Tyrphostins Are Inhibitors of Guanylyl and Adenylyl Cyclases†

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ABSTRACT: Guanylyl cyclase C (GC-C), the receptor for guanylin, uroguanylin, and the heat-stable enterotoxin, regulates fluid balance in the intestine and extraintestinal tissues. The receptor has an extracellular domain, a single transmembrane spanning domain, and an intracellular domain that harbors a region homologous to protein kinases, followed by the C-terminal guanylyl cyclase domain. Adenine nucleotides can regulate the guanylyl cyclase activity of GC-C by binding to the intracellular kinase homology domain (KHD). In this study, we have tested the effect of several protein kinase inhibitors on GC-C activity and find that the tyrphostins, known to be tyrosine kinase inhibitors, could inhibit GC-C activity in vitro. Tyrphostin A25 (AG82) was the most potent inhibitor with an IC_{50} of ~15 μM. The mechanism of inhibition was found to be noncompetitive with respect to both the substrate MnGTP and the metal cofactor. Interestingly, the activity of the catalytic domain of GC-C (lacking the KHD) expressed in insect cells was also inhibited by tyrphostin A25 with an IC_{50} of ~5 μM. As with the full-length receptor, inhibition was found to be noncompetitive with respect to MnGTP. Inhibition was reversible, ruling out a covalent modification of the receptor. Structurally similar proteins such as the soluble guanylyl cyclase and the adenylyl cyclases were also inhibited by tyrphostin A25. Evaluation of a number of tyrphostins allowed us to identify the requirement of two vicinal hydroxyl groups in the tyrphostin for effective inhibition of cyclase activity. Therefore, our studies are the first to report that nucleotide cyclases are inhibited by tyrphostins and suggest that novel inhibitors based on the tyrphostin scaffold can be developed, which could aid in a greater understanding of nucleotide cyclase structure and function.

Membrane-associated guanylyl cyclases serve as receptors for a variety of peptide ligands. These receptors are found as dimers or higher order oligomers in the basal state (*1*−*3*). Ligand binding to the extracellular domain induces activation of the C-terminal catalytic domain that results in elevation of intracellular cGMP levels and subsequent signaling events, with no apparent change in the oligomeric status of the receptors. The intracellular juxtamembrane region bears significant sequence similarity to protein kinases and is called the kinase homology domain (KHD).[1] The C-terminus harbors the guanylyl cyclase catalytic domain. The KHD modulates signaling by these receptors, and its essentiality for ligand-mediated activation of guanylyl cyclase A and guanylyl cyclase C (GC-C) has been described (*3*−*5*).

GC-C is the receptor for a family of bacterial heat-stable enterotoxins and the guanylin family of peptides. GC-C is predominantly expressed in the intestine, but its expression has also been demonstrated in a number of extraintestinal tissues (*6*−*9*). Despite the presence of the KHD in GC-C, it is unlikely that this domain has protein kinase activity, since critical residues required for phosphotransfer activity in many kinases are absent from GC-C. Deletion of the KHD results in a form of GC-C that is no longer sensitive to ligand addition, indicating that the KHD plays a structural and regulatory role in the receptor and serves as a link between the extracellular domain and the intracellular catalytic domain (*10*). GC-C shows ATP-mediated potentiation of ligand-stimulated activity and ATP-mediated inhibition of detergent-stimulated activity (*3*, *11*−*13*). In our efforts to understand molecular aspects of the regulation of GC-C and with the evidence that GC-C activity is regulated by the KHD, we modeled the KHD of GC-C and showed that it can adopt a structure similar to protein kinases (*3*). Mutation of a critical lysine residue in GC-C, which is present in a position equivalent to that shown in protein kinases to be important for interaction with ATP, resulted in loss of ligand-stimulated activity, suggesting that the KHD of GC-C is a site for ATP binding (*3*). Therefore, compounds that could interact with the KHD may serve as regulators of GC-C signaling. We decided to investigate the effects of different protein kinase inhibitors that act as either ATP analogues or substrate mimics on GC-C activity. This report describes the effect of tyrphostins, potent tyrosine kinase inhibitors, on GC-C activity. We show here that tyrphostin A25, which is an inhibitor of receptor tyrosine kinases, is also an inhibitor of not only GC-C but also soluble guanylyl cyclase and adenylyl cyclases. Tyrphostin A25 binds to the catalytic domain of GC-C and inhibits the activity in a noncompetitive manner

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[1] Abbreviations: 2-ME, 2-mercaptoethanol; GC-C, guanylyl cyclase C; GC-C-Catbac, catalytic domain of GC-C expressed in insect cells; IgG, immunoglobulin G; IPTG, β-thiogalactopyranoside; KHD, kinase homology domain; PMSF, phenylmethanesulfonyl fluoride; RIA, radioimmunoassay; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; STh, stable toxin of the human variety.

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with respect to metal-GTP and free metal. Indeed, tyrphostin A25 is the most potent inhibitor of receptor guanylyl cyclases described to date. Using structurally diverse tyrphostins, it appears that a vicinal hydroxyl group is required for efficient inhibition. These results therefore extend the range of enzymes that are inhibited by tyrphostins and suggest that novel inhibitors of nucleotide cyclases could be obtained on the basis of the tyrphostin backbone.

MATERIALS AND METHODS

Tissue culture media were from Life Technologies. All fine chemicals were from Sigma-Aldrich. Protein G beads and ECL Plus Western blotting detection reagent were obtained from Amersham Biosciences. [^{125}I]Iodine and Western blot chemiluminescence reagent were from NEN Life Science Products. Stable toxin of the human variety (STh) and a mutant form of the ST peptide, ST_{Y72F}, were purified as described earlier (*14*, *15*). Restriction enzymes were obtained from New England Biolabs and MBI Fermentas. T84 cells (CCL 247) were obtained from the ATCC (Rockville, MD). Tyrphostins were obtained from Calbiochem.

Preparation of Cellular Extracts. A stable cell line of HEK293 cells expressing wild-type GC-C was generated, and the crude membrane fraction was isolated from cells as described earlier (*16*). Protein was estimated using a modification of the Bradford protein assay (*17*). To monitor the activity of the soluble guanylyl cyclase, the cytosolic fraction prepared from HEK293 cells was prepared as described earlier (*18*). Briefly, cell lysates were prepared from HEK293 cells, sonicated, and centrifuged at 100000*g* for 1 h at 4 °C. The supernatant (cytosol) was taken for further experiments.

Receptor-Binding Analysis. ST_{Y72F} was iodinated with Na^{125}I as described earlier (*15*). Membrane protein (20−30 μg) was incubated with ^{125}I-labeled ST_{Y72F} (100000 cpm) for 1 h at 37 °C in binding buffer (50 mM Hepes, pH 7.5, 4 mM $MgCl_2$, 0.1% BSA, 10 μg/mL leupeptin, 10 μg/mL aprotinin) in the absence or presence of unlabeled STh (100 nM). Following incubation, the reactions were filtered through GF-C filters, the filters were dried, and the associated radioactivity was counted in a γ counter.

Purification of GC-C-IDbac. Procedures for the expression of the intracellular domain of GC-C in Sf21 cells have been described earlier (*19*). The recombinant protein was purified essentially as described earlier and used for guanylyl cyclase assays in the absence or presence of tyrphostin A25 and MnGTP as substrate as described earlier (*19*) and below.

Guanylyl and Adenylyl Cyclase Assays. Guanylyl cyclase assays were performed as described earlier (*16*) in a buffer consisting of 60 mM Tris-HCl (pH 7.6), containing 500 μM isobutylmethylxanthine and a GTP regenerating system of 20 μg of creatine phosphokinase and 7.5 mM creatine phosphate in a total volume of 100 μL. All control assays included 1% DMSO to account for the carryover of the solvent in experiments performed with tyrphostins or other kinase inhibitors.

To monitor the effect of kinase inhibitors on the activity of GC-C with MnGTP as a substrate, 10 μg of membrane protein was incubated in assay buffer in the absence or presence of ATP (1 mM) and kinase inhibitors genistein (100 μM), tyrphostin A25 (100 μM), and H7 (100 μM) for 10 min at 4 °C. Substrate (1 mM GTP and 10 mM $MnCl_2$) was added and incubation continued at 37 °C for 10 min. For assays that monitored tyrphostin effects on ligand-stimulated activity, membrane protein was first incubated in the absence or presence of tyrphostin for 10 min at 4 °C, then 100 nM STh along with substrate (1 mM GTP and 10 mM $MgCl_2$) was added, and incubation was continued at 37 °C for 10 min. The reaction was terminated by the addition of 50 mM sodium acetate (pH 4.6), samples were heated in a boiling water bath for 5 min, and the supernatant was assayed for cGMP by a modified RIA procedure as described earlier (*15*).

Soluble guanylyl cyclase (sGC) was assayed with 20 μg of protein of the cytosol prepared from HEK293 cells after preincubation in the presence of varying concentrations of tyrphostin A25 for 10 min at 4 °C, followed by stimulation with 100 μM sodium nitroprusside (SNP) along with substrate (1 mM GTP and 10 mM $MgCl_2$). Incubation was continued for a further 10 min at 37 °C, the reaction was terminated by the addition of 50 mM sodium acetate buffer (pH 4.6), and samples were taken for RIA as described above.

Adenylyl cyclase assays were performed with membranes from HEK293 cells, prepared as described above. Membrane protein (5−10 μg) was incubated with 1 mM metal-ATP and 10 mM free metal in the presence of an ATP regenerating system in the presence or absence of tyrphostin A25. Assays were performed at 37 °C as described earlier (*20*) in a buffer consisting of 50 mM Hepes (pH 7.5), 10% glycerol, and 1 mM dithiothreitol in a total volume of 50 μL and an ATP regenerating system as described above. The reaction was terminated by the addition of 50 mM sodium acetate (pH 4.6), and cAMP produced was measured by RIA as described earlier (*20*).

Guanylyl cyclase assays performed with GC-C-Catbac (see below) were performed at 25 °C in the presence of 20 mM free Mn^{2+} and varying concentrations of MnGTP as indicated and initiated by the addition of enzyme. Assays were incubated for 10 min and stopped by the addition of 50 mM sodium acetate buffer as described above.

Assays for assessing the interactions of tyrphostin and the substrates (metal or nucleotide) were carried out after accounting for the binding of metal to the nucleotide that generates metal nucleotide, which is the true substrate. WinmaxC [http://www.stanford.edu/~cpatton/maxc.html (*21*)] was used for calculations of concentrations of metal nucleotide and free metal during assay conditions.

Experiments presented were repeated at least three times. Nonlinear regression analysis of the data was performed using Graph Pad Prism 4 Software (San Diego, CA).

Expression of the Catalytic Domain of GC-C. Expression of GC-C-Catbac was achieved using the Bac-Bac baculovirus expression system (Gibco BRL). The catalytic domain of GC-C including the C-terminal domain and part of the linker region (beginning from Pro732 in full-length human GC-C and continuing to the C-terminus of the protein) known as GCC:GCD was generated as described earlier (*22*). A *Xho*I−*Hin*dIII fragment from this clone was excised and cloned into pFastBac HTb to generate a clone that would express the intracellular domain of GC-C, fused to a hexahistidine tag at the N-terminus, present in the vector. The recombinant bacmid DNA in *Escherichia coli* DH10Bac was generated as per the manufacturer's instructions. Bacmid DNA was

transfected into Sf21 cells with Cellfectin reagent (Invitrogen), and virus was harvested from the culture supernatant 72 h following transfection. Infected Sf21 cells were harvested 72 h following infection, and extracts were prepared as described earlier (*19*). In brief, cells grown in a 10 cm dish were lysed in 1 mL of lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 5 mM 2-mercaptoethanol, 5 μg/mL leupeptin, and 5 μg/mL aprotinin). Cells were briefly sonicated and centrifuged at 12000*g* for 45 min at 4 °C. The supernatant containing the expressed protein was taken for guanylyl cyclase assays. Expression of the catalytic domain of GC-C was confirmed by Western blot analysis using IgG (5 μg/ mL) purified from an antiserum raised to the C-terminus of GC-C that has been described earlier (*3*).

Assessment of the Reversible Nature of Tryphostin Binding to GC-C-Catbac. Enzyme preparations were preincubated with or without tyrphostin A25 (50 μM) for 10 min at 25 °C and then diluted 10-fold in assay buffer. Samples were then assayed immediately, or after a further incubation for 10 min at 25 °C, in the absence or presence of 5 μM tyrphostin A25. The reaction was initiated by addition of substrate (20 mM $MnCl_2$ and 250 μM GTP) and allowed to proceed for 10 min at 25 °C. cGMP produced was measured by radioimmunoassay as described above.

Cloning, Expression, and Purification of the IC_1-IIC_2 Fusion Protein in E. coli. The pTrc(273)IC_1IIC_2 L3 plasmid [kind gift of Dr. A. G. Gilman, University of Texas (*23*)] was used to release a *Bsp*HI and *Hin*dIII fragment which was ligated to the *Nco*I- and *Hin*dIII-digested pPRO-ExHTA vector (Invitrogen Life Technologies) to obtain the plasmid p$PROIC_1IIC_2$ L3. Protein was produced using the *E. coli* Rosetta(DE3)pLysS strain and purified by immobilized metal affinity chromatography. Cells were grown at 25 °C and induced using 50 μM IPTG for 18 h. Cells were lysed in buffer containing 25 mM Tris-HCl, pH 8.2, 5 mM 2-ME, 100 mM NaCl, 2 mM PMSF, 5 μg/mL soybean trypsin inhibitor, and 5 μg/mL aprotinin by sonication. The supernatant obtained following centrifugation at 30000*g* was bound to Ni-NTA−agarose (Quiagen). The column was washed with buffer containing 500 mM NaCl followed by washes with buffer containing 200 mM Tris-HCl, pH 8.2, 5 mM 2-ME, and 10 mM imidazole. Protein was eluted with 500 mM Tris-HCl, pH 8.2, 5 mM 2-ME, 500 mM imidazole, and 10% glycerol and desalted into 25 mM Tris-HCl, pH 8.2, and 5 mM 2-ME using a HiTrap desalting column on an AKTA FPLC system (Amersham Biosciences). The purified IC_1IIC_2 protein was stored in 11 mM CHAPS, 2 mM $MgCl_2$, and 1 mM EDTA at −70 °C prior to use in assays. Assays were carried out using 200 ng of total protein in a buffer containing 60 mM Hepes−NaOH, pH 7.5, 1 mM DTT, and 10% glycerol at 30 °C for 10 min. Assays contained 1 mM metal-ATP and 10 mM free metal Mn). Western blot analysis was carried out following SDS−PAGE in 12% gels and using an anti-hexahistidine monoclonal antibody (Amersham Biosciences) and enhanced chemiluminescence.

RESULTS

Effect of Protein Kinase Inhibitors on GC-C Activity. Earlier reports have suggested that ATP inhibits the guanylyl cyclase activity of GC-C when MnGTP is used as substrate (*3, 24*). Other nucleotides such as ATPγS and AMP-PNP also have similar effects on GC-C. Since ATP is believed to bind to the KHD of GC-C, we tested the catalytic activity of GC-C in the presence of protein kinase inhibitors that have been shown to act as ATP analogues. The concentrations used were those that are employed routinely in studies involving these inhibitors. Using membranes prepared from HEK293−hGC-C cells, we observed that neither genistein (a tyrosine kinase inhibitor) nor H7 (a general serine-threonine kinase inhibitor) inhibited cGMP production when MnGTP was used as a substrate (Figure 1). Interestingly, tyrphostin A25 (an inhibitor of the EGF receptor) inhibited the guanylyl cyclase activity almost completely.

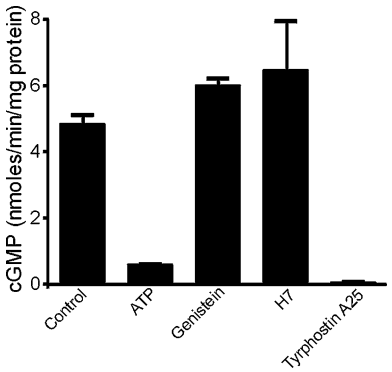


FIGURE 1: Effect of various kinase inhibitors on MnGTP-mediated GC activity. Membrane protein (10 μg) prepared from HEK293−hGCC cells was incubated with the indicated kinase inhibitors (100 μM) or ATP (1mM) for 10 min at 4 °C prior to addition of 1 mM GTP and 10 mM $MnCl_2$ as substrate. Incubation was continued for 10 min at 37 °C, and the amount of cGMP produced was monitored by RIA. Experiments were repeated three times in duplicate, and values represent the mean ± SEM.

T84 cells harbor high levels of GC-C and show a high fold increase of guanylyl cyclase activity in the presence of ligand (*15*). Tyrphostin was able to inhibit ligand-stimulated guanylyl cyclase activity with an IC_{50} of ∼18 μM with MgGTP as substrate, without affecting the ability of ST to bind to the receptor (Figure 2A). As shown in Figure 2B, the IC_{50} for inhibition was ∼16 μM using MnGTP as substrate.

Mechanism of Inhibition of Guanylyl Cyclase Activity by Tyrphostin A25. The catalytic mechanism of class III nucleotide cyclases is believed to involve two metal ions, one required for complex formation with GTP, which generates the true substrate (metal-GTP), and the second free metal ion involved in proton abstraction during the cyclization reaction (*25*). We therefore decided to perform assays in the presence of a fixed concentration of the MnGTP substrate at different concentrations of free metal in the absence or presence of tyrphostin A25. We observed that the IC_{50} for tyrphostin A25 inhibition did not change appreciably under these conditions (Figure 3), indicating that tyrphostin A25 did not compete for the binding site of the free metal.

To assess the behavior of tyrphostin A25 with respect to MnGTP, assays were performed by varying MnGTP concentrations at a fixed concentration of free metal (20 mM Mn) and in the absence or presence of tyrphostin A25. As shown in Figure 4, analysis of the reaction kinetics indicated slight cooperativity (Hill coefficient of 1.2−1.4) for all conditions. There was also no significant change in the

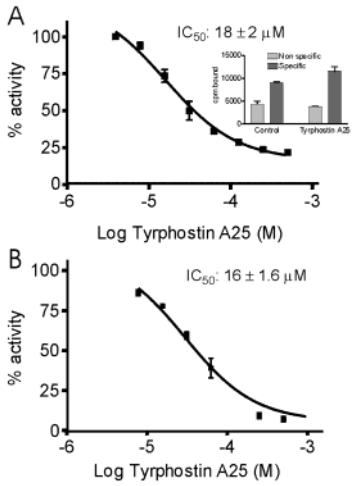


FIGURE 2: Effect of tyrphostin A25 GC-C activity. (A) Membrane protein (10 μg) prepared from T84 cells was incubated with varying concentrations of tyrphostin A25 at 4 °C, STh (10^{-7} M) was added along with 1 mM GTP and 10 mM $MgCl_2$, and incubation was continued at 37 °C for 10 min. The activity observed is shown as the percentage of the activity in the absence of tyrphostin A25, which was 700 pmol of cGMP produced min^{-1} (mg of protein)$^{-1}$. Experiments were repeated three times in duplicate, and values represent the mean ± SEM. Inset: T84 (50 μg) membrane was incubated in the absence or presence of tyrphostin A25 (200 μM) for 1 h at 4 °C, followed by the addition of radiolabeled ST_{Y72F}, in the presence (nonspecific) or absence (specific) of unlabeled STh. Bound radioligand was monitored. Experiments were repeated three times in duplicate, and values represent the mean ± SEM. (B) Membrane protein (10 μg) prepared from HEK293−hGCC cells was incubated with varying concentrations of tyrphostin A25 at 4 °C, and 1 mM GTP and 10 mM $MnCl_2$ as substrate were added. Incubation was continued at 37 °C for 10 min. Activity detected is the percentage of the activity observed in the absence of tyrphostin A25, which was 3600 pmol of cGMP produced min^{-1} (mg of protein)$^{-1}$. Experiments were repeated three times in duplicate, and values represent the mean ± SEM.

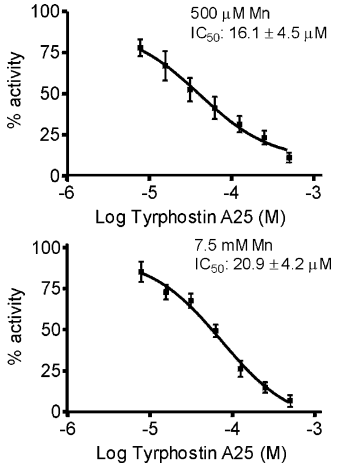


FIGURE 3: Effect of free manganese on tyrphostin A25-mediated inhibition of guanylyl cyclase activity. Membrane protein prepared from HEK293−hGCC cells was incubated with varying concentrations of tyrphostin A25 for 10 min at 4 °C and then further incubated at 25 °C with two fixed concentrations of free metal (500 μM and 7.5 mM $MnCl_2$) at the same concentration (580 μM) of MnGTP. Activity detected is depicted as the percentage of the activity observed in the absence of tyrphostin A25, which was 300 pmol of cGMP produced min^{-1} (mg of protein)$^{-1}$ in the presence of 500 μM $MnCl_2$ and 1200 pmol of cGMP min^{-1} (mg of protein)$^{-1}$ in the presence of 7.5 mM $MnCl_2$. Experiments were repeated twice in duplicate, and values represent the mean ± SEM. Note the similarity in IC_{50} in both cases and in Figure 2C.

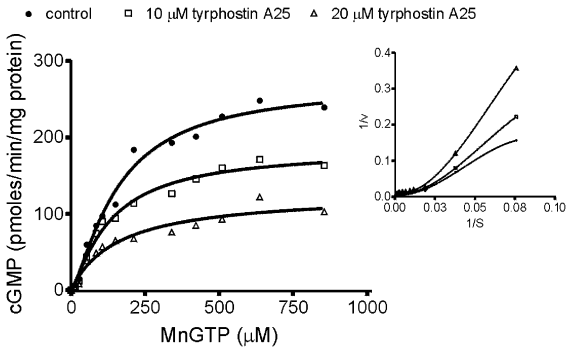


FIGURE 4: Mechanism of inhibition of guanylyl cyclase activity by tyrphostin A25. Kinetic analysis of GC-C catalytic activity in the presence of tyrphostin A25. Membrane protein (10 μg) was assayed at 25 °C, in the presence of 20 mM free Mn and varying concentrations of MnGTP as indicated, in the absence or presence of either 10 or 20 μM tyrphostin A25. Data shown are representative of assays performed three times. Nonlinear regression analysis of the data was performed using GraphPad Prism4 and fitted to the Hill equation. Inset: Double reciprocal plot of data is shown as inset.

apparent K'_{MnGTP} (control, 147 ± 12 μM; 10 μM tyrphostin A25, 123 ± 5 μM; 20 μM tyrphostin A25, 135 ± 12 μM). However, a significant decrease in the apparent V_{max} was observed in the presence of tyrphostin A25, indicating that the inhibition was noncompetitive.

To show that the inhibition of cyclase activity by tyrphostin A25 was by direct binding to GC-C and not via a protein that could associate with GC-C, we purified the intracellular domain of GC-C which was expressed in insect cells along methods that we had described earlier. Figure 5 shows that tyrphostin A25 was able to inhibit the activity of the purified protein with an IC_{50} of 13 μM, indicating that tyrphostin A25 was able to bind directly to GC-C and did not require interaction with an associated protein which in turn regulated GC-C.

The noncompetitive inhibition observed suggested that binding of tyrphostin possibly occurs at a site distinct from the substrate-binding site, perhaps to the KHD of GC-C. This hypothesis is plausible, since there are a number of reports that show that the KHDs of receptor guanylyl cyclases regulate the catalytic domain. To test this hypothesis, we proceeded to clone and express a protein comprising only the catalytic domain of GC-C.

Tyrphostin A25 Interacts with the Catalytic Domain of GC-C. Since the catalytic domain proved problematic to express in bacterial cells in an active form (data not shown), we expressed the protein (GC-C-Catbac) in insect cells. Lysates prepared from Sf21 cells infected with baculovirus encoding the catalytic domain of GC-C showed significant guanylyl cyclase activity, and a protein of the expected size (M_r 43000) was detected by Western blot analysis with an antibody to the C-terminal domain of GC-C (Figure 6A). The expressed protein possessed guanylyl cyclase activity, and interestingly, tyrphostin A25 was able to inhibit the activity with an IC_{50} of ~5 μM (Figure 6B).

Kinetic analysis was performed in the presence of varying concentrations of MnGTP as substrate, with a fixed concentration of free metal ion (20 mM), in the presence and absence of varying concentrations of tyrphostin A25 (Figure 7). The enzyme interestingly showed a greater degree of cooperative behavior than the full-length receptor (Hill coefficient for control, 2.4 ± 0.4; 5 μM tyrphostin, 2.4 ±

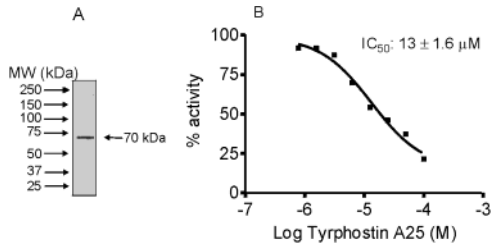


FIGURE 5: Tyrphostin can interact directly with GC-C. Purification of GC-C-IDbac from Sf21 cells was performed as described earlier (*19*). (A) Coomassie-stained gel of the purified protein. (B) Purified protein was used for assays in the presence of varying concentrations of tyrphostin A25 as indicated and using MnGTP as substrate. Data shown in representative of assays repeated three times.

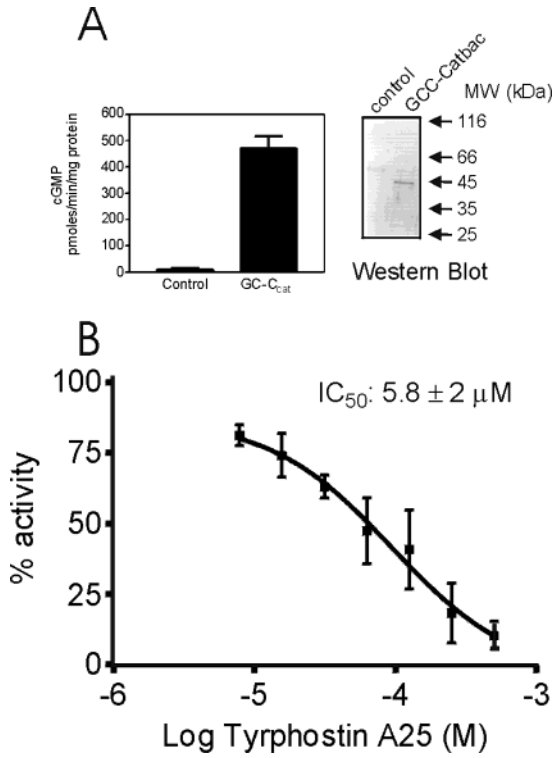


FIGURE 6: Tyrphostin A25 interacts with the catalytic domain of GC-C. (A) Guanylyl cyclase activity was monitored with MnGTP as substrate, with lysate (10 μg of protein) prepared from infected and noninfected cells, prepared 72 h after infection. Experiments were repeated twice in duplicate, and the data shown are the mean ± SEM. Western blot analysis to monitor the expression of the catalytic domain of GC-C in Sf21 cells was performed with crude lysate protein (20 μg) prepared from cells infected with recombinant virus. The antibody was raised to the C-terminal domain of GC-C (*16*). A protein of M_r 43000 was detected only in lysates prepared from infected cells and was not seen in uninfected Sf21 cells. (B) Crude lysate protein (10 μg) from infected cells was incubated in the presence of varying concentrations of tyrphostin A25 for 10 min at 4 °C and further incubated with 1 mM GTP and 10 mM $MnCl_2$ at 37 °C. Activity detected is depicted as the percentage of the activity observed in the absence of tyrphostin A25 [~200 pmol of cGMP min^{-1} (mg of protein)$^{-1}$]. Assays were performed three times with duplicate determinations, and data shown are the mean ± SEM.

0.2; 10 μM tyrphostin, 2 ± 0.1) while the apparent K'_{MnGTP} remained similar at all concentrations (control, 89 ± 14.1 μM; 5 μM tyrphostin, 89 ± 7.7 μM; 10 μM tyrphostin, 90 ± 6.1 μM). There was a decrease in the apparent V_{max} in the presence of tyrphostin, showing that the inhibition was noncompetitive with respect to the substrate MnGTP, in a manner similar to the full-length receptor. Therefore, the

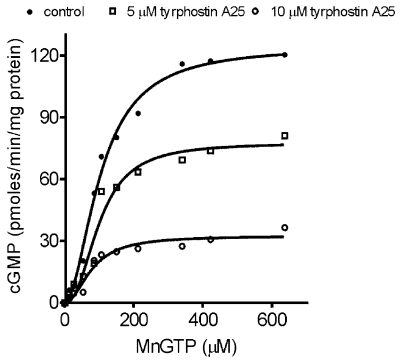


FIGURE 7: Kinetic analysis of activity of the guanylyl cyclase domain of GC-C in the presence of tyrphostin A25. Crude lysate protein (10 μg) from infected Sf21 cells was taken for assay in the presence of the indicated concentration of tyrphostin A25 and varying concentrations of MnGTP and 20 mM free $MnCl_2$ in the presence or absence of 5 or 10 μM tyrphostin as indicated. Data shown are representative of assays performed three times.

binding site for tyrphostin A25 lay in the catalytic domain of GC-C, probably distinct from the region where the substrate binds. The lowering in the apparent K'_{MnGTP} for the catalytic domain protein when compared to the full-length receptor could be reflective of the increase in the cooperativity of the catalytic sites and a likely consequence of this alteration in the Hill coefficient.

Tyrphostin Binds Reversibly to GC-C. The noncompetitive inhibition of the guanylyl cyclase activity observed with tyrphostin A25 could be due to irreversible binding of tyrphostin, leading to covalent modification of the receptor. To investigate this, we preincubated GC-C-Catbac with 50 μM tyrphostin A25 (a concentration that inhibits the activity by ~90%; Figure 6B) for 10 min at 25 °C. The preparation was then diluted 10-fold and assayed immediately or after incubation of the diluted enzyme−inhibitor mix for a further 5 min prior to addition of substrate. Controls included enzyme that had not been preincubated with tyrphostin A 25 and then assayed in the presence of 5 μM tyrphostin A25. As shown in Table 1, the >90% inhibition observed in the presence of 50 μM tyrphostin A25 was appreciably reduced on dilution and further incubation and approached the inhibition that would be expected in the presence of 5 μM tyrphostin A25. This indicated the reversible nature of interaction of tryphostin A25 with the enzyme.

Tyrphostin A25 Inhibits both Soluble Guanylyl Cyclase Activity and Adenylyl Cyclase Activity. Since tyrphostin A25 inhibition of GC-C is brought about by binding to the cyclase domain, we were prompted to investigate whether proteins which have structurally similar domains i.e., the class III nucleotide cyclase domain (*26*), were also inhibited. We investigated the effect of tyrphostin A25 on the cytosolic soluble guanylyl cyclases (sGCs) that are activated by nitric oxide. Lysates from HEK293 cells that express sGC were prepared, and sGC was activated by addition of SNP. As shown in Figure 8A, the guanylyl cyclase activity could be inhibited by tyrphostin A25, with an IC_{50} of ~34 μM. These results show for the first time that sGCs are also targets for tyrphostin A25, extending observations that were made with retinal GC earlier (*27*).

Given the similarity in primary amino acid sequence among guanylyl cyclases, it could be expected that the tyrphostin-binding site in the soluble guanylyl cyclase could

Table 1: Inhibition of GC-C-Catbac Activity by Tyrphostin A25 Is Reversible by Dilution[a]

tyrphostin A25 concn			
during preincubation (μM)	during guanylyl cyclase assay (μM)	incubation prior to assay	cGMP [pmol min^{-1} (mg of protein)$^{-1}$]
0	0	−	31.2 ± 0.5
0	0	+	28.7 ± 0.9
0	50	−	4.0 ± 0.7
0	50	+	3.7 ± 0.7
0	5	−	17.2 ± 0.7
0	5	+	17.2 ± 0.4
50	5	−	6.7 ± 0.2
50	5	+	15.9 ± 0.9

[a] Lysates from infected Sf21 cells were assayed directly (i.e., without preincubation) in the absence or presence of tyrphostin A25 (5 and 50 mM) or after incubation with tyrphostin A25 (5 and 50 mM) for 10 min at 25 °C prior to assay. In a parallel set, enzyme was preincubated with 50 mM tyrphostin A25 for 10 min at 25 °C, diluted 1:10 times with assay buffer, and then either assayed directly or after incubation at 25 °C for a further 10 min prior to assay. All assays were initiated by addition of substrate (20 mM $MnCl_2$ and 250 mM GTP). Assays were performed twice in duplicate, and data shown are the mean ± SEM.

be conserved in receptor guanylyl cyclases. However, there is also significant similarity between the adenylyl and guanylyl cyclases (~30% identity between human GC-C and human membrane bound adenylyl cyclases) which allows these enzymes to be classified as members of the class III nucleotide cyclase family (*28*, *29*). Since tyrphostin A25 could bind to the catalytic domain of guanylyl cyclases, we investigated if tyrphostin A25 could inhibit adenylyl cyclase activity. HEK293 cells have been shown to express all isoforms of adenylyl cyclases except ACIV and ACVIII (*30*). Adenylyl cyclase assays were therefore performed with membrane preparations from HEK293 cells in the presence of tyrphostin A25 with MnATP as substrate. As shown in Figure 8B, tyrphostin A25 inhibited the adenylyl cyclase activity with an IC_{50} of 119 μM. This value could be high due to the expression of multiple adenylyl cyclase isoforms in HEK293 cells (*30*) or because of differences in the structure of the tyrphostin-binding site in adenylyl cyclases.

All but one isoform (AC1) of mammalian adenylyl cyclases are activated by forskolin (*25*). Tyrphostin A25 was a poor inhibitor of forskolin-stimulated adenylyl cyclase activity, and as shown in Figure 8C, tyrphostin A25 inhibited the forskolin-stimulated adenylyl cyclase activity with an IC_{50} of ~1 mM.

Expression of a single mammalian adenylyl cyclase isoform, HEK293 cells, was inefficient, and the high level of activity observed in untransfected cells did not allow us to determine if tyrphostin A25 could inhibit a specific isoform of adenylyl cyclase (data not shown). Mammalian adenylyl cyclases contain two domains with similarity to each other, called C1 and C2, which dimerize to form the catalytic center (*31*). A recombinant protein consisting of the C1 domain of bovine type I adenylyl cyclase and the C2 domain of rat type II adenylyl cyclases fused in tandem has been shown to possess the properties of the full-length proteins from which the individual domains were derived (*23*, *32*, *33*). We therefore expressed this protein in *E. coli* fused to a hexahistidine tag at the N-terminus and partially purified the protein through metal affinity chromatography. Figure

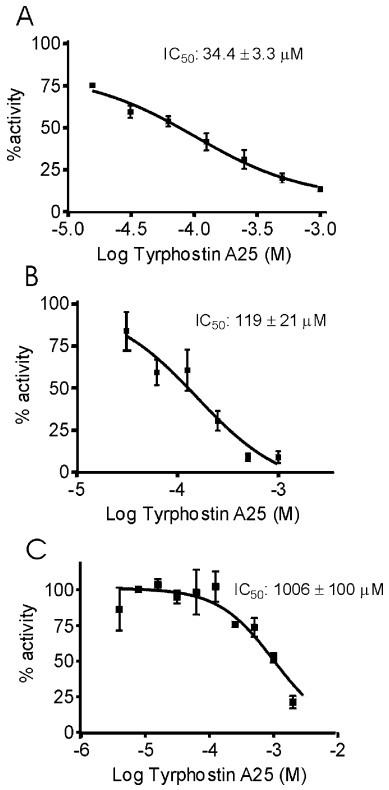


FIGURE 8: Effect of tyrphostin A25 on soluble guanylyl cyclase activity and adenylyl cyclase activity. Cytosol protein (20 μg) from HEK293 cells was preincubated with the indicated concentrations of tyrphostin A25 or alone for 10 min at 4 °C prior to addition and further incubation with 100 μM SNP, 1 mM GTP, and 10 mM Mn. The activity observed is shown as the percentage of the activity in the absence of tyrphostin A25, which was 200 pmol of cGMP produced min^{-1} (mg of protein)$^{-1}$. Assays were performed twice in duplicate, and data shown are the mean ± SEM. (B) Adenylyl cyclase assays were performed on membranes (10 μg of protein) from HEK293 cells using 1 mM MnATP and 10 mM free Mn at varying concentrations of tyrphostin A25. Activity is depicted as the percentage of activity observed in the absence of tyrphostin A25, which was 48 pmol of cAMP produced min^{-1} (mg of protein)$^{-1}$. Data shown are representative of assays performed three times. The IC_{50} value indicates the mean ± SEM from three independent experiments. (C) Adenylyl cyclase assays were performed on membranes (5 μg) in the presence of 100 μM forskolin and 10 mM free Mg and 1 mM MgATP as substrate in the presence of varying concentrations of tyrphostin A25. Activity is depicted as the percentage of activity observed in the absence of tyrphostin A25, which was ~800 pmol of cAMP produced min^{-1} (mg of protein)$^{-1}$. Data shown are the mean and range of duplicate determinations, representative of experiments performed three times.

9A shows a silver-stained gel of the purified protein and a Western blot using anti-His antibody. A protein of MW ~58 kDa, corresponding to the size of the recombinant protein, was present in the purified preparation, along with a band of MW ~30 kDa, which presumably represents the C1 fragment, which has been shown to proteolyze from the fused C2 protein in earlier reports (*33*). As shown in Figure 9B, tyrphostin A25 was able to inhibit the activity of this C1−C2 construct with an IC_{50} of ~16 μM, which was lower than that for the mixture of full-length mammalian proteins expressed in HEK293 cells. Whether this is due to a differential interaction of tyrphostin A25 with different isoforms of adenylyl cyclases or due to a slightly altered structure of the "artificial" C1−C2 domain protein, these results indicate that binding of tyrphostin A25 appears to be

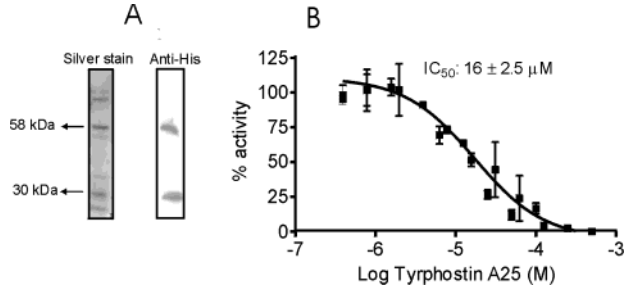


FIGURE 9: Activity of a C1−C2 fusion protein is inhibited by tyrphostin A25. A protein corresponding to the C1 domain of dog ACI and the C2 domain of rabbit ACII was expressed in *E. coli* and partially purified. (A) Silver staining and Western blot analysis of the purified preparation using anti-H6 antibody. (B) Assays were performed with partially purified protein using manganese as the metal cofactor in the presence of varying concentrations of tyrpostin A25. Activity is depicted as the percentage of activity observed in the absence of tyrphostin A25, which was ~1 nmol of cAMP produced min^{-1} (mg of protein)$^{-1}$. Data shown are the mean and range of duplicate determinations and are representative of experiments performed three times.

specific to a structural feature in the catalytic site of nucleotide cyclases in general.

Tyrphostin A25 inhibited the forskolin-stimulated activity of the C1−C2 construct very poorly with an IC_{50} of 500 ± 90 μM (data not shown).

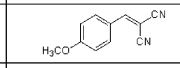
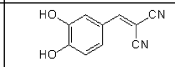
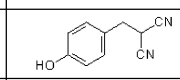
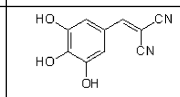
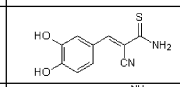
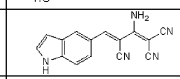
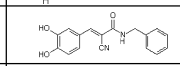
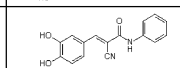
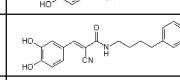
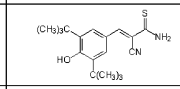
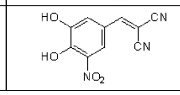
Extent of Inhibition Is Determined by the Structure of the Tyrphostin. To evaluate the structural determinants required for inhibition of guanylyl cyclase activity by tyrphostins, we monitored the effects of a variety of tyrphostins in bringing about the inhibition of the guanylyl cyclase activity of the catalytic domain of GC-C. As shown in Table 2, some tyrphostins inhibited the activity poorly even at concentrations >200 μM, whereas others were able to inhibit cGMP production significantly at lower concentrations. Tyrphostin A25 has three hydroxyl groups (at positions 3, 4, and 5) on the cyanocinnamonitrile scaffold that seem to be important in bringing about most effective inhibition. Derivatives of this or related compounds with two hydroxyl groups such as AG18 and AG213 (OH at positions 3 and 4) were effective to similar extents. AG43 with a single hydroxyl group at position 4 was ineffective. Modifications, such as the presence of the indole group (AG370) or di-*tert*-butyl groups (AG879), were not tolerated. Importantly, compounds with intact 3,4-dihydroxyl groups (AG490, AG494, and AG556) were effective despite their large structures. The importance of the 5-hydroxyl group of tyrphostin A25 is evident from the fact that a change to 5-nitro, as in the AG1288 compound, makes it less effective as an inhibitor.

We tested this by using AG9 (tyrphostin 1) that lacks all three hydroxyl groups and has a 4-methoxy group and found it to be inactive against both guanylyl and adenylyl cyclases (data not shown). The hydroxyl groups probably act by forming critical hydrogen bonds at the tyrphostin-binding site and appear to be essential for inhibition. Therefore, our results suggest that tyrphostins could be used as templates to design novel inhibitors of nucleotide cyclases.

DISCUSSION

Tyrphostins have been extensively used as inhibitors of protein tyrosine kinases, and a large number of compounds are available with different specificities for different kinases.

Table 2: Inhibition of GC-C by Various Tyrphostins[a]

Name	Structure	IC_{50} (μM)
AG 9		>200
AG 18		8 ± 1
AG 43		>200
AG 82 (Tyrphostin A25)		5.8 ± 2
AG 213		8.1 ± 1.3
AG 370		>200
AG 490		6.4 ± 1.1
AG 494		11.7 ± 3.3
AG 556		10.8 ± 3.1
AG 879		>200
AG 1288		9.2 ± 0.05

[a] The GC-C-Catbac protein was assayed in the presence of various concentrations of the indicated tyrphostins. The IC_{50} was calculated for the compounds that showed a significant extent of inhibition. Values represent the mean ± SEM of experiments performed three times.

It was noted early on that different tyrphostins inhibited a variety enzymes. Competitive inhibition with an IC_{50} of 20−60 μM for AG18, AG10, and AG112 was observed for calcineurin, where it was argued that the tyrphostins were similar to the substrate, which can be a phosphotyrosine residue (*34*). HIV integrase was inhibited with an IC_{50} of 0.5 μM for AG1717 and AG1718, and inhibition was competitive with respect to DNA binding (*35*). Topoisomerase I was inhibited by AG18 and AG555 with an IC_{50} of 100−500 μM (*36*). GTP utilizing enzymes such at retinal guanylyl cyclase and transducin were inhibited by AG18, AG82, and AG217 with an IC_{50} of ~26 μM (*27*), but the authors did not elaborate on the mode of inhibition. As is apparent from earlier results and our studies described here, chemically distinct tyrphostins are effective in inhibiting the activity of different enzymes.

Only tyrphostins with two or three hydroxyl groups could inhibit GC-C, and large modifications were not tolerated (Table 2). Studies with a spectrum of 11 tyrphostins indicate that the presence of the 3,4,5-trihydroxyl groups of tyrphostin A25 are most important for bringing about inhibition. 3,4-Substituted and 4-monosubstituted tyrphostins were less effective. Tyrphostin A1, with a 4-methoxy group, was inactive against the class III cyclases. Interestingly, tyrphostin A1 was not effective in inhibition of activity of the epidermal growth factor receptor or the GTPase activity of transducin (*27*, *37*). Since the three-dimensional structures of the kinase

domain of the epidermal growth factor receptor, nucleotide cyclases, and transducin are very different, only a small region of structural similarity is sufficient for the interaction of tyrphostin in these different proteins. The only common property among these enzymes is the ability to bind nucleotides. It is therefore likely that the tyrphostin-binding site may lie in the vicinity of the nucleotide-binding pockets of these enzymes.

2-Chloroadenosine has been shown to inhibit GC-C and the soluble guanylyl cyclase in a noncompetitive manner, and the authors suggested that an additional ATP-binding site is present in the guanylyl cyclase catalytic domain (*18*). Tyrphostin A25 inhibits GC-C at much lower concentrations in comparison to 2-chloroadenosine (~5 vs 200 μM). It is interesting to note that the mechanism of inhibition of tyrphostin A25 is similar to that seen for the adenosine analogues, and it is conceivable that the two compounds could bind to the same site. Studies along these lines would be of interest in the future.

A number of compounds have been known to inhibit soluble guanylyl cyclases, and these include LY 83583 (*38*) and ODQ (*39*). These have IC_{50} values in the range of 20 nM to 2 μM. However, both compounds are suggested to interfere with the action of the heme moiety in the α-subunit of the soluble enzymes. Indeed, we saw no inhibition of GC-C activity using Ly 83583 (data not shown). In addition, none of these compounds are likely to inhibit adenylyl cyclases. Therefore, the tyrphostins appear to be able to bind to a common site in the catalytic domain of all nucleotide cyclases, in contrast to the more precise requirements for specific inhibitors of adenylyl and guanylyl cyclases.

Tyrphostin A25 did not inhibit ST-mediated activation of GC-C when applied to intact cells (data not shown). A similar lack of inhibition was observed with other tyrphostin analogues and has been attributed to the poor availability of the compound within cells, since other proteins can interact with tyrphostin and prevent its binding to the target of interest (*40*). We have monitored uptake of tyrphostin A25 in HEK cells and found that the compound is rapidly degraded in the extracellular medium, with a rapid but minimal uptake into cells (data not shown), suggesting that the lack of in vivo effects may be attributed to its poor cellular availability.

The binding site for tyrphostin remains unknown at present, and our results do not preclude an additional binding site for tyrphostin in the KHD of GC-C. Apart from the adenine nucleotides, there is evidence to show that additional compounds can interact with the KHD of receptor guanylyl cyclases. Amiloride has been shown to modulate the binding of atrial natriuretic factor to guanylyl cyclase A and requires an intact KHD in this receptor to demonstrate these effects (*41*, *42*). It is therefore possible, though we have not directly demonstrated it, that an additional binding site for tyrphostin is present in the KHD of GC-C. However, with the results that we have obtained from kinetic analysis of the enzyme activity, tyrphostin A25 may bind at a site distinct from the MnGTP and free metal binding sites in the catalytic core. Since it has been reported that tyrphostin A25 can interact with metals directly (*43*), we suggest that the site for tyrphostin binding could be positioned in such a way that the hydroxyl groups are close enough to the free metal to coordinate the metal atom. This could prevent the metal from participating in the cyclization reaction, therefore inhibiting the adenylyl or guanylyl cyclase activity.

The nucleotide-binding pocket in cyclases is lined by hydrophobic residues (*31*), and it is possible that the tyrphostin ring may be accommodated in this region of the enzyme. In addition, the hydrophobicity of the phenyl ring in tyrphostins may allow additional interactions that can lead to alterations in the juxtapositioning of the two-polypeptide chains in the cyclases. We have recently shown that stacking interactions between hydrophobic groups are involved in formation of a homodimer of the catalytic domain of a bacterial adenylyl cyclase (*20*). This mycobacterial adenylyl cyclase (Rv1625c) was also inhibited by tyrphostin A25 with an IC_{50} of ~30 μM (data not shown). Forskolin binds the pseudosymmetric site in mammalian adenylyl cyclases, indicating that a hydrophobic binding pocket does exist in these enzymes (*25*).

It is interesting to note that adenylyl cyclases are inhibited by a group of adenine nucleotide analogues that have an intact purine group with substitutions on the ribose moiety. These P-site compounds are noncompetitive with MnATP or uncompetitive with MgATP as substrates and generate a dead-end species upon binding to the enzyme−product complex (*44*). They do not affect substrate binding, though they bind to the same region as metal-ATP. Further structural studies may show whether tyrphostins are also able to bind in the same pocket as the P-site inhibitors.

This study raises the possibility that a specific inhibitor may be obtained for nucleotide cyclases by synthetic refinements and can be rationally designed on the tyrphostin core. More specifically, the results described here may facilitate the design of inhibitors as biological and structural tools for a better understanding of GC-C signaling.

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