Tyrosine Kinase Inhibitors Suppress the Growth of Non-Hodgkin B Lymphomas

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ABSTRACT

Non-Hodgkin lymphomas usually become resistant to chemotherapy and relapse due to the their intense antiapoptotic robustness. Furthermore, the slow growth of these malignancies limits the effectiveness of drugs aimed mainly at the proliferative pathways. Because protein tyrosine kinases (PTKs) play a key role in both proliferative and antiapoptotic pathways we screened our library of PTK inhibitors for agents that induce growth arrest and apoptosis in non-Hodgkin B cell lymphoma cell lines. Herein, we describe the identification of a family of PTK inhibitors whose most potent member is AGL 2592. This agent induces growth arrest and massive apoptosis in a number of non-Hodgkin lymphoma cell lines. We also show that the lymphoma cell lines are much more sensitive to this class of agents compared with other malignant carcinoma cells. AGL 2592 induces a dose-dependent and time-dependent inhibition of tyrosine phosphorylation of numerous proteins, including Stat3, and an increase of Bcl-2 phosphorylation, both biochemical hallmarks of growth inhibition and apoptosis.

The majority of non-Hodgkin's lymphomas like acute lymphoblastic leukemias (ALLs) are of B-cell lineage (B-NHL) (Harris et al., 2000). The B-cell lymphomas are heterogeneous in terms of histology, clinical presentations, response to treatment, and prognosis (Coiffier et al., 1991; Potter, 1992). Despite recent advances in therapy, many cases still relapse, remain refractory to conventional chemotherapy and even to high dose polychemotherapy, followed by peripheral blood stem cell transplantation (Salzman et al., 1997; Cheson et al., 1999; Bosly et al., 2001). Immunotherapy and the use of biological modifiers such as interferon and interleukins have also been introduced to treat disease but have not changed significantly the overall prognosis, and over 50% of the patients relapse (Piris et al., 1994; Canellos, 1998). A chimeric monoclonal antibody (Rituximab) has been introduced as monotherapy to treat B-cell lymphomas and is presently evaluated in combination with chemotherapy (Davis et al., 1999, 2000a,b). More recently an idiotype pulsed dendritic cells vaccination was shown to induce T-cell and humoral anti-idiotype immune responses and durable tumor regression in patients with B-cell lymphoma (Timmerman et al., 2002). Still, the development of novel approaches to therapy remains a formidable challenge. One of the potential useful approaches to therapy is signal transduction therapy (Levitzki, 1999), namely, to block key signaling pathways essential for the survival and/or growth of cancer cells. Because enhanced PTKs activity is the hallmark of most cancers as well as of other proliferative diseases, it is likely that such inhibitors may become useful for the management of B-NHL (Levitzki, 1992, 1999). The success of some PTK inhibitors as blockers of chronic myelogenous leukemia (CML) and lessons learned from the development of an Abl tyrosine kinase inhibitor for CML (Carlo-Stella et al., 1999; Druker and Lydon, 2000) and preB-ALL (Meydan et al., 1996) prompted us to search systematically for PTK blockers aimed at B-NHL. Because unlike CML and preB-ALL the kinases involved in enhanced growth and resistance to apoptosis in B-NHL are unknown, we chose a different approach to search for effective inhibitors. We screened the library of tyrphostins that we possess (~2500 compounds, represented by eight families) for agents that induce growth arrest and apoptosis in B-NHL cell lines, representing various stages of B-cell differentiation (Ben-Bassat et al., 1987). Using this screening we identified a family of bis-tyrphostins as the most effective agents inducing growth arrest and cell death. Within

ABBREVIATIONS: ALL, acute lymphoblastic leukemia; B-NHL, non-Hodgkin B lymphoma; PTK, protein tyrosine kinase; CML, chronic myelogenous leukemia; FCS, fetal calf serum; Med, RPMI 1640 medium without phenol red, with 10% fetal calf serum and antibiotics; DMSO, dimethyl sulfoxide; XXT, 2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxyanilide; PMS, phenazine methosulfate; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; DAPI, 4,6-diamidino-2-phenylindole.

that family we identified AGL 2592 as the most effective agent. In this article, we describe the biological activity of AGL 2592 and its potential therapeutic use.

Materials and Methods

Materials. The following antibodies were used to monitor the levels and state of various signaling proteins: for phosphorylated tyrosine 4G10, mouse monoclonal (Upstate Biotechnology, Lake Placid, NY); Jak2, rabbit polyclonal anti-mouse (Upstate Biotechnology); Bcl-2, mouse monoclonal anti-human (Upstate Biotechnology); Stat3, rabbit polyclonal IgG, anti-human (Upstate Biotechnology); and for phosphorylated Stat3, rabbit anti-phospho-Tyr 705 synthetic peptide of mouse Stat3 (New England Biolabs, Beverly, MA). Fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO) was used for immunofluorescence staining. Fetal bovine serum was from Invitrogen (Carlsbad, CA). Tissue culture media and antibiotics were from Biological Industries (Beit Haemek, Israel). Tissue culture reagents and growth supplements were from Sigma-Aldrich.

Cells. The cell lines were propagated in RPMI 1640 medium, supplemented with 20% fetal bovine serum (FCS) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were maintained at 37°C in a humidified incubator containing 5 to 8% CO_2. Logarithmically growing cells were used in the experiments (Ben-Bassat et al., 1987).

Experimental Design. Cells were seeded at subconfluent densities in 96-well microplates in RPMI 1640 medium without phenol red, with 10% FCS and antibiotics (abbreviated Med) and with tyrphostin at the appropriate concentrations. Stock solutions of tyrphostins were 10 mM in DMSO. For the experiments tyrphostins were diluted in Med. Controls were cells grown in Med only and in Med with DMSO. DMSO concentration in the controls was equal to the concentration in the tyrphostin containing Med. For each tyrphostin concentration the appropriate DMSO concentration was taken as 100%. The highest concentration of DMSO used was 0.1%, which by itself had negligible effect (see below). The cells were treated with one application of tyrphostin for 72 h.

Automated Microculture XTT-PMS Assay. Cell growth was determined by the XTT-PMS assay for cell growth and drug sensitivity. Fifty microliters of XTT and 0.15 μg of PMS per 200 μl/well were added to the tyrphostin-treated and control cultures and incubated for 4 h (stock solutions: 1 mg/ml XTT in RPMI 1640 medium alone, with no phenol red, 1.53 mg/ml PMS in PBS). After thorough mixing on a mechanical plate-mixer, absorbance at 450 nm was measured with a microplate reader (model htII; Anthos Labtec Instruments, Salzburg, Austria). Titration experiments showed linear reading for 1×10^3 to 1×10^5 cell/well. Each point of the growth curve experiments is calculated from eight wells.

Calculation of Growth Inhibition. For each tyrphostin concentration used, the appropriate Med containing only DMSO was used as control. Thus, for each concentration the control was taken as 100% growth. The highest DMSO concentration used in this study is 0.1%, which by itself had no significant effect on cell growth (data not shown). A dot above the column marks the significant results.
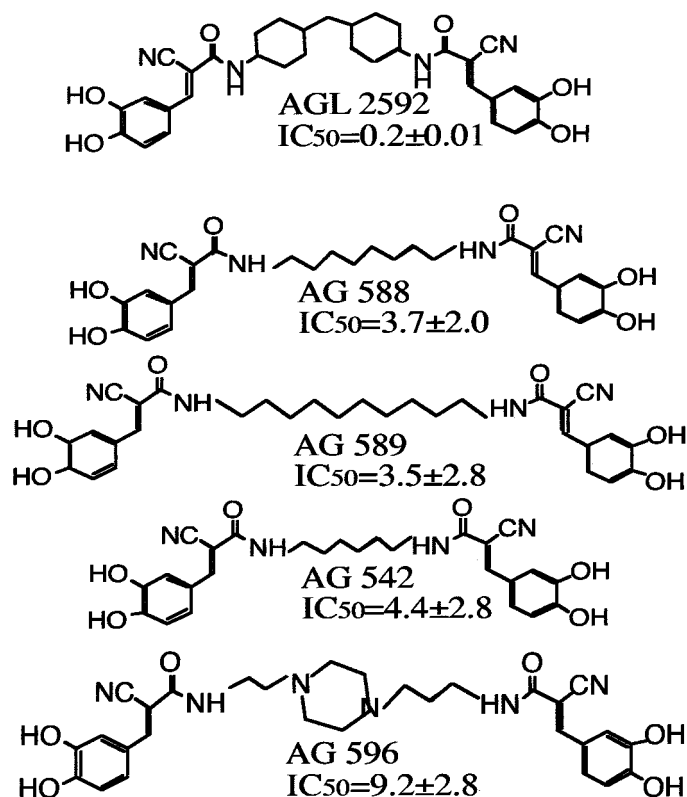
Growth in Methyl Cellulose. The Farage cell line was used in these experiments (Ben-Bassat et al., 1992). Farage cells (5×10^4 cells/six-well

dish) were suspended in methylcellulose medium (1.05% final concentration) containing selected concentrations of AGL 2592. Colonies number per well is calculated from four wells after 7 days in culture.

FACS Analysis and DNA Content. Selected samples of cell culture suspensions treated with tyrphostin concentrations for predetermined periods were stained with ethidium iodide. Cell cycle analysis of the cell cycle populations was carried out with FACS FPAR-PLUS (BD Biosciences, San Jose, CA). A dot above the column marks the significant results.

Determination of Apoptotic Cells. Cells undergoing apoptosis were visualized by DAPI staining. Briefly, cell cultures were washed twice with PBS, fixed with 4% formaldehyde for 20 min at room temperature, washed extensively with PBS, stained with 0.05 mg/ml DAPI (Sigma-Aldrich) for 30 min in the dark, and again washed extensively and examined with a UV microscope. Trypan blue exclusion to determine percentage of dead cells was performed in part of the experiments. The percentage of apoptotic cells was determined also by FACS analysis of the cell population. A dot above the column marks the significant results.

Western Blot Analysis and Phosphotyrosine. Farage cells (Table 1) were seeded at 5×10^5 cells/ml in Med in flasks. After 20 h tyrphostin at the appropriate concentration was added for predetermined periods. The reaction was stopped by placing the cultures on ice and washing them with ice-cold PBS. Whole cells were lysed with buffer, boiled for 5 min, run on 7 to 15% SDS-polyacrylamide gel for 4 h, and then transferred to nitrocellulose paper, at room temperature overnight. Thereafter, the samples were incubated with the appropriate antibody or with monoclonal anti-phosphotyrosine antibody 4G10, following the manufacturer's recommendations. Goat anti-mouse or anti-rabbit fluorescent antibody was added (3 μl/30 ml) for 30-min incubation at room temperature. The nitrocellulose membrane was washed with PBS-Tween and the enhanced chemiluminescence

TABLE 1
NHL-B cell lines

Line	Origin
KM 3	Prepre B
Nalm 6	PreB-ALL
Farage	B-NHL (large cell)
Raji	Burkitt lymphoma (BL)
DG 75	Burkitt lymphoma (BL)
Bjab	Burkitt lymphoma (BL)
ARH 77	Myeloma (MM)
Monga LBL	Lymphoblastoid-healthy donor

Fig. 1. Structure of bis-tyrphostins and their efficacy against Farage. Farage cells were exposed to the tyrphostin for 3 days. The tyrphostins were added once after seeding. Growth was determined using the XTT-PMS assay, and the IC_{50} values for growth inhibition of the cells by these compounds were calculated.
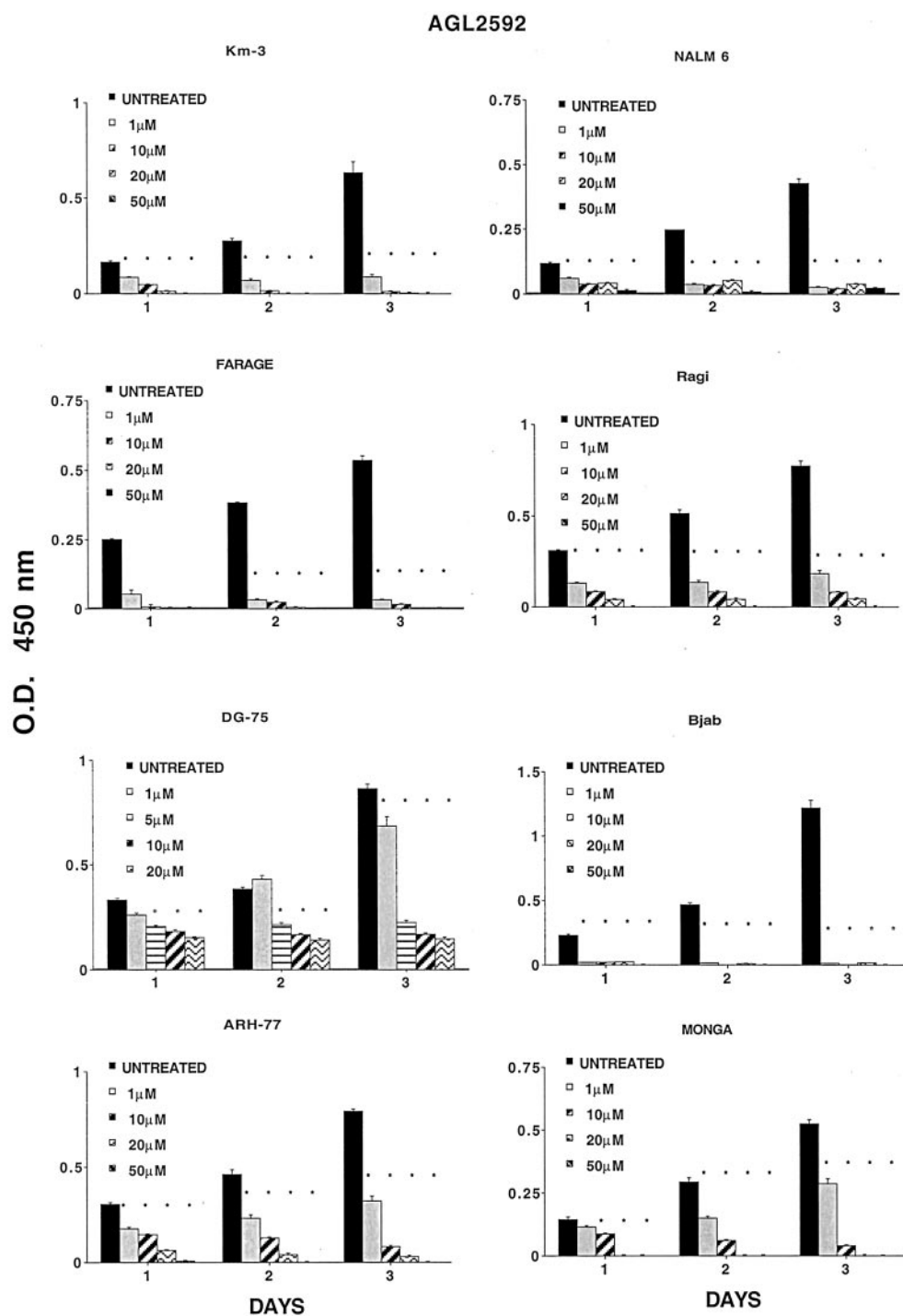
Fig. 2. Growth inhibition by AGL 2592 of B-NHL cell lines. The cells of B-NHL lines at various stages of differentiation were seeded at subconfluent densities in 96-well microplates in RPMI 1640 medium without phenol red, with 10% FCS and antibiotics, and with tyrphostin at the appropriate concentrations. Tyrphostins were added only once after seeding and growth was compared with cells grown in the presence of DMSO. Cells were exposed to the tyrphostin for 3 days, followed by determination of growth. Additional experimental details are given in the text.

system applied, and then the membrane was exposed to X-rays films in cassettes. To clarify the Western blot results, relevant and significant bands were quantified and optical density was determined with the Multi-Analyst/PC, version 1.1 (Bio-Rad, Hercules, CA).

Statistical Analysis. The results are based on two to three experiments, and each point is calculated from eight to 12 wells. Student's t test was used to analyze the results and determine ± S.D.

Results

Tyrphostins Suppress B-NHL Cell Growth

We conducted a screening of the different families of PTK inhibitors (tyrphostins) where their efficacy was measured by

their potency to inhibit the growth of human leukemia-lymphoma cells. We analyzed cell lines of prepreB, preB-ALL, or B-NHL, representing various stages of B-cell differentiation (Table 1). They were assigned to their respective compartments of the differentiation scheme according to a combination of positive expression of surface markers (Ben-Bassat et al., 1987). In parallel, we examined the effect of these compounds on the growth of a human normal B-EBV immortalized, lymphoblastoid line. Among the tyrphostins six were found to possess IC_{50} values below 10 μM; these belong to the family of bis-tyrphostins (Fig. 1).

Tyrphostins were added only once after seeding and

growth was compared with cells grown in the presence of DMSO and with cells in the absence of the vehicle. Cells were exposed to the tyrphostin for 3 days, followed by determination of growth using the XTT-PMS assay.

The effect of AGL 2592 on the B-NHL cell lines is depicted in Fig. 2. It can be seen that 1 μM AGL 2592 effectively suppressed the growth of the prepreB, preB, B-leukemia-lymphoma, and the EBV immortalized B cells. We chose Farage cells (Ben-Bassat et al., 1992) for a more detailed study of AGL 2592. The IC_{50} values for growth inhibition of Farage cells by these compounds is summarized in Fig. 1. It can be seen that AGL 2592 possesses the lowest IC_{50} value ($<0.25 \pm 0.01$ μM). On a molar basis the potency ratio of these tyrphostins was found to be AGL 2592 > AG 588 > AG 589 > AG 542 > AG 596 (Fig. 1). It should be noted that those IC_{50} values are likely to be overestimates of the true IC_{50} values, because the agents were added only once and the medium was not changed throughout the experiment, which was 72 h. Our experience with this class of compounds indicates that they degrade in the medium with half-lives of 16 to 30 h (A. Levitzki, unpublished data). We chose the most efficacious compound, AGL 2592, to conduct a more detailed analysis.

Growth Arrest and Rescue Experiments

In these experiments we measured the growth and the self-renewal capacity of B-NHL cells after treatment with AGL 2592. Km 3, Nalm 6, DG 75, and ARH 77 cells were treated with 0.1, 1, or 10 μM AGL 2592 for 3 days, washed, replated, and their proliferative capacity determined. AGL 2592 was applied once and the status of the cells examined after 72 h. The results are summarized in Fig. 3. Treatment with >10 μM AGL 2592 suppressed completely cell growth, and the cells did not recover after wash and replating without the compound. Additional experiments with lower con-

centrations of AGL 2592 confirmed the high efficacy of this tyrphostin (Fig. 4). After treatment of Farage cells with 0.3 μM AGL 2592 growth inhibition was greater than 80% (Fig. 4A). After washing and replating of the cells treated with 0.2 or 0.5 μM AGL 2592, growth was resumed and almost complete survival was obtained (Fig. 4B). With 2 or 5 μM AGL 2592, complete growth inhibition was obtained and the cells did not regain their proliferative capacity (Fig. 4B). We then examined the colony-forming ability on methylcellulose of Farage cells treated with various concentrations of AGL 2592; 0.1 μM AGL 2592 reduced significantly (>60%) the cloning efficiency of Farage cells, and with 0.5 μM AGL 2592 or higher, complete inhibition was obtained and no colonies were formed (data not shown).

AGL 2592 Is Less Effective against Other Cells

Intrigued by the high efficacy of AGL 2592 to suppress growth of B-NHL cells we examined its inhibitory effect on other human malignant cells: an ovarian carcinoma cell line (OV1063), a breast carcinoma cell line (MCF 7), an osteosarcoma cell line (Saos2), and a human myeloma cell line (U 266), as well as on human normal cells of epithelial origin, including keratinocytes and an EBV immortalized human B-lymphoid cell line (Monga). Table 2 shows that AGL 2592 is more effective against Farage cells compared with the other types of cells. We also examined the ability of the treated cells to recover after 72-h exposure to the agent, as was done for lymphoma cells. Table 2 shows that these three malignant cell lines can withstand significantly higher concentrations of AGL 2592 and fully recover compared with Farage cells.

AGL 2592 Induces Massive Apoptosis

To explore more directly the mechanism of growth suppression by AGL 2592, cell cycle analysis was performed on Km 3,
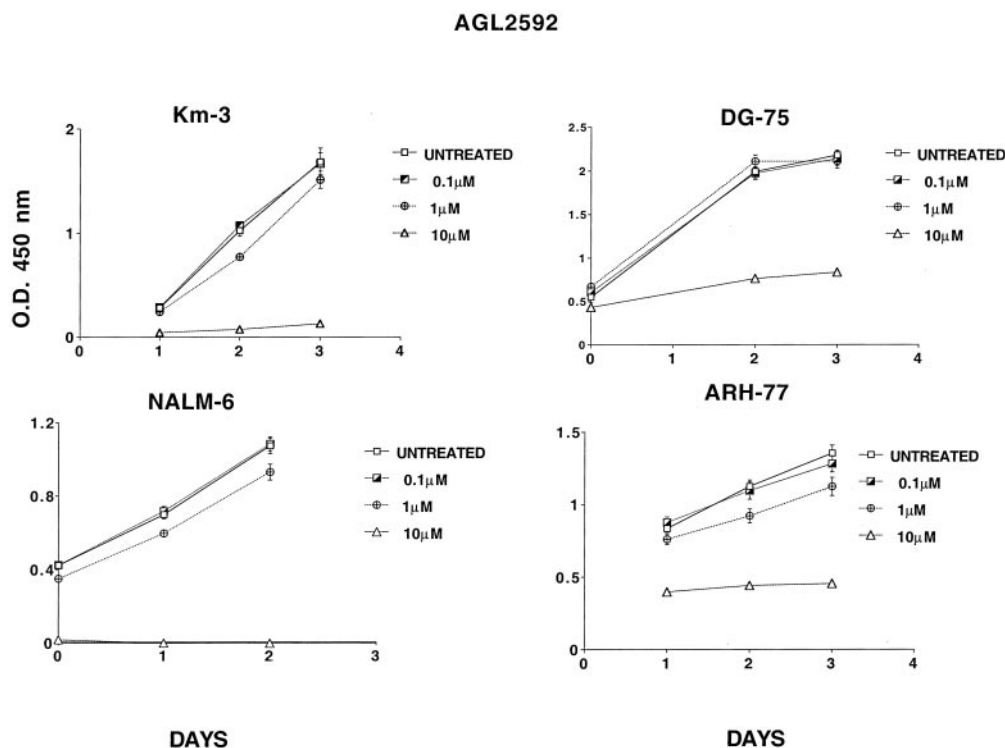
AGL2592

Fig. 3. Self-renewal capacity: rescue of B-NHL cell lines after treatment with AGL 2592. Cells of B-NHL lines at various stages of differentiation were treated with AGL 2592 for 3 days. Thereafter, the cells were washed and replated and the self-renewal capacity (rescue) determined. Experimental details are given in the text.
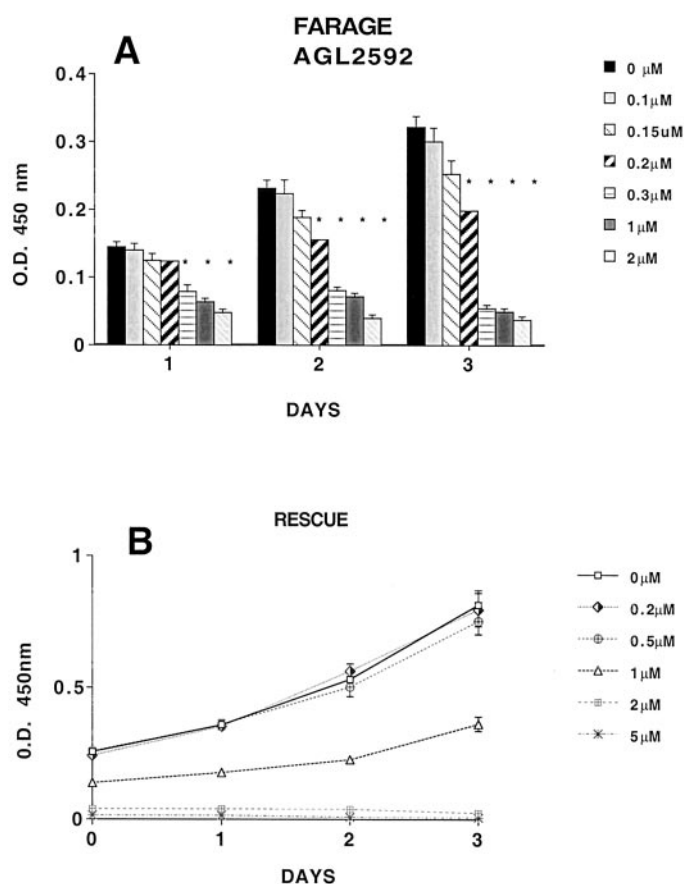
Fig. 4. Inhibition of Farage cells growth by AGL 2592 and rescue from the agent. A, inhibitory effect on cell proliferation: Cells were seeded at subconfluent densities in 96-well microplates in RPMI 1640 medium without phenol red, with 10% FCS and antibiotics, and with AGL 2592 at the appropriate concentrations. The tyrphostin was added only once after seeding and growth was compared with cells grown in the presence of DMSO. Cells were exposed to the tyrphostin for 3 days, followed by determination of growth. B, rescue after treatment: Farage cells were treated with AGL 2592 for 3 days. Thereafter, the cells were washed and replated and the self-renewal capacity (rescue) determined. Experimental details are given in the text.

TABLE 2
Growth inhibition of different cell lines compared with Farage cell line
The data are based on two to three experiments, 8 to 12 wells/point. Student's *t* test was used to calculate S.D.

Cells	Origin	IC$_{50}$	Complete Inhibition
		μM	
Farage	B-NHL	0.25 ± 0.1	Below 2
OV 1063	Ovarian carcinoma	6.0 ± 0.5	At 10
MCF 7	Breast carcinoma	3.0 ± 0.3	At 5
Saos 2	Osteosarcoma	1.0 ± 0.3	At 5
Keratinocytes	Human normal	4.2 ± 0.4	At 10
U266	Myeloma	4.7 ± 0.5	At 10
Monga	Lymphoblastoid-healthy donor	3.8 ± 0.4	At 5

Nalm 6, Farage, and Bjab lines. Cells exposed to this compound were assessed by FACS analysis at 6, 24, and 48 h after initiating treatment. AGL 2592 alters the cell cycle distribution of these cell lines in a concentration- and time-dependent manner (Fig. 5A). Already after 6 h of treatment, an alternation in the cell cycle distribution is observed, with a significant effect noted even with 1 μM AGL 2592. After 6 h at 1 and 5 μM AGL 2592 the proportion of cells in G$_1$ is

increased and the proportion of cells in G$_2$/M decreased, with no effect on the apoptotic cell fraction. At 24 h the increase in the cells in G$_1$ was still evident but the decrease in S was more pronounced with a significant increase in the apoptotic cell fraction. The increased fraction of cells in G$_1$ and the decrease in the number of cells S, G$_2$/M phases is concomitant to the increase in the proportion of apoptotic cells. The effect becomes more pronounced on day 2. These findings suggest that the cells exit to apoptosis from both S and G$_2$/M phase of the cell cycle. Up to 50% of the cells undergo apoptosis, whereas the rest remain irreversibly arrested, mostly at G$_1$ (Fig. 5A). Microscopic examination of the Farage cells also showed cells with the typical nuclear apoptotic morphology at treatment with $\geq$1 μM AGL 2592 for 24 h that became more pronounced after 48 h of treatment (Fig. 5B).

### Effects of AGL 2592 with Ritoximab on Farage Cells Are Additive

We examined the effect of AGL 2592 on CD20 surface expression, growth inhibition, and possible synergism or additivity with Rituximab (a chimeric anti-CD20 monoclonal antibody currently used in human therapy of B-NHL; Davis et al., 2000a) on the Farage cells. CD20 surface expression on Farage cells was not affected after treatment with AGL 2592 (data not shown). Ritoximab inhibited the growth of Farage cells in a dose-dependent manner. The maximal growth suppression attained was ~40%, using 1 μg/ml for treatment of 3 days (Fig. 6). Ritoximab at 1 μg/ml has been previously reported to inhibit cell proliferation of the B-NHL SU-DHL-4 line (Maloney et al., 1997). Combining Ritoximab with 0.25 μM (0.15 μg/ml) AGL 2592 (its IC$_{50}$ value), resulted in 72% growth inhibition, suggesting that the effect of the two agents is additive (Fig. 6).

Biochemical Activities of AGL 2592 on Farage Cells

Bcl-2 phosphorylation. Because treatment with AGL 2592 of the B-NHL Farage cells resulted in growth arrest and apoptosis, we examined the possibility that Bcl-2 could be linked to the process. Figure 7 shows that AGL 2592 induces a shift in the electrophoretic mobility of Bcl-2, at concentrations of $\geq$5 μM already after 24 h of treatment, also evident at 48 and 72 h at >1 μM. Time-course analysis shows that slower mobility forms of Bcl-2 were not formed at 4-h exposure to AGL 2592 (data not shown). This finding is in accordance with the FACS analysis results and microscopic examination showing cells with apoptotic nuclear morphology at treatment with $\geq$1 μM AGL 2592 for 24 h, which were not detected at 6 h (Fig. 5, A and B, respectively). It has previously been suggested that the mobility changes of Bcl-2 are due to phosphorylation of the protein (Haldar et al., 1995, 1997). After 48 and 72 h of treatment with 5, 10, and 50 μM AGL 2592 the increase in the slower mobility form of the Bcl-2 protein is more pronounced (Fig. 7).

AGL 2592 Inhibits Tyrosine Phosphorylation. Although the target(s) of AGL 2592 has not been identified, it inhibits the tyrosine phosphorylation of a number of proteins. Because the inhibitory effect was found to be already at 24 h but maximal at 72 h after addition of AGL 2592 we examined the pattern of tyrosine phosphorylation in Farage cells at these time points. Figure 8, A and B, shows that the phosphorylation of a number of proteins is inhibited in a time- and
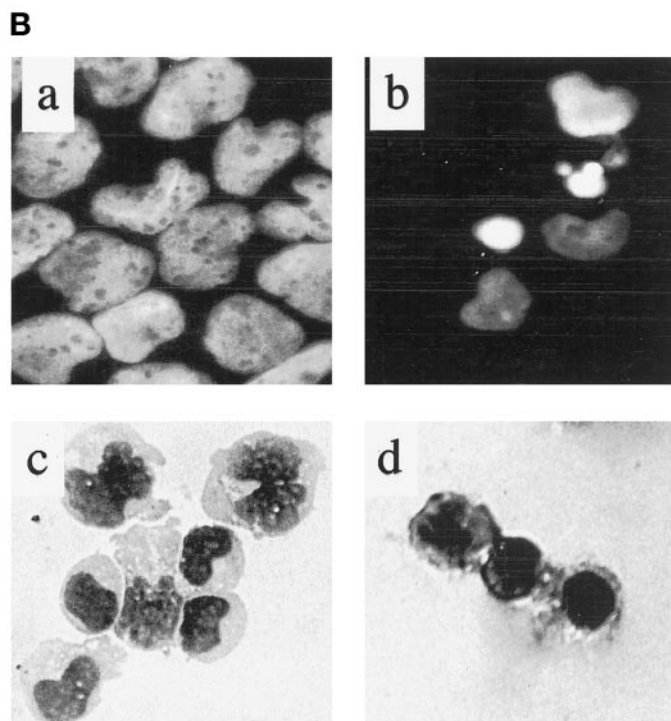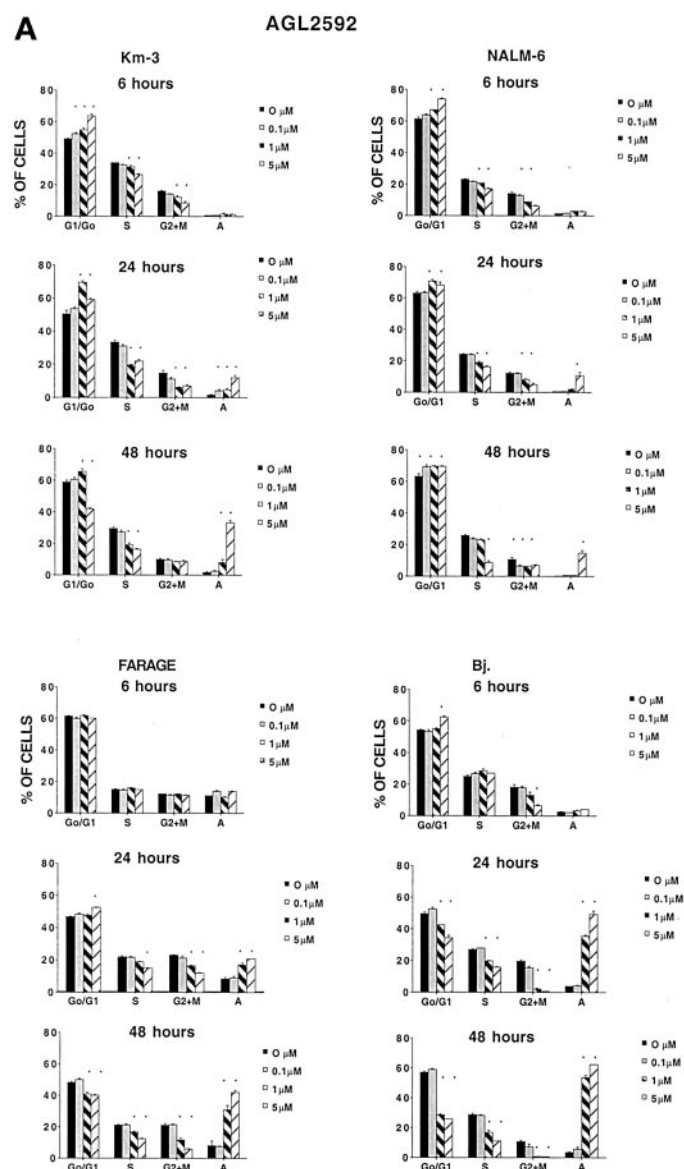
Fig. 5. AGL 2592 affects cell cycle distribution and apoptosis of B-NHL lines. A, cell cycle distribution and apoptosis of B-NHL lines at various stages of differentiation after treatment with AGL 2592. Cells exposed to this compound were assessed by FACS analysis at 6, 24, and 48 h after initiating treatment. AGL 2592 alters the cell cycle distribution of these cell lines in a concentration- and time-dependent manner. The cells were prepared for FACS analysis, as described under *Materials and Methods*. B, morphological changes typical for apoptotic cell nuclei. Microscopic examination of Farage cells showed the typical nuclear apoptotic morphology at treatment with ≥1 μM AGL 2592 for 24 h that became more pronounced after 48 h. a and b, DAPI staining: untreated control cells (a) and cells treated with 1 μM AGL 2692 for 48 h (b). c and d, Giemza staining: untreated control cells (c) and cells treated with 1 μM AGL 2692 for 48 h (d).

dose-dependent manner. One of the proteins whose phosphorylation is inhibited is Stat3.

Inhibition of Stat3 Phosphorylation. Farage cells express constitutively phosphorylated Stat3 (Fig. 8, C and D). AGL 2592 inhibits phosphorylation of Stat3 in a dose- and time-dependent manner, with no significant changes in the protein level (Fig. 8, C and D). The dose-dependent inhibition of Stat3 phosphorylation is already evident at 30 min and at 4 h with >50 μM AGL 2592 treatment (Fig. 8C) and with >5 μM at 24 and 48 h of treatment (Fig. 8D). Stat3 phosphorylation was 53% at treatment with 50 μM AGL 2592 at 30 min and 30% at 4 h compared with control. Stat3 phosphorylation was 65% at treatment with >5 μM AGL 2592 at 24 h and 50% at 48 h compared with control.

Jak2. Treatment of Farage cells with AGL 2592 does not affect significantly Jak2 phosphorylation, even at 72 h of treatment. Only in the presence of 50 μM AGL 2592 a slight decrease in the level of phosphorylated Jak2 is observed at 24, 48, and 72 h of treatment (Fig. 9).

Discussion

The present results identify a family of bis-tyrphostins (Fig. 1) as effective agents inducing growth arrest and apoptosis of B-NHL cells. Within this family AGL 2592 is the most effective compound, inducing irreversible growth arrest, changes in the cell cycle distribution, and cell death by apoptosis in human B lymphoid malignancies, at various stages of the B-cell develop-
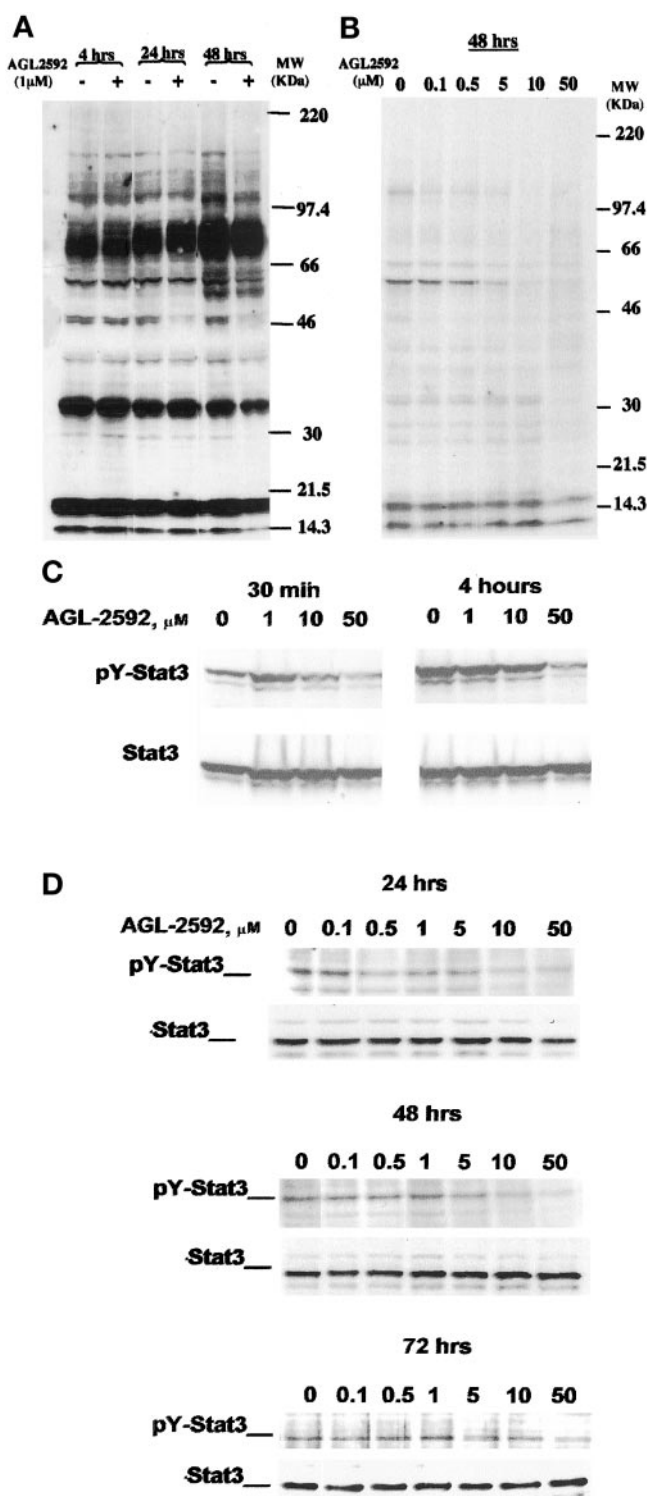
ADITIVITY OF AGL2592 AND RITUXIMAB
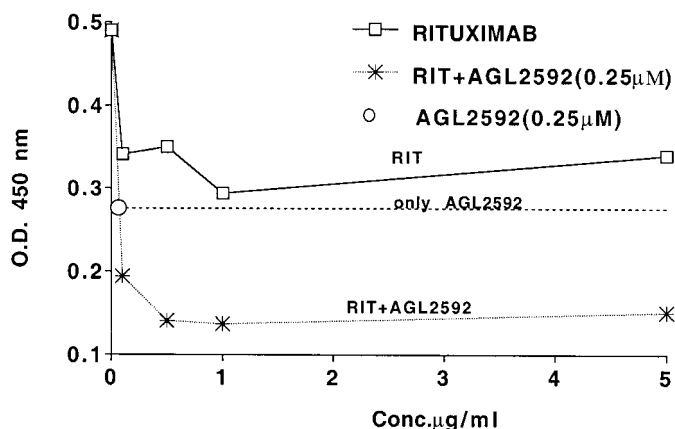
Fig. 6. Additivity of AGL 2592 and Ritoximab: inhibitory effect on Farage cells growth. Farage cells were treated with increasing concentrations of Ritoximab alone or in combination with 0.25 μM (0.15 μg/ml) AGL 2592 (its IC$_{50}$ value). Cell growth was determined by the XTT-PMS method after 3 days treatment.
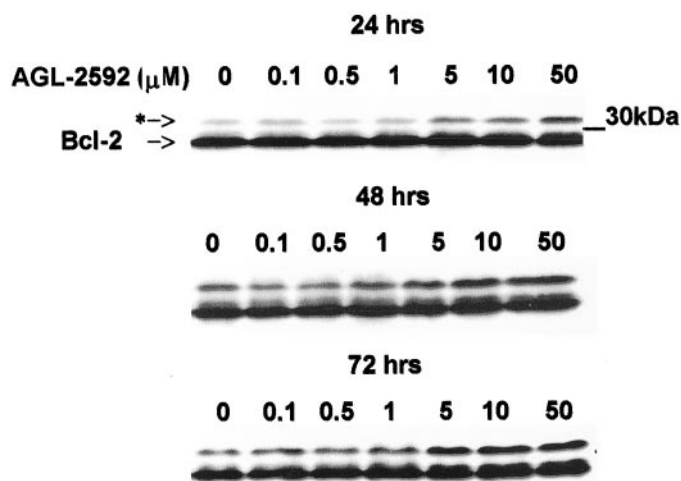
Fig. 7. Time-related changes in the Bcl-2 protein expression of Farage cells after AGL 2592 treatment. Immunoblotting of cell lysates, using monoclonal antibody against Bcl-2, was performed as described under *Materials and Methods*. Asterisk-arrow indicates modified (phosphorylated) form of Bcl-2 protein.
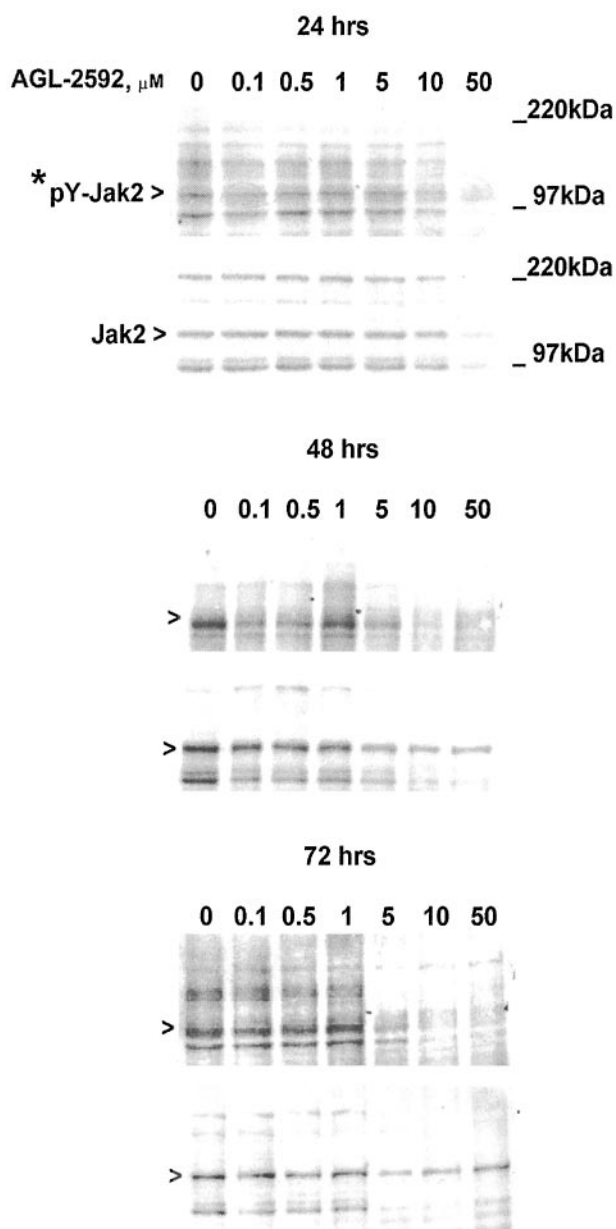

Fig. 8. AGL 2592 inhibits overall tyrosine phosphorylation and Stat3 phosphorylation. A and B, AGL 2592 inhibits overall tyrosine phosphorylation in a dose- and time-dependent manner. Details are given under *Materials and Methods*. C and D, AGL 2592 inhibits Stat3 phosphorylation in a time-and dose-dependent manner. Western blot analysis of cell lysates. Cells were treated with AGL 2592 for 30 min or 4, 24, 48, or 72 h, and the expression of Stat3 protein and its phosphorylated form was followed.

ment. In general, these cellular responses are dose- and time-dependent. AGL 2592 is very effective in suppressing growth and inducing apoptosis in a B-NHL cell line, Farage, pathologically defined as diffuse large cell lymphomas, which are not usually curable (Dumontet et al., 2000). They represent 40% of adult NHL and 80% of the mortality due to transformation from follicular lymphomas (Ye, 2000). A single dose of less than 1.0 μM AGL 2592 induces irreversible growth arrest and apoptosis in the Farage cells, after 2 days of treatment (Fig. 4). Therefore, the prospect of using tyrphostins in combination with chemotherapy or immunotherapy is of interest (Levitzki, 1999). We show herein that combining Ritoximab treatment (Davis et al., 2000a,b), which by itself is moderately effective with maximal inhibitory effect of 60% yields 72% growth inhibition when combined with 0.25 μM (IC$_{50}$) AGL 2592 (Fig. 6). The results suggest that the effect of the two agents is additive.

The growth arrest induced by AGL 2592 seems to occur

24 hrs

AGL-2592, μM   0   0.1 0.5 1   5   10   50

*pY-Jak2 >

Jak2 >

48 hrs

0   0.1 0.5 1   5   10   50

72 hrs

0   0.1 0.5 1   5   10   50

Fig. 9. No effect of AGL 2592 treatment on Jak2 phosphorylation and protein level of Farage cells. Western blot analysis of cells lysates treated of with AGL 2592 shows no significant changes in Jak2 phosphorylation, even at 72 h of treatment. Only in the presence of 50 μM AGL 2592 a slight decrease in the level of phosphorylated Jak2 is observed at 24, 48, and 72 h of treatment.

mainly at the G_1/S phase of the cell cycle. The increase in the fraction of cells in G_0/G_1 and the decrease in S, and G_2/M phases are evident as early as 6 h after exposure to AGL 2592 in most of the B-lymphoma cell lines studied (Fig. 5A). The changes in the cell cycle persist, are still evident on day 2, and occur concomitantly to the increase in the proportion of apoptotic cells (Fig. 5A). These findings suggest that the cells exit to apoptosis from both S and G_2/M phases of the cell cycle.

The favorable efficacy of AG 2592 on a wide range of B-NHL cells (Fig. 2) prompted us to assess which signaling pathways are affected by this tyrphostin. Little, however, is known about the signaling molecules in general, and on

PTKs in particular, which play the key role in the oncogenic and antiapoptotic signaling pathways in NHLs (Reed et al., 1992; Haldar et al., 1995; Reed, 1998; Ye, 2000). Because expression of the antiapoptotic Bcl-2 protein is not restricted to B-NHL with t(14;18) but is expressed in a broad spectrum of lymphoid malignancies (Pezzella et al., 1990; Zutter et al., 1991; Adams and Cory, 1998), we examined the possibility that AGL 2592 induces apoptosis in a Bcl-2-dependent manner. The present results show that AGL 2592 induces the generation of modified forms of the Bcl-2 protein (Fig. 7) in a dose- and time-dependent manner in the B-NHL lymphoma Farage cells. These results support observations demonstrating that mobility changes in Bcl-2 are due to phosphorylation of the protein and that phosphorylation of Bcl-2 seems to inhibit its ability to interfere with apoptosis (Haldar et al., 1995, 1997). Immunoblotting experiments showed that Bcl-2 is phosphorylated on serine, with an additional protein band and apparent molecular weight of 35 kDa, which is phosphorylated on tyrosine (Fig. 7). Another protein that has been shown to play a key role in oncogenesis is Stat3 (Catlett-Falcone et al., 1999a; Bowman et al., 2000). Farage cells as well as other B-NHL cells (data not shown) express constitutively phosphorylated Stat3. AGL 2592 inhibits phosphorylation of a number of yet unidentified substrates (Fig. 8, A and B) as well as the phosphorylation of Stat3 in a dose- and time-dependent manner, with a minor reduction in the protein level (Fig. 8, C and D). This action of AGL 2592 may be at least partially responsible for its proapoptotic activity because the activation of Stat3 is highly correlated with oncogenesis and the antiapoptotic robustness of tumor cells (Bowman and Jove, 1999; Bowman et al., 2000). It has been demonstrated that disruption of Stat3 signaling not only increases apoptosis but also confers sensitivity to some proapoptotic agents such as *cis*-diamminedichloroplatinum(II) (cisplatin) (Nagane et al., 1998; Karni and Levitzki, 2000) and Fas ligand.(Catlett-Falcone et al., 1999b). It has also been shown that Stat1 and Stat3 are constitutively activated in primary lymphoid and myeloid leukemia cells and in EBV-related lymphoma cell lines (those producing interleukin-10), suggesting a role of Stat activation in leukemogenesis (Weber-Nordt et al., 1996). Gene therapy studies have demonstrated that blocking Stat3 signaling induces potent antitumor activity in vivo (Niu et al., 1999). These observations and our results suggest that a combination of AGL 2592 with other antitumor agents may be a useful therapeutic approach to treat B-NHLs. We have not yet identified the protein tyrosine kinase that is responsible for Stat3 phosphorylation, but it is quite clear that Jak2 is not involved (Fig. 9).

In summary, in this study we show that AGL 2592 induces persistent growth arrest, changes in the cell cycle distribution, and apoptosis in human B-lymphoid malignancies, at various stages of the B-cell development. It is especially effective in suppressing growth and inducing apoptosis in diffuse large cell lymphomas (Farage line), which are not usually curable and represent 80% of the mortality due to transformation from follicular lymphomas. Another feature of the study, which may be used in the future, is the approach we used to identify the AGL 2592 family of compounds. In most cancers one actually does not know a priori the relative importance of the signaling pathways that are important for the particular oncogenic phenotype at hand. Yet, we do know the potential signaling pathways that might play a role.

These include various PTKs and cyclin-dependent kinases. Thus, one can use restricted chemical libraries focused on known scaffolds that inhibit these pathways to find lead compounds.

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