blockers. J Cell Biol 113: 857
7. Faaland CA, Mermelstein FH, Hayashi J, Laskin JD (1991) Rapid uptake of tyrphostin into A431 human epidermoid cells is followed by delayed inhibition of epidermal growth factor (EGF)-stimulated EGF-receptor tyrosine kinase activity. Mol. Cell Biol 11, 2697
8. Gazdar AF (1992) Molecular markers for the diagnosis and prognosis of lung cancer. Cancer 69 [Suppl]: 1592
9. Gazit A, Yaish P, Gilon C, Levitzki A (1989) Tyrphostins. 1. Synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem 32: 2344
10. Gazit A, Osherov N, Posner I, Yaish P, Poradosu E, Gilon C, Levitzki A (1991) Tyrphostins. 2. Heterocyclic and α-substituted benzylidenemalononitrile tyrphostins as potent inhibitors of EGF-receptor and ErbB2/neu tyrosine kinases. J Med Chem 34: 1896.
11. Gullick WJ (1991) Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers. Br Med Bull 47: 87
12. Koch-Weser J, Sellers EM (1976) Binding of drugs to serum albumin, part 1. N Engl J Med 294: 311

13. Koch-Weser J, Sellers EM (1976) Binding of drugs to serum albumin part 2. N Engl J Med 294: 526
14. Levitzki A (1992) Tyrphostins: tyrosine kinase blockers as novel antiproliferative agents and dissectors of signal transduction. FASEB J 6: 3275
15. Levitzki A, Gazit A, Osherov N, Posner I, Gilon C (1991) Inhibition of protein-tyrosine kinases by tyrphostins. Methods Enzymol 201: 347
16. Li P-M, Fukazawa H, Yamamoto C, Mizuno S, et al. (1993) Method of identifying inhibitors of oncogenic transformation: selective inhibition of cell growth in serum-free medium. Oncogene 8: 1731
17. Lyall RM, Zilberstein A, Gazit A, Gilon C, Levitzski A, Schlessinger J (1989) Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. J Biol Chem 264: 14503
18. Macaulay VM (1992) Insulin-like growth factors and cancer. Br J Cancer 65: 311
19. McPherson GA (1985) Analysis of radioligand binding experiments: a collection of computer programs for the IBM PC. J Pharmacol Methods 14: 213
20. Munson PJ, Rodbard D (1980) LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107: 220–239.
21. Ohmichi M, Pang L, Ribon V, Gazit A, Levitzki A, Saltiel AR (1993) The tyrosine kinase inhibitor tyrophostin blocks the cellular actions of nerve growth factor. Biochemistry 32: 4650
22. Powis G (1991) Signalling targets for anticancer drug development. Tips Rev Trends Pharmacol Sci 12: 188
23. Reddy KB, Mangold GL, Tandon AK, Yoneda T, Mundy GR, Zilberstein A (1992) Inhibition of breast cancer cell growth in vitro by a tyrosine kinase inhibitor. Cancer Res 52: 3636
24. Reinard T, Jacobsen H-J (1989) An inexpensive small volume equilibrium dialysis system for protein-ligand binding assays. Anal Biochem 176: 157
25. Yaish P, Gazit A, Gilon C, Levitzki A (1988) Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science 242: 933
26. Yoneda T, Lyall RM, Alsina MM, Persons PE, Spada AP, Levitzki A, Zilberstein A, Mundy GR (1991) The antiproliferative effects of tyrosine kinase inhibitors tyrophostins on a human squamous cell carcinoma in vitro and in nude mice. Cancer Res 51: 4430
27. Young SW, Poole RC, Hudson AT, Halestrap A, Denton RM, Tavare JM (1993) Effects of tyrosine kinase inhibitors on protein kinase-independent systems. FEBS Lett 316: 278