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# Tyrphostin-induced differentiation of mouse erythroleukemia cells

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Inhibitors of protein-tyrosine kinases (TPKs) from the tyrphostins family induce terminal erythroid differentiation of mouse erythroleukemia (MEL) cells. The most potent tyrphostin was found to be AG-568 which was therefore investigated in more detail. Just prior to differentiation the inhibition of tyrosine phosphorylation of a pp97 protein band was noted. We also found that AG-568 treatment induces the appearance of a putative differentiation factor which could induce tyrphostin-independent differentiation in untreated cells. Our study suggests that the inhibition of tyrosine phosphorylation by AG-568 leads to the production of differentiating factor(s) which induce the MEL cells to differentiate.

## 1. INTRODUCTION

Protein tyrosine kinases (PTKs) are involved in the regulation of cell proliferation and differentiation [1]. Oncogenic mutations in tyrosine kinases block normal cell differentiation and induce cellular transformation [1]. Furthermore, blockers of tyrosine kinase activity, such as tyrphostins [2] reverse the transformed phenotype of *src* transformed cells [3] and strongly inhibit the growth of squamous cell carcinoma which overexpresses the EGF receptor [4]. In this communication we report on the potency of tyrphostins to induce differentiation of mouse erythroleukemia (MEL) cells, probably due to its tyrosine kinase inhibitory activity.

MEL cells have been widely used as a model for cell differentiation [6]. These cells can differentiate into erythroid cells in vitro in response to a number of compounds, including dimethyl sulfoxide [5] and hexamethylene-*bis*-acetamide [6]. Herbimycin A, a non-selective PTK blocker has also been reported to induce differentiation of MEL cells [7]. Two other tyrosine kinase blockers, ST638 and genistein were found to induce differentiation of MEL cells only when added to the culture medium together with agents which damage DNA such as mitomycin C, or with inhibitors of topoisomerases [8,9].

In this study we screened tyrphostins in an attempt to identify non-toxic PTK blockers which by themselves can induce MEL differentiation and which exhibit specificity towards the MELC.

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## 2. MATERIALS AND METHODS

### 2.1. *Cell and culture conditions*

MEL cells were cultured in RPMI 1640, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 10 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere, with 5% $CO_2$.

### 2.2. *Tyrphostin synthesis*

The synthesis of tyrphostins is described elsewhere [11–13].

### 2.3. *Assay for cell growth and erythroid differentiation*

Cells ($1\times10^5$/ml) were incubated with increasing concentrations of tyrphostin in a final volume of 2.0 ml. As control we have used the medium containing identical concentrations of the tyrphostin solvent, DMSO. The number of live cells was measured by staining the cells with Trypan blue [15]. Erythroid differentiation of the cells was evaluated by benzidine staining as reported previously [16]. Tyrphostin was replenished every 72 h. Cells were centrifuged and resuspended in the desired concentration of the drug or the tyrphostin solvent as specified in the figures.

Supernatants were prepared by sedimenting the cells (1,200 rpm), and the supernatant diluted with two volumes of fresh medium before addition to untreated cells.

### 2.4. *Analysis of tyrosine phosphorylated proteins*

Exponentially growing MEL cells (approximately $7\times10^5$/ml), were treated with nontoxic levels of AG-568 for increasing periods of time. The cells were then washed twice with Hank's buffered solution, and the pellet was resuspended with protein lysis buffer containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM NaF, 1% Triton X-100, 2 mM penylmethylsulfonyl fluoride, 100 KIU/ml aprotinin, 5 μg/ml leupeptin and 100 μM sodium vanadate. Cell lysates were prepared on ice for 1 h with short vortexing every 10 min. Cells lysates were cleared by centrifugation at 12,000×*g* for 30 min. The protein concentration of the supernatants was measured using the Bio-Rad protein reagent. Cell lysates were boiled for 6 min in SDS gel sample buffer prior to electrophoresis. Sixty micrograms of protein were loaded on each lane of 7.5% SDS polyacrylamide gel, electrophoresed and blotted onto nitrocellulose paper (0.2 mm, Schleicher and Schuell Inc.). Protein blots were blocked overnight with 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride and 0.05% Tween-20, 5% bovine serum albumin and 1% chicken egg albu-


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min and then incubated for 2 h at room temperature with phosphotyrosine antibodies (Zymed Inc.) in blocking solution. The filters were washed and incubated with peroxidase-protein A for 40 min at room temperature, washed again and subjected to ECL reaction (Amersham Inc.) and autoradiography.

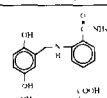
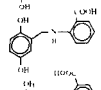
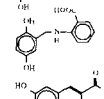
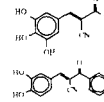
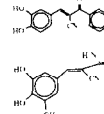
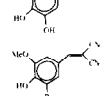
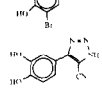
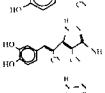
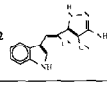
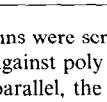
## 3. RESULTS

### 3.1. *Screening tyrphostins for their ability to induce differentiation of MEL cells*

Over 40 tyrphostins from different families [2, 12–14] were examined for their ability to induce erythroid differentiation of MEL cells. Table I also shows for comparison, the potency of the various tyrphostins tested for their potency to block EGFR kinase as compared to the blocking activity against $p210^{bcr\text{-}abl}$ kinase in vitro, using previously described protocols [10]. Only one tyrphostin, AG568, (Table I) was found to be effective and to induce over 80% of the cells in the culture to differentiate. The onset of erythroid differentiation parallel to cell growth was followed for 15 days subsequent to AG568 application (Fig. 1). Between days 14 and 15 of the experiment an abrupt reduction of approximately 40% in the benzidine positive cells was found concomitantly with a 30% reduction of living cells. From day 5, cell differentiation was accompanied by growth arrest, and its degree correlates with the level of cell differentiation. Fig. 2 shows that treatment for four days with an AG568 concentration below 100 $\mu$M has no effect on cell proliferation or differentiation. On the other hand, after twelve days of treatment, differentiation levels reach 80% of the treated cells at 25–50 $\mu$M AG568, concomitant to growth arrest. Growth arrest and differentiation follow a parallel dose–response curve with respect to AG568. The fact that AG568 concentrations below 100 $\mu$M had no effect on cell proliferation at day four of the experiment (Fig. 2A) and the correlation between the differentiation and growth arrest (Fig. 2B) suggest that the concentrations of AG568 effective in inducing differentiation are not toxic to the cells. Fig. 3 shows the characteristic picture of AG568 treated cells compared to untreated cells.

Table I

Screening of in vitro active tyrphostins for their ability to induce differentiation of K562 and MEL cells

| tyrphostin | | purified kinase $IC_{50}$, ($\mu$M) | | Inducer for differentiation | |
|---|---|---|---|---|---|
| Name | Structure | $p210^{bcr\text{-}abl}$ | EGF R | K562 cells | MEL cells |
| AG-952 |  | 0.77±0.23 | 0.28 | - | - |
| AG-956 |  | 1.3±0.1 | 0.28 | - | - |
| AG-896 |  | 1.9±0.2 | 0.58 | - | - |
| AG-775 |  | 2.7±0.3 | 2.8 | - | - |
| AG-538 |  | 4±0.5 | 0.37 | - | - |
| AG-183 |  | 3.2±0.64 | 0.8 | - | - |
| AG-124 |  | >>20 | 143 | + | - |
| AG-514 |  | 1.3±0.2 | 94 | + | - |
| AG-568 |  | 1.8±0.4 | 11 | + | + |
| AG-1112 |  | 10.2±3.5 | 18.5 | ++ | -/+ |

Tyrphostins were screened for their potency to inhibit EGFR kinase activity against poly $Glu_6$ $Ala_3$ Tyr [11] and $p210^{bcr\text{-}abl}$ kinase activity [14]. In parallel, the compounds were examined for their potency to induce K562 differentiation [14] and differentiation of MEL cells as described in Section 2.

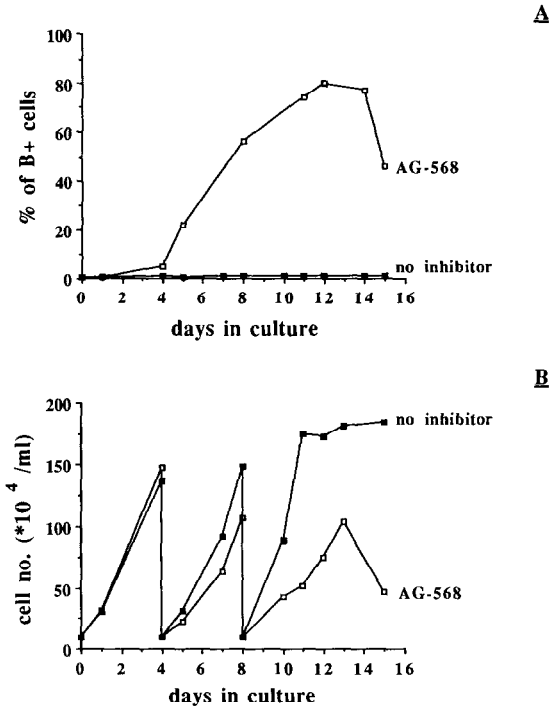



Fig. 1. Kinetics of MEL cells differentiation induced by AG568. MEL cells ($1\times10^5$/ml) were treated with 50 $\mu$M AG-568. At days 4 and 8, the cells were diluted with fresh medium and tyrophostin for their initial concentration ($1\times10^5$/ml). As control we treated the cells with medium that contained a comparable concentration of the tyrphostin solvent (0.1% DMSO). Part A is the kinetics of differentiation expressed by the percent of hemoglobin containing cells, stained with benzidine (B+ cells). Part B represents the growth kinetics.

### 3.2. *The effect of the supernatant of AG568-treated cells on cell differentiation*

During our experiments, analyzing AG568 as differentiation inducer, we examined several types of treatments as schematically shown in Fig. 4. In the pro-

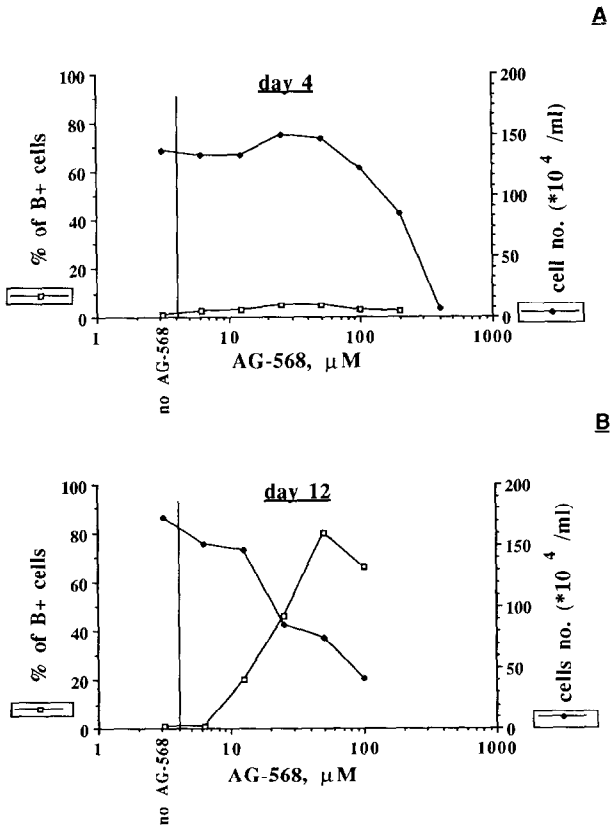



Fig. 2. The effect of various concentrations of AG-568 on MEL cells differentiation and growth. The percent of hemoglobin-producing cells (B+ cells) and concentration of live cells in the culture were analyzed at day 4 (A) and day 12 (B) subsequent to AG-568 application. Day 4 (previous to differentiation) and day 12 (up to 80% of differentiated cells) are both 4 days after the cells were diluted to their initial concentration ($1\times10^5$ cells/ml). The concentrations of 200 $\mu$M and above are toxic to cells, and hence do not appear in (B).

cedure designed 'unwashed cells' 75% of the culture was replaced every two or three days with fresh tyrphostin-containing medium. This was the routine procedure we used for screening the effect of various tyrphostins. In a different procedure, designed 'washed cells' 25% of the culture were sedimented and resuspended with fresh AG568-containing medium. Fig. 4B shows that while AG568 used in the 'unwashed cells' procedure induces differentiation of MEL cells, it fails to induce differentiation in the alternative procedure. We therefore suspected that the tyrphostin induces the production of a differentiating factor. Thus we collected the medium at least two days after the last medium renewal and treated MEL cells previously not exposed to tyrphostin. As shown in Fig. 5, the supernatant (S.N.) of cells treated with 50 $\mu$M AG568 using the 'unwashed cells' procedure induces the differentiation of MEL cells. In contrast, the supernatant of cells treated using the 'washed cells' procedure does not induce differentiation. The supernatant from cells which were treated using the 'unwashed cells' procedure but without tyrphostin also does not induce cell differentiation. We also find that AG568 incubated in medium alone in the absence of cells, under identical conditions does not induce differentiation (data not shown). These results suggest that the differentiation is produced by MEL cells as a result from AG568 treatment.

### 3.3. *AG568 inhibits the tyrosine phosphorylation of pp97 in intact MEL cells*

Using antiphosphotyrosine antibodies, a number of tyrosine phosphorylated proteins could be identified in Western blots of lysates of MEL cells (Fig. 6A). The tyrosine phosphorylation of one band, pp97, diminished following treatment with AG568, at a concentration effective in inducing cell differentiation concomitant to growth arrest. The 170 and 53 kDa bands were not significantly affected by AG568. The onset of inhibition of pp97 tyrosine phosphorylation precedes growth arrest (Fig. 6B). AG775, a highly potent tyrphostin in vitro [12], neither induces MEL cells to differentiate (Table I) nor does it inhibit the tyrosine phosphorylation of any of the above bands (Fig. 6A). Similarly, other tyrphostins potent in vitro but ineffective in inducing MEL cell differentiation (Table I) also fail to block pp97 phosphorylation.

## 4. DISCUSSION

In this study we present evidence that the tyrphostin AG568, a specific inhibitor for tyrosine kinases, induces erythroid differentiation of MEL cells (Fig. 1–5) following the inhibition of tyrosine phosphorylation of at least one protein, pp97. Other potent tyrphostins, such as AG775 (Table I), which do not induce cell differentiation also fail to inhibit tyrosine phosphorylation of proteins observed in the phosphorylation blot (Fig. 6). This

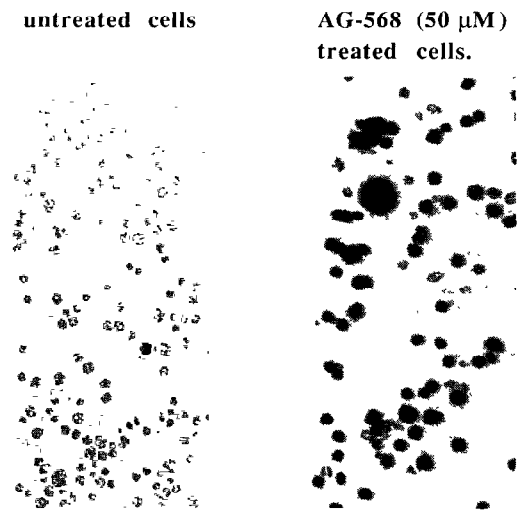
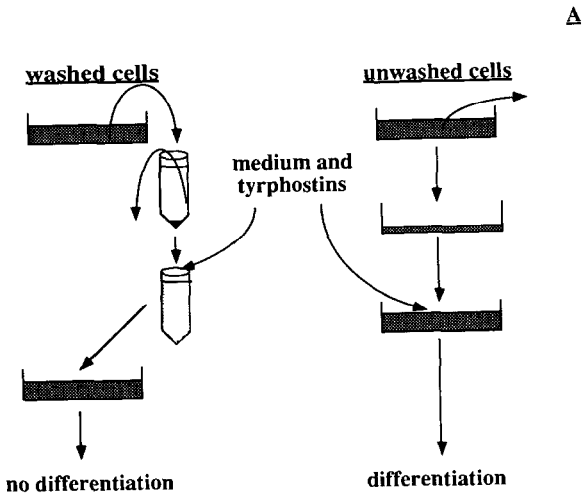



Fig. 3. MEL cells treated with AG-568. MEL cells were treated for 12 days with 50 $\mu$M AG-568. A sample from the culture was stained with benzidine, and photographed at magnitude ×200.



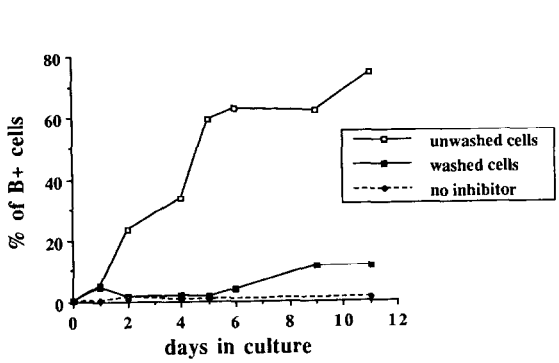




Fig 4. AG568 induced production of a putative differentiation factor. MEL cells were treated with 50 $\mu$M AG-568 using two alternative procedures as schematically described in (A). In the procedure designated 'unwashed cells' 75% of the culture was removed every 2 days and tyrphostin-containing medium in the same volume was added. In the procedure designated 'washed cells' the cells were sedimented, the old medium was removed completely, and the cells were resuspended with fresh medium containing tyrphostin As control the cells were treated using 'unwashed cells' procedure, but in the absence of tyrphostin. (B) shows that cells treated by the 'unwashed cells' procedure were differentiated, whereas cells whose supernatant was washed were not.

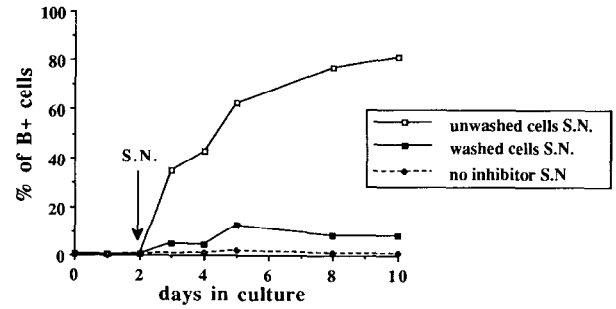
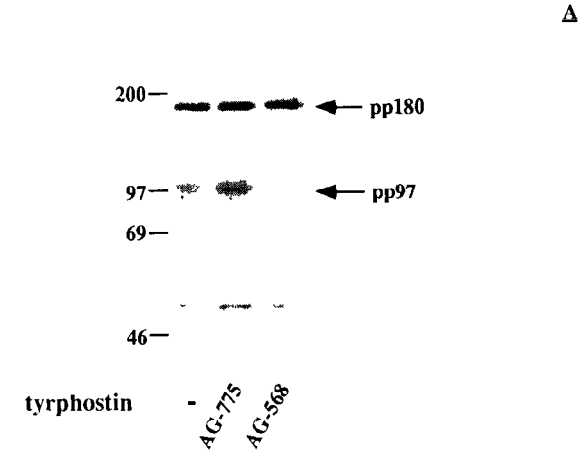



Fig. 5 The effect of supernatant from cells treated with AG-568 on the differentiation of untreated MEL cells. MEL cells were treated with supernatant from cells treated with AG568 as described in Fig. 4, two days after AG-568 application. The supernatant of both washed and unwashed cells contains the same amount of AG-568 left over from previous treatments As a control we have used supernatant from cells treated with the 'unwashed cells' procedure in the same conditions, but without tyrphostin.

result suggests a correlation between the inhibition of tyrosine phosphorylation in intact MEL cells and the onset of differentiation. AG568 seems to be a selective tyrosine kinase blocker since it inhibits the tyrosine phosphorylation of pp97, but not of other proteins in



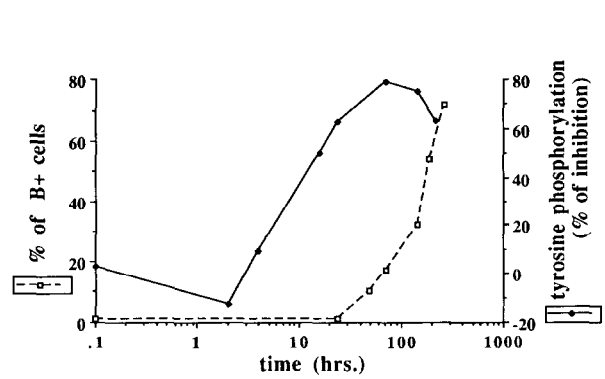




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Fig. 6. AG-568 inhibits pp97 phosphorylation in intact MEL cells, prior to differentiation. (A) The tyrosine phosphorylation levels in MEL cells was analyzed by antiphosphotyrosine antibodies. For a control we have used AG-775, non-differentiated but in vitro effective tyrphostin, or medium containing the tyrphostin solvent alone. (B) Kinetics of inhibition of the tyrosine phosphorylation by AG-568. The intensity of the phosphotyrosine-containing bands was analyzed using a Macintosh scanner. The value measured for pp97 was standardized to compare with the AG-568 uninfluenced band, pp180, in each lane. The percent of inhibition was calculated compared to the intensity of standardized pp97 of the untreated cells. Benzidine-positive cells (B+ cells) were analyzed as described.

intact MEL cells. AG568 was also found to induce erythroid differentiation of the K562 cell line concomitantly with the inhibition of $p210^{bcr\text{-}abl}$ tyrosine phosphorylation in intact cells [14]. One of the derivatives of AG568, AG1112, was found to be even more effective in inducing K562 differentiation and in inhibiting tyrosine phosphorylation of $p210^{bcr\text{-}abl}$ [14]. Interestingly, AG1112 was ineffective in the MEL cell system (Table I, data not shown) suggesting that the latter is a more selective tyrphostin towards K562 cells and the $p210^{bcr\text{-}abl}$ kinase. The differentiation of MEL cells begins 2–5 days after, subsequent to AG568 application. Fig. 1 shows that after 80% of the cells differentiated, a significant fraction of the differentiated cells die. This result suggests that AG568 induces terminal differentiation of MEL cells. Kinetic and dose–response analysis demonstrates a correlation between growth arrest and differentiation (Figs. 1 and 2). Furthermore, growth arrest is induced by AG568 concentration, well below its toxic level (Fig. 2). Thus, growth arrest is not a result of general toxicity of AG568, but rather a consequence of the differentiation process.

AG568 was also found to inhibit the tyrosine phosphorylation of $p210^{bcr\text{-}abl}$ in intact K562 cells after one hour of application [14]. In MEL cells, the inhibition of pp97 phosphorylation is a secondary event induced by this tyrosine kinase blocker, probably due to the inhibition of important but rare tyrosine kinase(s).

We have found that MEL cells treated with AG568, produce a putative differentiation agent. We cannot eliminate, at this stage, the possibility that the differentiating agent is a cell-mediated modification of AG568 which elevates the activity of the original compound. The appearance of AG568-mediated differentiation agent, is probably part of the differentiation process induced by AG568 and requires a more elaborate investigation. We would like to suggest that the production of this agent occurs as a result of the tyrosine kinase inhibitory activity of AG568 where the inhibition of pp97 phosphorylation (Fig. 6) may be involved.

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