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Tyrphostin induced growth inhibition: correlation with effect on p210$^{bcr-abl}$ autokinase activity in K562 chronic myelogenous leukemia

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We have examined a series of tyrosine kinase inhibitors structurally related to erbstatin (tyrphostins) for inhibition of p210$^{bcr-abl}$ autokinase activity *in vitro* and for growth inhibition of chronic myelogenous leukemia (CML) K562 cells. Of the tyrphostins with IC_{50} for growth < 50 μM, AG814, AG946, AG952, AG896, AG953, AG956 and AG957 (structurally related to lavendustin A and piceatannol) completely inhibited p210$^{bcr-abl}$ kinase activity in an immune complex kinase assay. Another group of tyrphostins (AG807, AG568, AG763, AG1076, AG490, AG1318, AG556, AG1319, AG555 and AG1111) inhibits growth of K562 cells but not p210$^{bcr-abl}$ tyrosine kinase activity. Of the compounds which inhibit growth and p210$^{bcr-abl}$ tyrosine kinase activity, AG957 inhibits DNA synthesis as early as 2 h (60% inhibition at 20 μM of AG957), a time and concentration of drug where RNA and protein synthesis were not affected. AG957 inhibits p210$^{bcr-abl}$ tyrosine phosphorylation in living cells by 1 h without an inhibition of total protein phosphorylation. Growth inhibition by AG957 was reversible after 4 h of exposure, but irreversible after 24 h. AG957 can be considered as an important lead structure for the development of anti-*bcr–abl* tyrosine kinase antagonists. These data also raise the possibility that *bcr–abl* kinase activity is directly linked to maintenance of DNA synthesis in Philadelphia chromosome positive (Ph$^+$) CML cells.

Key words: bcr–abl, chronic myelogenous leukemia, tyrosine kinase.

Introduction

Patients with chronic myelogenous leukemia (CML) frequently have a cytogenetic abnormality (the Philadelphia chromosome) resulting from reciprocal translocation between chromosome 9 and chromosome 22.[1,2] This translocation results in the transfer of the c-*abl* non-receptor protein tyrosine kinase protoncogene from its normal position on chromosome 9 into the *bcr* gene on chromosome 22.[3–6] A specific 8 kb mRNA transcript[7] of the *bcr–abl* fusion gene is translated into a chimeric *bcr–abl* fusion protein of 210 kDa (p210$^{bcr-abl}$) that exhibits constitutive protein tyrosine kinase activity (EC 2.7.1.112).[8] The normal, untranslocated c-*abl* protoncogene product has considerably lower constitutive protein tyrosine kinase (PTK) activity than p210$^{bcr-abl}$.[9] Therefore the increased PTK activity on the part of p210$^{bcr-abl}$ suggests an important role for p210$^{bcr-abl}$ tyrosine kinase activity in the pathogenesis of CML and raises the possibility that specific PTK inhibitors directed at p210$^{bcr-abl}$ could be of use to patients with CML. A major question is whether compounds with relatively selective inhibitory potential for p210$^{bcr-abl}$ tyrosine kinase activity exist and what effects these compounds may have on the growth of neoplastic cells.

Compounds which inhibit phosphotyrosine kinase activity include quercetin,[10] the isoflavone genestein,[11] and other naturally occurring compounds such as erbstatin,[12] herbimycin A,[13] lavendustin A[14] and aeroplysinin-1.[15] In an effort to design inhibitors directed at the protein substrate, Gazit *et al.*[16] have created a series of inhibitors (tyrphostins) initially modeled on the compound erbstatin but later patterned after novel leads. Initial studies with PTK inhibitors in CML cells have revealed that certain tyrphostins can induce erythromyeloid differentiation of human myelogenous leukemic, Philadelphia chromosome positive (Ph$^+$) K562 cells.[17,18] With regard to effects on p210$^{bcr-abl}$, some tyrphostins can discriminate in inhibition of the normal *abl* protein p140^{c-abl} as compared with the putatively oncogenic counterpart p210$^{bcr-abl}$.[19] However, in each of these cases whether the tyrphostin under consideration could

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inhibit cell growth as a primary result of inhibiting p210$^{bcr-abl}$, or due to additional unrelated effects on cell metabolism, has also not been elucidated. Only one family represented by AG1112[20] was found to block p210$^{bcr-abl}$ kinase in intact K562 cells and inducing them to differentiate and die.

In the experiments reported in this paper we have sought to define novel structural prototypes which would inhibit p210$^{bcr-abl}$ PTK activity *in vitro* as well as in K562 cells. We have identified the tyrphostin AG957 as such a compound and demonstrate further that inhibition of p210$^{bcr-abl}$ tyrosine phosphorylation in K562 cells precedes decreased cell growth. We discriminate these effects of AG957 from other tyrphostins, which can inhibit K562 cell growth yet do not apparently inhibit p210$^{bcr-abl}$ tyrosine kinase activity.

Materials and methods

Inhibitors

Tyrphostins were synthesized as described by Gazit *et al.*[21,22] The tyrphostin stock solutions were prepared in dimethylsulfoxide (DMSO). Their structures are depicted in Tables 1 and 2.

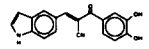
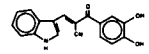
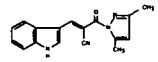
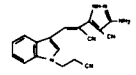
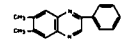
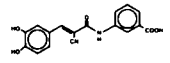
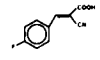
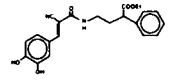
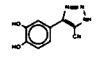
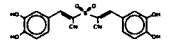
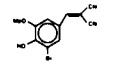
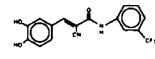
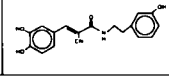
Cell culture and cell growth assay

Human leukemia Philadelphia chromosome positive CML cell line K562 was from ATCC (Rockville, MD). Cells were cultured in RPMI medium containing 10% fetal calf serum, 2 mM glutamine, and 100 units/ml of penicillin and 100 μg/ml streptomycin. Cells (2×10^3 cells/well) were incubated with increasing concentrations of tyrphostins in a final volume of 200 μl in 96-well plates. Control cells were incubated with medium containing identical concentrations of the tyrphostin solvent, DMSO. Growth of K562 cells was quantitated after 6 days by ability of living cells to reduce the yellow dye 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product.[23]

Reversibility of tyrphostin effect

Cells were exposed to 15 and 25 μM concentrations of the indicated compounds for 1, 4 and 24 h. At each time, cells were washed three times with medium and resuspended in fresh medium. Cells were counted with a hemocytometer and plated in 6-well plates (12 500 cells/2 ml). Cells for continuous exposure to drug were plated identically in medium with the appropriate drug concentration. Cells were counted by hemocytometer on day 6 and were checked for viability using Trypan blue.

Table 1. Inhibition of growth and p210$^{bcr-abl}$ kinase activity by tyrphostins in K562 cells

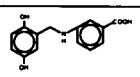
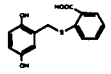
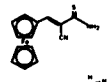
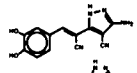
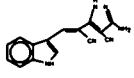
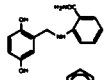
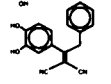
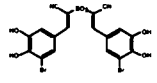
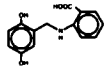
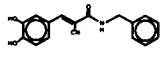
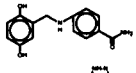
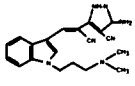
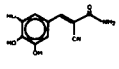
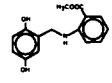
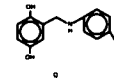
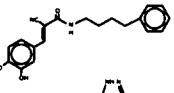
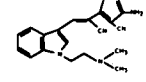
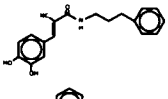
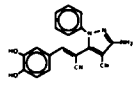
Tyrphostin	Structure	IC_{50}* μM±S.E.	p210$^{bcr-abl}$ Inhibition**
AG805		>100	Partial
AG808		>100	Partial
AG1370		>100	No effect
AG1332		>100	No effect
AG1295		>100	No effect
AG822		>100	Partial
AG135		97 ± 21	Partial
AG561		88 ± 16	No effect
AG514		76 ± 10	No effect
AG982		70 ± 9	Partial
AG124		70 ± 8	Partial
AG1109		58 ± 6	Partial
AG981		56 ± 2	Partial

*6 day MTT assay.
**Immune complex kinase assay at 50 μM drug concentration. Complete = 100%; partial = ~10–80%; no effect = <10% inhibition.

Macromolecular synthesis

K562 cells were plated at a density of 5000 cells per well in 96-well plate in 100 μl of medium. Cells were exposed to tyrphostins for the indicated periods and pulsed with [^{3}H]thymidine, L-[^{3}H]leucine and

Table 2. Inhibition of growth and p210$^{bcr-abl}$ kinase activity by tyrphostins in K562 cells

Tyrphostin	Structure	IC_{50}* μM±S.E.	p210$^{bcr-abl}$ Inhibition**
AG814		50 ± 3	Complete
AG946		46 ± 6	Complete
AG807		42 ± 5	No effect
AG568		42 ± 3	Partial
AG1112		35 ± 11	Complete
AG952		35 ± 3	Complete
AG763		33 ± 7	No effect
AG1076		32 ± 2	Partial
AG896		30 ± 11	Complete
AG490		29 ± 4	Partial
AG953		22 ± 6	Complete
AG1318		21 ± 3	Partial
AG775		19 ± 6	Complete
AG956		16 ± 3	Complete
AG957		15 ± 4	Complete
AG556		14 ± 3	No effect
AG1319		12 ± 3	Partial
AG555		9.2 ± 2	No effect
AG1111		8 ± 1	No effect

*6 d MTT assay.
**Immune complex kinase assay at 50 μM drug concentration. Complete = 100%; partial = ~10–80%; no effect = <10% inhibition.

[^{3}H]uridine at 5 μCi/ml for the last 2 h of drug exposure or 10 μCi/ml for the last 30 min of the drug exposure. Cells were harvested and incorporation of label assessed as described in Kaur *et al.*[24]

ATP levels

Ten million cells were collected by centrifugation and washed once with phosphate buffered saline (PBS). To the cell pellet was added 500 μl of 60% methanol. The contents were mixed, heated at 95°C for 1.5 min, clarified by centrifugation and analyzed by ion-exchange HPLC on a Partisal SAX column using gradient elution with ammonium phosphate buffers.[25]

Cell extraction and p210$^{bcr-abl}$ immunoprecipitation

Exponentially growing K562 cells (1×10^7 cells) were washed twice in PBS, and then the cell pellet was lysed in 1.0 ml of ice-cold kinase-lysis buffer [10 mM Na_2HPO_4–NaH_2PO_4 (pH 7.0), 1% Triton X-100, 0.05% sodium dodecyl sulfate (SDS), 150 mM NaCl containing 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 10 μg/ml of pepstatin], briefly vortexed and centrifuged at 35 000 r.p.m. for 90 min. To the clear cell extract leupeptin was added to a final concentration of 50 μg/ml. Each 1 ml of clarified extract was incubated with 5 μl of anti-*bcr–abl* sera (Ab-2, Oncogene Science Uniondale, NY) or with antiserum which had been incubated with immunizing peptide (10 times) at room temperature for 2 h prior to addition to extract. Incubation with antisera was overnight (16 h) at 4°C with gentle shaking. To harvest the immune complex, 15 μl packed volume of preswollen Protein A–Sepharose beads (per 1 ml of extract) was added and extracts were incubated for another 2 h at 4°C with gentle shaking. Beads were pelleted by centrifugation.

In vitro auto-phosphorylation reaction

The p210$^{bcr-abl}$ protein immunoprecipitates were washed twice with extraction buffer lacking SDS. Precipitates were washed once with 50 mM Tris (pH 7.0) and resuspended in 20 μl of 20 mM PIPES [piperzine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 7.0) plus 20 mM $MnCl_2$. In some reactions, acid denatured rabbit muscle enolase (5 μg/5 μl) was added

as an exogenous substrate for the p210$^{bcr-abl}$ kinase. Five microliters of tyrphostins were added at eight times final concentration to each reaction mixture. Reactions were initiated with the addition of 10 µl of [γ-^{32}P]ATP (10 µCi per sample, 3000 Ci/mmol; Amersham Corp.), incubated for 20 min at 30°C, stopped by addition of 10 µl of 5 × SDS gel loading buffer, heated at 95°C for 5 min and analyzed by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) and by autoradiography.[26]

[^{32}P]orthophosphate labeling, immunoprecipitation and phosphotyrosine immunoblotting

Cells (1×10^7) were exposed to tyrphostins for 1, 6 and 24 h. Cells were labeled for 1 h with 1 mCi of carrier-free [^{32}P]orthophosphate in 5 ml phosphate-free medium containing 10% dialyzed serum and appropriate concentrations of the drug. Cells were centrifuged at 1000 r.p.m. for 5 min, washed three times, and lysed in 600 µl of 10 mM sodium phosphate (pH 7.5), 100 mM NaCl, 5 mM NaF, 100 µM Na_3VO_4, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin and 10 µg/ml leupeptin. Cell lysates were centrifuged at 14 000 r.p.m. for 15 min. Supernatant was removed and proteins determined by the method of Bradford;[27] phosphorylated proteins (15 µg) were separated by 7.5% SDS-PAGE, silver stained and autoradiographed.[26] Labeled cell lysate protein (600 µg) was immunoprecipitated with Ab-2. Immunoprecipitated proteins were separated by 7.5% SDS–polyacrylamide gels and transferred to Immobilin-P® in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11.0), 10% methanol at 0.5 A for 2 h at 4°C. Phosphotyrosine was detected by Western blotting with a mouse monoclonal anti-phosphotyrosine antibody (05-321; UBI, Lake Placid, NY) followed by alkaline phosphatase detection or with analogously prepared unlabeled cell extracts by [^{125}I]Protein A.

Results

Inhibition of growth and *in vitro* p210$^{bcr-abl}$ autokinase activity

To correlate K562 growth inhibition with p210$^{bcr-abl}$ kinase inhibition, K562 cells were exposed to several tyrphostins for 6 days followed by estimation of cell number using the colorimetric MTT assay. The same compounds were also studied for their capacity to inhibit p210$^{bcr-abl}$ kinase activity in an immune complex autokinase assay from untreated cells. This assay examines the capacity of the p210$^{bcr-abl}$ to phosphorylate itself on tyrosine. Figure 1 illustrates an example of tyrphostin with complete (AG957), partial (AG1318) or no capacity (AG555) to inhibit p210$^{bcr-abl}$ autokinase. All the tyrphostins with IC_{50} for growth of 50 µM or more for K562 growth have only partial or no inhibitory effect on p210$^{bcr-abl}$ autokinase activity at 50 µM of drug (Table 1). Among the tyrphostins with an IC_{50} less than 50 µM for growth of K562 cells, several also inhibit autokinase activity only partially (AG568, AG1076, AG490, AG1318 and AG1319) or were without effect (AG807, AG763, AG556, AG555 and AG1111). However, of the compounds with IC_{50} less than 50 µM for growth, complete inhibition of p210$^{bcr-abl}$ autokinase activity was observed in a number of cases, including AG814, AG946, AG1112, AG952, AG896, AG953, AG775, AG956 and AG957 (Table 2).

Tyrphostin AG957 is of particular interest because previous studies have demonstrated that it can inhibit p210$^{bcr-abl}$ kinase activity using the exogenous substrate poly-glu–tyr, with an IC_{50} of 1 µM.[19] The results obtained here therefore also indicate that AG957 inhibits p210$^{bcr-abl}$ autokinase activity. In addition, it is of interest that the structurally related compounds AG814, AG946, AG952, AG896, AG953 and AG956 also inhibit p210$^{bcr-abl}$ autokinase activity, and also inhibit K562 cell growth with moderate potency.

These experiments also show that tyrphostins which inhibit cell growth (IC_{50} < 50 µM) yet do not

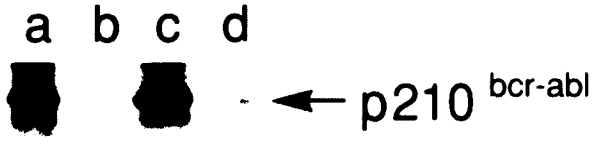


Figure 1. Effect of tyrphostin on p210$^{bcr-abl}$ autophosphorylation. *In vitro* phosphorylation of p210$^{bcr-abl}$ protein. Human K562 cells (10^7 cells/lane) were extracted and immunoprecipitated with anti-*bcr–abl* serum (Ab-2). Immune complexes were collected on Protein A–Sepharose beads and incubated with [γ-^{32}P]ATP for 20 min at 30°C as described in Materials and methods, in the presence of: a, no addition; b, 50 µM AG957 (complete inhibition); c, 50 µM AG555 (no inhibition); d, 50 µM AG1318 (partial inhibition). After incubation, the immunoprecipitates were heat-denatured, supernatant recovered, electrophoresed by 7.5% SDS–PAGE and dried. The phosphorylated p210$^{bcr-abl}$ protein was detected by autoradiography.

affect p210$^{bcr-abl}$ kinase activity also exist. For example, AG555 has an IC_{50} of 9.2 μM for growth, yet does not inhibit the p210$^{bcr-abl}$ kinase activity *in vitro*. When phosphorylation of enolase as an exogenous substrate of p210$^{bcr-abl}$ was monitored, no compound emerged which was substantially better in inhibiting phosphorylation of the exogenous as compared with autokinase reaction (data not shown).

AG957 and AG555: effect on macromolecular synthesis

Since the foregoing experiments suggested that inhibition of p210$^{bcr-abl}$ autokinase activity might be related in some cases to inhibition of growth, we further characterized the cellular effects of AG957 as an example of a cell growth and p210$^{bcr-abl}$ kinase-inhibiting compound, and AG555 as an example of a cell growth inhibiting-tyrphostin which did not inhibit p210$^{bcr-abl}$ kinase. To determine if the growth inhibitory action of AG957 could be related temporally to inhibition of p210$^{bcr-abl}$ tyrosine kinase activity, we compared AG957 and AG955 with respect to cellular effects shortly after drug addition. Exposure of K562 cells to AG957 for 24 h inhibits DNA, protein and RNA synthesis completely at 25 μM (Figure 2A). Tyrphostin AG555 also inhibits DNA and RNA synthesis by 80% at 25 μM, but protein synthesis is less affected (only about 50% inhibition) even at 50 μM concentration of the drug (Figure 2B). Of interest, cells whose growth is arrested after 24 h of exposure to drug are clearly viable as measured by Trypan blue exclusion (data not shown) and by capacity to reduce MTT (Figure 2A and B), as reduction of the MTT dye depends on intact mitochondrial electron transport.[23] Figure 2(C) further demonstrates that after 24 h of exposure to growth inhibitory concentrations of AG957 and AG555, K562 cells maintain comparable levels of ATP with a similar ATP/ADP ratio to untreated or vehicle treated cells. Thus, inhibition of cell growth and macromolecular synthesis does not occur with gross alterations of cellular metabolic capacity.

Time course for inhibition of DNA synthesis

The preceding results raised the question of whether inhibition of nucleic acid synthesis might be an early effect of AG957. We, therefore, followed the time course of inhibition of [^{3}H]thymidine incorporation. Figure 3(A) demonstrates that AG957 inhibits [^{3}H]thymidine incorporation by 60 or 90% after 2 h exposure to AG957 at 20 or 40 μM, respectively; [^{3}H]uridine and L-[^{3}H]leucine incorporation are maintained at above 80% after 2 h exposure to the same concentrations of AG957, and even at 8 h of exposure to drug, L-[^{3}H]leucine incorporation is largely unaffected while [^{3}H]uridine incorporation is 60% of control (Figure 3B and C). Therefore, AG957 appears to manifest growth inhibition in conjunction with an early decrease in DNA synthesis.

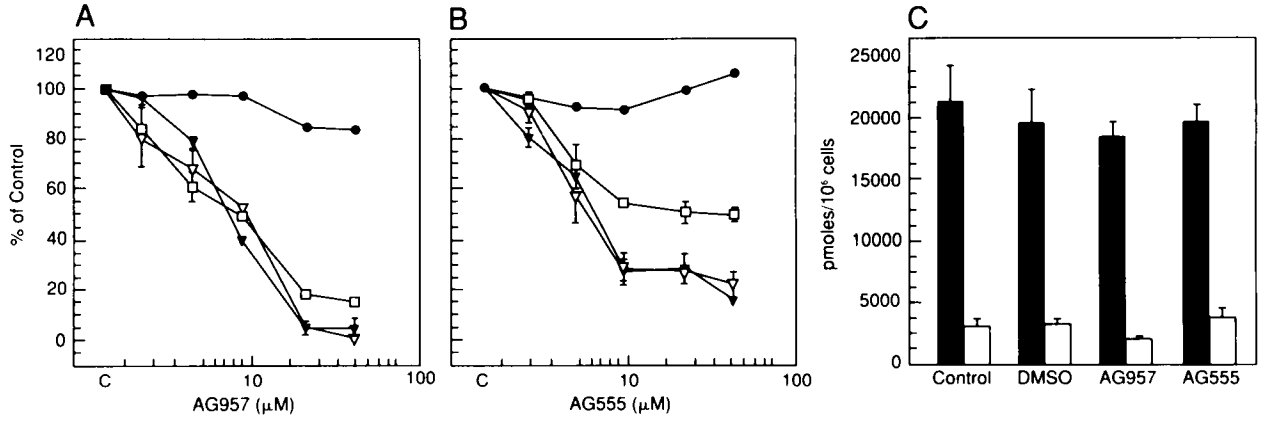


Figure 2. Effect of AG957/AG555 on K562 cells. Tyrphostin AG957 (A) and AG555 (B) mediated inhibition of macromolecular synthesis. Five thousand cells plated in a 96-well plate were exposed to tyrphostins for 18 h. Cells were pulsed with [^{3}H]thymidine (▽), [^{3}H]leucine (□) and [^{3}H]uridine (▼) at a final concentration of 5 μCi/ml for the last 2 h of drug treatment or were incubated with MTT for 4 h and A570 nm determined (●). Cells were harvested and counted as described in Materials and methods. In (C), ATP (■) and ADP (□) levels by mass were determined after exposure to 25 μM AG957, 15 μM AG555, no addition or vehicle as described.

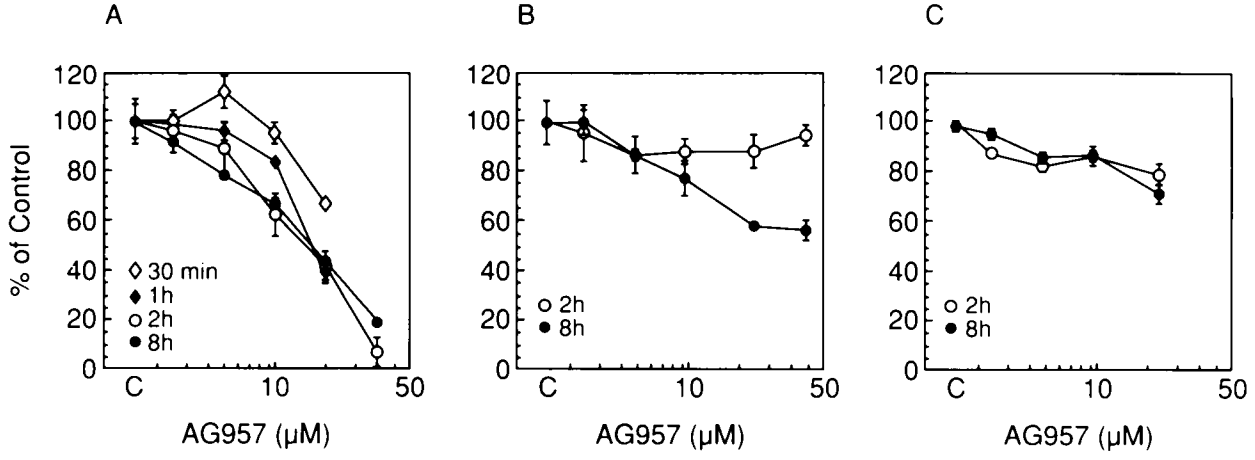


Figure 3. Time course of AG957 effect on macromolecular synthesis. Five thousand cells plated in a 96-well plate were exposed to AG957 for 30 min (◇), 1 h (◆), 2 h (○) and 8 h (●). Cells were pulsed with (A) [^{3}H]thymidine, (B) [^{3}H]uridine or (C) L-[^{3}H]leucine at a final concentration of 5 μCi/ml for the last 2 h (2 and 8 h samples) or 10 μCi/ml for last 30 min (30 min and 1 h samples) of drug treatment. At the end of the incubation cells were harvested and radioactivity counted as described elsewhere.[23]

Effect of AG957/AG555 on total phosphorylation of proteins and inhibition of p210$^{bcr-abl}$ phosphorylation in K562 cells

As AG957 and AG555 are both potential tyrosine kinase antagonists, we examined the effect of the drugs on total protein and p210$^{bcr-abl}$ tyrosine phosphorylation in K562 cells. Neither AG957 (25 μM) nor AG555 (15 μM) after 24 h of drug exposure inhibits $^{32}PO_4$ incorporation into total proteins (Figure 4B). However, Figure 5(A) demonstrates that 1 h after addition of AG957 (but not AG555), there is decreased $^{32}PO_4$ labeling of the p210$^{bcr-abl}$ protein and also a decrease in the mass of phosphotyrosine detected by anti-phosphotyrosine antibodies using an alkaline phosphatase colorimetric (Figure 5B) or [^{125}I]Protein A (Figure 5C) detection technique. Both of these changes occur as a decrease in DNA synthesis is developing (Figure 3A), but before any significant decrease in RNA or protein synthesis (Figure 3B and C). Thus, p210$^{bcr-abl}$ tyrosine kinase inhibition may affect a pathway leading to continued DNA synthesis and by its inhibition AG957 could then inhibit cell growth. In contrast, AG555 does not ever inhibit p210$^{bcr-abl}$ kinase activity even as it inhibits cell growth.

Reversibility of K562 growth inhibition by AG957 or AG555

Since a useful therapeutic effect of a tyrphostin in CML might be achieved by intermittent exposure to drug, we assessed the degree to which K562 cells recover after exposure to AG957. Figure 6 shows that exposure to AG957 at 25 μM for 24 h, or 6 days of continuous exposure, results in analogous growth inhibition. In contrast, exposure for 1 or 4 h demonstrates considerable reversibility of drug effect after washout. AG555 was somewhat more reversible at 15 and 25 μM after 4 h of treatment as compared with AG957. This experiment therefore suggests that a brief exposure (<4 h) to these concentrations will not lead to sustained growth

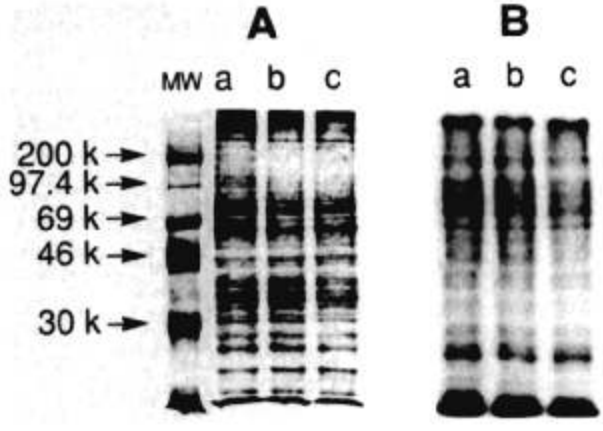


Figure 4. Effect of AG957 and AG555 on protein phosphorylation. Cells (10^7) were treated with AG957 (25 μM) and AG555 (15 μM) for 24 h. Cells were pulsed with [^{32}P]orthophosphate as described in Materials and methods. Samples were analyzed on 7.5% SDS gel. Gels were silver stained (A), dried and autoradiographed (B) at −70°C. Panel (A) shows the silver stained gel of untreated cells (a) and after 24 h exposure to AG957 (b) or AG555 (c). Panel (B) displays the phosphorylation of protiens after 24 h treatment with AG957 and AG555.

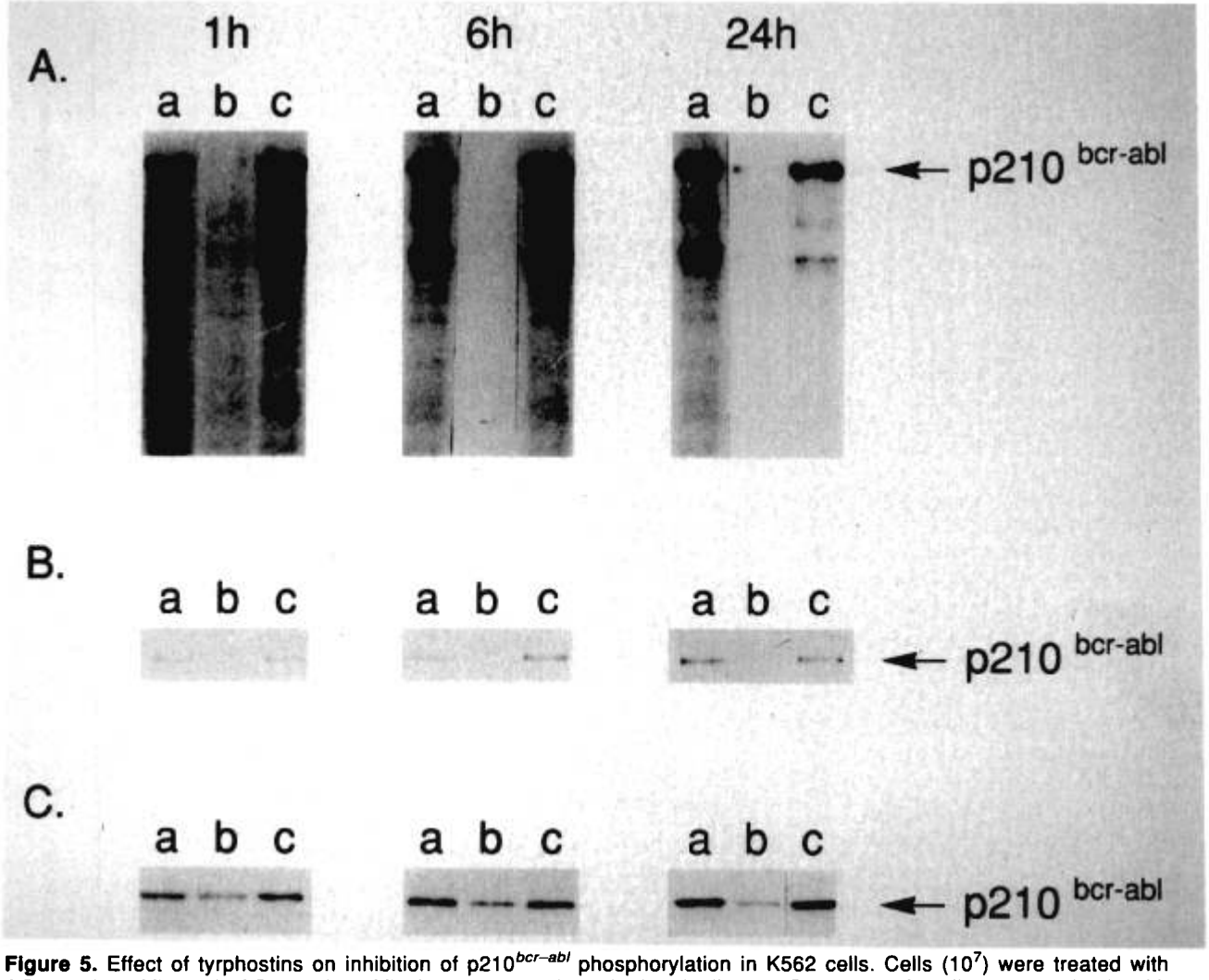


Figure 5. Effect of tyrphostins on inhibition of $p210^{bcr-abl}$ phosphorylation in K562 cells. Cells (10^7) were treated with AG957 (25 μM) and AG555 (15 μM) for 1, 6 and 24 h in duplicate. One set of cells was pulsed with [^{32}P]orthophosphate for 60 min prior to cell lysis. The $p210^{bcr-abl}$ protein was immunoprecipitated as described in Materials and methods. Immunoprecipitated proteins were separated by 7.5% SDS–PAGE, transferred to nitrocellulose membranes and autoradiographed (panel A). This membrane was probed with phosphotyrosine antibody and the bands were detected by alkaline phosphatase (panel B). Another set of membranes from cells not previously radiolabeled was probed with anti-phosphotyrosine antibody, detected by ^{125}I-labeled Protein A and autoradiography for 6 h (panel C).

inhibition but prolonged exposure (perhaps up to 24 h) will be necessary for persistent growth inhibition.

Discussion

The Philadelphia chromosome, which is the result of the chromosome 9:22 translocation and which results in the derived protein, $p210^{bcr-abl}$, is present at the earliest identifiable stages of CML. Its expression also causes a hematopoietic disease in animal models of CML.[28] Expression of $p210^{bcr-abl}$ is therefore possibly an initiating event in CML and therefore may be important in allowing the clonal dominance of stem cells carrying the translocation. In this paper, we have characterized a set of potential inhibitors of $p210^{bcr-abl}$ kinase with respect to inhibition of K562 growth and K562 $p210^{bcr-abl}$ kinase activity. We have identified tyrphostins in this series of compounds with an IC_{50} for K562 cell growth above 50 μM with partial or no influence on $p210^{bcr-abl}$ kinase activity (Table 1). Other tyrphostins (AG814, AG946, AG1112, AG952, AG896, AG953, AG775, AG956 and AG957) inhibit growth and $p210^{bcr-abl}$ kinase activity, while there are also those (AG807, AG568, AG763, AG1076, AG490, AG1318, AG556,

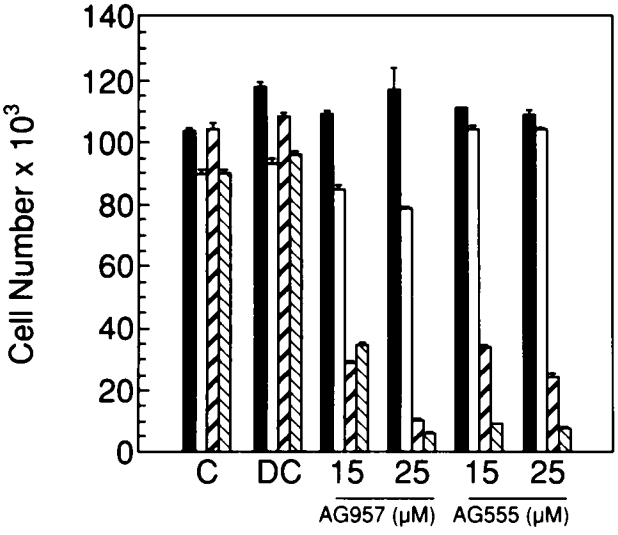


Figure 6. Cell growth reversal after AG957/555 treatment. Cells were exposed to AG957 and AG555 at indicated concentrations. At time periods of 1 h (■), 4 h (□) and 24 h (▨) cells were washed three times and resuspended in fresh media. Drug washed and continuous (▩) drug-exposed cells (12 500 cells/2 ml of media) were plated in a 6-well plate. Cell count was performed on day 6 and cells were checked for viability using the Trypan exclusion method.

AG1319, AG555 and AG1111) which inhibit growth, but not p210$^{bcr-abl}$ autokinase activity (Table 2). The ability of these latter compounds to inhibit other tyrosine kinase activities is currently under investigation.

Of interest to us are the anti-p210$^{bcr-abl}$ tyrosine kinase inhibitors which also might inhibit cell growth after limited exposure to the drug (AG814, AG946, AG1112, AG952, AG896, AG953, AG775, AG956 and AG957). Tyrphostin AG957 has previously been shown to have a K_i of 0.75 μM for p210$^{bcr-abl}$ using poly-glu–tyr as an exogenous substrate.[19] Surprisingly, and of interest to drug design, seven of the nine compounds which we found to inhibit p210$^{bcr-abl}$ autokinase activity completely and to inhibit cell growth with moderate potency have a structural similarity to Lavendustin A, whose active pharmacaphore is 2-hydroxy-5-(2,5-dihydroxy-benzyl) aminobenzoic acid.[14] These tyrphostins (AG814, AG946, AG952, AG896, AG953, AG956 and AG957) contain a 2,5-dihydroxy phenyl ring analogous to erbstatin[12] and the diaryl motif observed in piceatannol.[29] Erbstatin and piceatannol both are tyrosine kinase inhibitors found as natural products. Tyrphostins AG775 and AG1112 have a completely unrelated structure (Table 2) and inhibit cell growth; however AG775 inhibits p210$^{bcr-abl}$ kinase activity only *in vitro* whereas AG1112 inhibits p210$^{bcr-abl}$ kinase activity *in vitro* and in cells.[20]

The results obtained here therefore suggest that a systematic variation of common features of the AG957 structure may reveal compounds of greater growth inhibitory potential, assuming that growth inhibition is related to p210$^{bcr-abl}$ kinase inhibition. Important questions include whether a *para*-dihydroxy motif, or the function and rotation of the side chain, or both are of importance for inhibitory tyrosine kinase activity of these compounds (Table 2). As AG814, AG946, AG952, AG896, AG953, AG956 and AG957 might possibly undergo oxidation to the corresponding quinone, the extent to which this aspect of their structure is necessary for growth inhibition will also be of importance to establish.

To address the relevance of p210$^{bcr-abl}$ kinase inhibition to inhibition of cell growth, the experiments in this paper have also clearly demonstrated that an early effect of AG957 in K562 cells is inhibition of DNA synthesis (Figures 2 and 3). At the time and concentration when DNA synthesis is inhibited, cells have preserved ATP levels and growth inhibition was reversible upon removal of the drug after 4 h (Figure 4). At the same time and concentration of AG957, we also inhibit the p210$^{bcr-abl}$ phosphorylation *in vitro* and in K562 cells (Figure 6). Thus, our data also raise the interesting biologic question of whether active p210$^{bcr-abl}$ kinase activity is causally linked to maintained DNA synthesis. Further experiments to address this possibility, including a detailed analysis of the effects of AG957 on the regulation of dNTP pools and the enzymes that regulate DNA synthesis, are necessary, as well as a consideration of the effect of the drug on endogenous substrates of p210$^{bcr-abl}$ in K562 cells. Such substrates could include regulatory elements or the enzymatic machinery responsible for initiation or completion of DNA synthesis.

An additional issue which needs further clarification is the actual specificity for AG957 and the lavendustin-related tyrosine kinase antagonists identified here. It is possible that another as yet unidentified tyrosine kinase or kinases are important for the growth inhibition in addition to or indeed apart from effects on p210$^{bcr-abl}$, and obviously the effects of AG555 and the effect on other tyrosine kinase activities of other non-p210$^{bcr-abl}$ directed tyrphostins which inhibit cell growth will be of importance to consider on other tyrosine kinase activities. Our initial approach to this issue will be to characterize the tyrosine-phosphorylated substrates in K562 cells in addition to

p210$^{bcr-abl}$ which are affected by the growth-inhibitory tyrphostins identified here.

It has recently been documented that the first exon sequence of the *bcr* portion of p210$^{bcr-abl}$ specifically activates the tyrosine kinase and the transforming potential of *bcr–abl* protein.[30] The *bcr* first exon has the property to bind to *abl*-SH2 domain in a phosphotyrosine independent manner.[31] It has also been shown that the adapter protein Grb-2 binds directly to phosphotyrosine 177 of *bcr–abl* and plays a role in oncogenic transformation.[32] The existence of a *bcr–abl*–Grb2 complex[32] in K562 cells suggests that modulation of Ras function is a possible consequence of the *bcr–abl* tyrosine phosphorylation of the *bcr–abl* transformation pathway. Whether AG957 suppresses this complex formation or activation of downstream components of this pathway is of great interest, as the capacity of AG957 to inhibit DNA synthesis may be the result of its effects on such downstream events.

In summary, our results have identified AG957 as an important structure in modulating p210$^{bcr-abl}$ kinase action in living CML cells and further suggest that initial efforts to direct a tyrphostin toward treatment of CML would require the assessment of the effectiveness of 10–20 μM of AG957 in an appropriate CML animal model,[28] particularly if these levels can be maintained for 24 h. Alternatively, if more potent compounds are derived based upon common structural features of AG957 and other lavendustin related tyrphostins (AG814, AG946, AG952, AG896, AG953 and AG956), an enhanced therapeutic index may be possible.

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