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ORIGINAL ARTICLE

Synergistic effect of AS101 and Bryostatin-1 on myeloid leukemia cell differentiation *in vitro* and in an animal model

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We evaluated the synergistic activity of AS101 (ammonium trichloro-(dioxoethylene-0-0′)-tellurate) with the protein kinase C (PKC) activators, Bryostatin-1 and phorbol-12-myristate-13-acetate (PMA), on human myelocytic leukemia cell differentiation *in vitro*, and in a mouse model. Use of AS101 with Bryostatin-1 or with a low concentration of PMA resulted in the differentiation of HL-60 cell line to cells with characteristics of macrophages. A similar synergistic effect was found *in vivo.* Compared with mice treated with AS101 alone or with Bryostatin-1 alone, the infiltration of leukemic cells into the spleen and the peritoneum of mice treated with both compounds, as well as the number of the HL-60 colonies extracted from those organs, were markedly reduced. The antitumor effects were associated with significantly prolonged survival (100% for 125 days) of the treated mice. Finally, the mechanism of action of this antitumor effect was explored, and was found to involve the Ras/extracellular signal-regulated kinase signaling pathway. Combined treatment with AS101 and Bryostatin-1 synergistically increased $p21^{waf1}$ expression levels independently of p53. Upregulation of $p21^{waf1}$ was necessary for HL-60 cell differentiation, which was found to be both c-raf-1 and mitogen-activated protein kinase dependent. This study may have implications for the development of strategies to induce differentiation in myeloid leukemias, myelodysplasias and possibly in other malignancies.
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Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and the second most frequent leukemia in children.[1] Chemotherapy treatments fail to cure most patients with AML. The human promyeloblastic leukemia cell line, HL-60, exhibits a defect in its terminal differentiation and maturation. These cells have proved to be a particularly useful model system for investigation of cell growth and differentiation processes since they can be induced to mature into granulocyte or monocyte/macrophage lineages in the presence of various agents.[2–4] Several studies demonstrated that activation of protein kinase C (PKC) is necessary for differentiation of HL-60 cells, especially along the monocytic lineage.[5] The tumor promoting agent, phorbol-12-myristate-13-acetate (PMA) is a PKC activator and also induces differentiation of HL-60 towards the macrophage lineage.[6,7] Another activator of PKC, Bryostatin-1, displays a variety of immunomodulatory properties and exhibits both *in vitro* and *in vivo* antitumor and antileukemic activity in a number of model systems.[8–12] However, only a small number of studies investigated its differentiating activity on HL-60 cells or its activity in animal models. Like PMA, Bryostatin-1 potently stimulates PKC in HL-60 cells;[13] however, it fails to induce differentiation in some HL-60 sublines.[14,15] Unlike PMA, Bryostatin-1 does not act as tumor promoter when applied to mouse skin, but mimics many of the other effects of PMA in its ability to stimulate multiple cellular pathways.[16]

In HL-60 cells, PKC activates Raf-1 by direct phosphorylation;[17] Raf-1, in turn, activates the extracellular mitogen-activated protein kinases (MAPK, ERK1/2). Activation of the ERK pathway is essential for PMA and Bryostatin-1 – induced upregulation of the cyclin-dependent kinase (CDK) inhibitors, $p21^{waf1}$ and $p27^{kip1}$ and results in the inhibition of proliferation by arresting cells in the G_1 phase of the cell cycle.[18,19]

The non-toxic immunomodulator, ammonium trichloro (dioxoethylene-0-0′) tellurate (AS101), first developed by us,[20] has been found to have clear antitumor properties in a variety of tumor models.[21–23] AS101 was found to improve the survival of Madison lung carcinoma-bearing mice when given in combination with chemotherapy.[24] In addition, combined treatment of AS101 with low doses of paclitaxel (Taxol) significantly enhances survival of B16 melanoma tumor-bearing mice.[23] This synergistic effect results in increased expression of the cyclin-dependent kinase inhibitor $p21^{waf1}$ which is both c-raf-1 and MEK-dependent. Phase II clinical trials in non-small lung cancer patients treated with AS101 in combination with chemotherapy showed a significant reduction in the severity of neutropenia and thrombocytopenia that accompany chemotherapy.[25] We previously found that AS101 and PMA synergistically enhance the secretion of interleukin-2 (IL-2) and granulocyte-macrophage-colony stimulating factor (GM-CSF) from human lymphocytes,[26] and also found a synergistic effect between AS101 and Bryostatin-1 on secretion of various cytokines by normal human mononuclear cells.[27]

Given the ability of AS101 to interact synergistically with PKC activators, we examined here its potential role in interacting with Bryostatin-1 or with a low concentration of PMA on HL-60 cell differentiation *in vitro.* We also present new evidence of leukemic cell differentiation *in vivo* induced by combined treatment with AS101 and Bryostatin-1, using SCID mice bearing HL-60 myeloid leukemia cells.

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Materials and methods

Compounds and chemicals

AS101 was supplied by M Albeck (Department of Chemistry, Bar Ilan University) in phosphate-buffered saline (PBS), pH 7.4, and maintained at 4°C. Bryostatin-1 was extracted from the marine *Bugula neritina*; the organisms were collected in the Mediterranean Sea and Bryostatin-1 purified according to Pettit *et al*.[8] The fraction obtained after extraction was dissolved in dimethylsulfoxide (DMSO), and stored at −20°C. PMA (Sigma Chemical Co. St Louis, MO, USA) was dissolved in DMSO, at a concentration of 1 mg/ml and stored at −20°C.

Inhibitors and other reagents. Farnesyltransferase inhibitor II (FTI) and PD98059 were purchased from Calbiochem (La Jolla, CA, USA) and were dissolved in H_20 and DMSO, respectively. SB203580 was purchased from Alexis Biochemicals (San Diego, CA, USA) and dissolved in DMSO. RO 31-8220 and Rottlerin were purchased from BIOMOL Research Laboratories (Pymouth Meeting, PA, USA) and were dissolved in DMSO. Geldanamycin was obtained as a gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Progam (NCI, Bethesda, MD, USA).

Cell culture

The human promyeloblastic leukemia cell line HL-60 and the human monocytic leukemia U937 and THP-1 cell lines were grown in RPMI-1640 medium (GIBCO, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Biologic Industries, Kibbutz Beit Haemek, Israel) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

In vivo *tumor model*

Male six to seven-week-old, CB-17/ICR SCID mice were obtained from Harlan Laboratories (Indianapolis, IN, USA). Animal experiments were performed in accordance with approved Institutional protocols and were approved by the Institutional Animal Care and Use Committee. Mice were injected intravenously (i.v.) with 2×10^6 HL-60 cells/0.2 ml PBS. Control mice were injected i.v. with PBS. Beginning after 48 h, mice were treated with 10 μg AS101, 50 ng Bryostatin-1 or PBS by intraperitoneal injection (i.p.), every second day, or with 10 μg AS101 + 50 ng Bryostatin-1 on alternate days, for 65 days. For leukemic load and clonogenic assays, mice were killed after 30 days. Survival was evaluated 125 days after HL-60 cell transplantation. At that end point, healthy and moribund mice were euthanized.

Assessment of differentiation

Differentiation of monocytes/macrophages was assessed by the acquisition of adherence to plastic, secretion of inflammatory cytokines, induction of CD14 expression and non-specific esterase (NSE) activity. (i) *Quantitation of cell adherence:* HL-60 cells (1×10^6) were seeded in 60 mm tissue culture dishes and incubated with AS101. After 24 h, PMA (0.4–4.8 nM) or Bryostatin-1 (6.5, 25, 50 ng/ml) was added to the relevant culture for another 24 h. Inhibitors were added to the culture 2 h before the addition of AS101, or 2 h before Bryostatin-1/PMA were added. Cells were examined under an inverted binocular microscope. To quantify the adherent cells, adherent cells were separated from non-adherent cells, centrifuged and counted by using a Neubauer Hemocytometer. The number of adherent cells was calculated as a percentage of total cells (adherent + non-adherent). (ii) *Cytokine secretion:* Supernatants were collected from cells treated with AS101, PMA, Bryostatin-1 or with AS101 for 24 h followed by the addition of PMA/Bryostatin-1 for another 24 h. Secretion of human IL-1β, IL-10 and tumor-necrosis factor (TNF)-α was evaluated by enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). (iii) *CD14 expression*: The cells were treated as indicated in (ii), washed two times with PBS and incubated for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-human CD14 antibody (BD Biosciences, San Jose, CA, USA). Expression of the cell surface antigen CD14 was analyzed by a FACStar plus (Becton Dickinson, San Jose, CA, USA) flow cytometer. (iv) *NSE staining:* The cells were examined for NSE activity using α-naphthyl acetate as the substrate (Sigma). Subsequently, the number of positive (dark cells) and negative cells was counted and the percentage of NSE-positive cells calculated.

Clonogenic assay

The soft agar method, described by Pluznik and Sachs,[28] based on the preparation of two layers of agar at different concentrations, was used: AS101, PMA or Bryostatin-1 were incorporated into 2 ml of hard agar medium in a 35-mm Petri dish. HL-60 cells (1×10^4) in 1 ml of soft agar medium were plated above the hard agar. After 7 days incubation at 37°C, the colonies were identified, and colonies with >50 cells were counted using an inverted microscope. In *in vivo* experiments, peritoneal cells were extracted on day 30 from mice treated with PBS, 10 μg AS101, 50 ng Bryostatin-1 or with AS101 + Bryostatin-1, seeded at 1×10^4/dish and after 7 days of incubation, HL-60 cell colonies were identified and counted.

Cell-cycle distribution studies

HL-60 cells (1×10^6) were incubated with AS101, PMA, Bryostatin-1 or a combination of AS101 + Bryostatin-1/PMA. After 48 h, the cells were collected, washed two times in PBS (Ca^{2+}/Mg^{2+} free) and suspended in the dark for 30 min at 4°C in 0.5 ml PBS buffer containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X and 1 mg/ml RNase. DNA content was measured by using a FACStar plus flow cytometer and Cell Quest software.

Immunofluorescence staining

Splenocytes and peritoneal cells were harvested from SCID mice treated with PBS, 10 μg AS101, 50 ng Bryostatin-1 or with AS101 + Bryostatin-1, labeled with FITC anti-human CD45 monoclonal antibody (Ancell Corp. USA) for 45 min, and then washed two times. The $CD45^+$ population represented infiltrating HL-60 cells, while the $CD45^-$ population corresponded to normal murine cells. The number and fluorescence intensity of fluorescein-positive CD45 cells (HL-60 cells) extracted from the spleen and the peritoneum were quantitated.

Western blot analysis

Cell extracts were prepared by suspension in ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 1% Triton X, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotonin and 5 μg/ml leupeptin. The pellets were boiled for 5 min, electrophoresed on 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, transferred to nitrocellulose and immersed in a blocking solution containing

H_2O, 0.1% Tween-20, 5% milk, 5% FCS, 10 mM Tris (pH 7.4) and 100 mM NaCl, for 1 h. The nitrocellulose was blotted for 1 h with primary antibodies against $p21^{waf1}$, $p27^{kip1}$, actin, Raf-1, p-Raf-1 (Ser 338), ERK1/2 (Santa Cruz Biotech, Santa Cruz, CA, USA) and activated MAP kinase (ERK1/2) (Sigma). The blots were washed three times with H_2O containing 0.1% Tween-20, 10 mM Tris (pH 7.4), 100 mM NaCl, incubated for 1 h with secondary antibodies conjugated to horseradish peroxidase and re-washed three times. Blots were developed using the ECL detection system (Pierce, Rockford, IL, USA).

Gene array

The preparation of total RNA and the synthesis of complementary radiolabeled DNA by reverse transcription in the presence of [α-^{33}P] dCTP have been described.[29,30] Human OncoChip (6.5K) arrays from the NCI/Center for Cancer Research (CCR) microArray Center were used according to the original protocols distributed by the NCI/CCR microArray Center (Gaithersburg, MD, USA).

Hybridization

Before hybridization, the solution of labeled cDNA probes were denaturated at 100°C for 2 min after the addition of 4.5 μl. 20 × saline-sodium citrate (SSC), 2 μl 8 mg/ml polyA and 0.6 μl 10% SDS. The probe suspensions were pipetted (12 μl) onto arrays, coverslipped, placed in hybridization chambers (Corning, NY, USA) and incubated in a 65°C water bath for 16–24 h. The arrays were washed with 0.5 × SSC, 0.01% SDS, followed by 0.06 × SSC at room temperature for 10 min each on a shaker (60 r.p.m.). The slides were then dried in a centrifuge with horizontal rotor for 5 min at 1200 *g*. Each probe mixture was applied to two separate arrays.

Array scanning and expression ratio extraction

Hybridized arrays were read using a GenePix 4000A scanner (Axon, Foster City, CA, USA) and the resulting TIFF images were analyzed with IPLab software (Fairfax, VA, USA) ArraySuite, developed at National Human Genome Research Institute, NHGRI. The ratios of sample intensities to the reference intensities for all targets were computed, and then ratio normalization was performed to set the center of the ratio distribution to 1.0. To assess the reliability of each ratio measurement, a quality score ranging from 0 (low) to 1 (high) was determined for each spot location.

Gene expression pattern analysis

Gene expression data analysis and tree view was carried using the GEPAS gene expression pattern analysis suite v3.0. (http://gepas.bioinfo.cipf.es/cgi-bin/cluster).

Statistical analysis

Statistical analysis of the differences between treated cells and controls was assessed with the unpaired Student's *t*-test. Statistical significance was established at a value of $P<0.05$.

Results

Synergistic effect of AS101 with PMA or Bryostatin-1 on myeloid leukemia cell differentiation

To evaluate the potential role of AS101 to interact with the PKC activators, Bryostatin-1 and PMA, in inducing cell differentiation *in vitro,* the ability of different concentrations of PMA (0.4–12.8 nM), AS101 (0.1, 0.5, 1, 2.5 μg/ml) or Bryostatin-1 (6.5, 25, 50 ng/ml) to induce human myelocytic leukemia cell differentiation was examined. We found that 0.4 nM of PMA failed to induce HL-60 cell differentiation. AS101 alone (Figure 1a) or Bryostatin-1 alone (Figure 1b) were also ineffective in inducing HL-60 cells to differentiate. However, HL-60 cells treated with AS101 for 24 h, followed by an additional 24 h of treatment with an inactive concentration of PMA (0.4 nM) (Figure 1a) or with Bryostatin-1 (Figure 1b), resulted in the appearance of differentiated cells with the characteristics of macrophages. Evidence of this effect was also seen in the development of adhesive cell clusters and in the secretion of cytokines characteristic of macrophages, including TNF-α, IL-1β and IL-10 (Figure 1c). In addition, expression of the membrane marker CD14 and the presence of NSE were significantly increased (Table 1). An optimal synergistic effect was attained in HL-60 cells at a concentration of 0.5 μg/ml AS101 with 0.4 nM PMA or with 6.5 ng/ml Bryostatin-1. The combined treatment (AS101 for 24 h, followed by an additional 24 h of treatment with PMA or Bryostatin-1) resulted in 60 and 85% of cells adhering to the plastic dish, respectively (Figure 1a and b). The monocytic cell lines U937 and THP1 were also examined but no significant changes were observed in their ability to differentiate following the combined treatment (data not shown).

The inhibition of HL-60 cell proliferation, as expressed in the decreased ability to form HL-60 cell colonies in soft agar can be seen in Figure 2a and b. AS101 (0.5, 1 μg/ml) alone or Bryostatin-1 (6.5 ng/ml) alone had some inhibitory effect ($P<0.05$ or $P=0.07$, respectively) on HL-60 cell clonogenicity, while combined treatment of AS101 + Bryostatin-1 (at all concentrations) (Figure 2b) or with 0.4 nM PMA (Figure 2a) revealed a significant (~10-fold) inhibition of HL-60 clone formation. These results suggest that the synergistic activity of AS101 with either PMA or Bryostatin-1 on HL-60 cells induces macrophage-like differentiation resulting in the cessation of cell division.

The synergistic effect of AS101 with Bryostatin-1 or PMA results in G_1 arrest of HL-60 cells and in upregulation of $p21^{waf1}$ and $p27^{kip1}$ expression

The ability of HL-60 cells to differentiate into macrophages as a consequence of high concentrations of PMA has been shown to involve growth arrest of the proliferating cells in G_1 phase.[31] We wished to determine whether combined treatment of HL-60 cells with AS101 and Bryostatin-1 or 0.4 nM PMA results in a similar G_1 cell cycle arrest. As can be seen in Figure 3a, the cell-cycle distribution of HL-60 cells was unaffected by incubation with AS101 alone, Bryostatin-1 alone or with an inactive concentration of PMA alone for 48 h. However, combined treatment using AS101 with Bryostatin-1, or AS101 with a low concentration of PMA resulted in arrest in G_1 phase, with significant reduction in the percentage of cells in the S phase. We wished to assess whether the G_1 arrest induced by the combined treatment of AS101 with Bryostatin-1/PMA was related to increased expression of $p21^{waf1}$ and $p27^{kip1}$, which were shown to play a critical role in the control of cell-cycle progression and differentiation.[32] Exponentially growing HL-60 cells (control) or cells treated with Bryostatin-1 alone expressed no detectable $p21^{waf1}$ and $p27^{kip1}$ (Figure 3b), but showed a mild increase in $p21^{waf1}$ expression with AS101 or 0.4 nM PMA treatment. However, combined treatment of HL-60 cells with AS101 + Bryostatin-1/PMA resulted in a significant increase in $p21^{waf1}$ and $p27^{kip1}$ expression. An active concentration of PMA

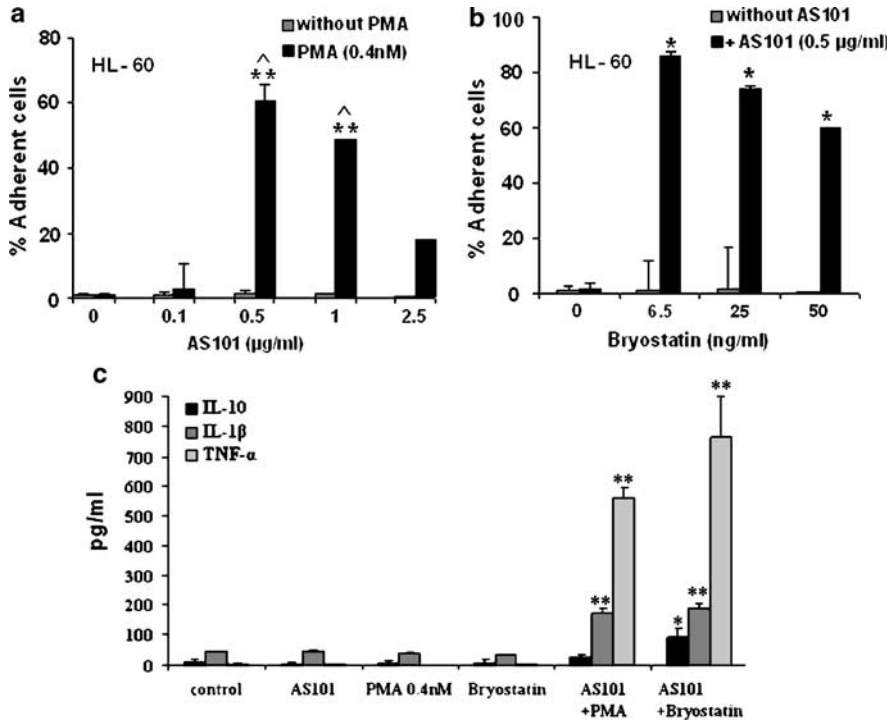

Figure 1 Synergistic effect of AS101 with PMA or Bryostatin-1 on myeloid leukemia cell differentiation. HL-60 cells were cultured with medium, AS101, PMA, Bryostatin-1 or in combination of AS101 for 24 h and then PMA (**a**) or Bryostatin-1 (**b**) were added for another 24 h. The percentage of differentiated cells was scored (as described in Materials and methods). $^{**}P<0.01$, increase vs 0.5 or 1 μg/ml AS101 alone; $^{\wedge}P<0.01$, increase vs 0.4 nM PMA alone. $^{*}P<0.001$, increase vs AS101 alone or Bryostatin-1 alone. Supernatants were collected from cells treated with AS101 (0.5 μg/ml), PMA (0.4 nM), Bryostatin-1 (6.5 ng/ml), or with AS101 for 24 h, followed by the addition of PMA/Bryostatin-1 for another 24 h. Cytokines secretion of human IL-1β, IL-10 and TNF-α was evaluated by ELISA kits (**c**). Results represent mean $\pm$ s.e. of three different experiments. $^{*}P<0.005$ and $^{**}P<0.001$ increase vs each compound alone. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PMA, phorbol-12-myristate-13-acetate.

Table 1 Induction of CD14 expression and NSE activity by AS101+PMA/Bryostatin-1

	% NSE-positive cells	*% CD14 expression*
Control	5.3±1.8	4.4±1.5
AS101 (0.5 μg/ml)	7±3.2	6.3±2.4
PMA (0.4 nM)	8±2	6.4±2
Bryostatin (6.5 ng/ml)	4±1.5	5.1±2
AS101+PMA	97±1.9	95±3
AS101+Bryostatin	98±1.3	96±2.2

Abbreviations: AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; NSE, non-specific esterase; PMA, phorbol-12-myristate-13-acetate.
Differentiation into macrophage-like cells was assessed by induction of CD14 expression, which was analyzed by a FACScan flow cytometer, and by using NSE activity staining.
The number of positive (dark cells) and negative cells was counted and the percentage of NSE-positive cells was calculated.

(6.4 nM) was used as a positive control. Thus, G_1 arrest of HL-60 cells induced by AS101 with Bryostatin-1/PMA is associated with an upregulation of cell-cycle inhibitory proteins, particularly $p21^{waf1}$.

Querying the patterns of gene expression changes in response to AS101 and PMA using RNA microarray

To characterize further the genes that may be associated with the differentiation phenotype, we investigated the expression profiles of HL-60 cells treated with AS101, PMA and the combination of AS101 with PMA for 1 and 6 h. Microarray data were analyzed by regression models. Many patterns of gene expression could be visually identified in the combined treatment for 1 h and were much more significant after 6 h (Figure 4a). The expression of 260 genes was found to be modulated differentially in treated vs untreated cells (control). Among them, 251 genes were upregulated and only nine genes were downregulated by the combined treatment with AS101 and PMA for 6 h. Analysis of early mRNA changes identified several major pathways with a fourfold increase following the combined treatment for 6 h, compared to AS101 or PMA alone. These include differentiation markers (ITGAM (CD11b), I-CAM-1, MNDA, MST1), actin-cytoskeleton changes (ARPC2, EPB41), calcium signaling (ERBB2), and the RAS/ERK (GRB7, EGFR, RAB2L, MAP2K3) – and JNK (JIP1, MKK4) signaling pathways. To confirm that AS101 activates the RAS/ERK pathway, we briefly incubated HL-60 cells with AS101 and evaluated activated ERK expression by western blot. We could note that after 5 min of incubation, the ERKs were markedly activated (Figure 4b). This activation is directly induced by AS101, since pretreatment with the FTI, a Ras inhibitor, prevented the activation of ERK1/2-induced by AS101 (Figure 4c).

Activation of the Ras/MAPK signaling pathway during HL-60 cell differentiation induced by AS101 + Bryostatin-1/PMA

The results we obtained from the RNA microarray analysis of HL-60 differentiation induced by AS101 plus PMA prompted us to evaluate the mechanism of the synergistic effect observed. To

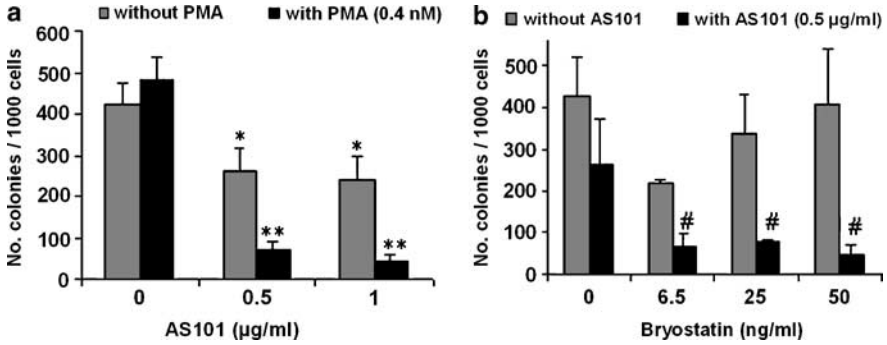

Figure 2 Synergistic effect of AS101 with PMA/Bryostatin-1 in reduces colony formation. HL-60 cells (1×10^4) were seeded in soft agar culture in the presence of AS101 (0.5 or 1 μg/ml), PMA (0.4 nM) or Bryostatin-1 (6.5, 25 and 50 ng/ml), or a combination of AS101 plus PMA (**a**) or AS101 plus Bryostatin-1 (**b**). Colonies were scored after 7 days of incubation at 37°C. Results represent mean ± s.e. of six different experiments. $^*P<0.05$, decrease vs control (medium); $^{**}P<0.05$, decrease vs AS101 alone; $^{\#}P<0.05$, decrease vs Bryostatin-1 alone. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PMA, phorbol-12-myristate-13-acetate.

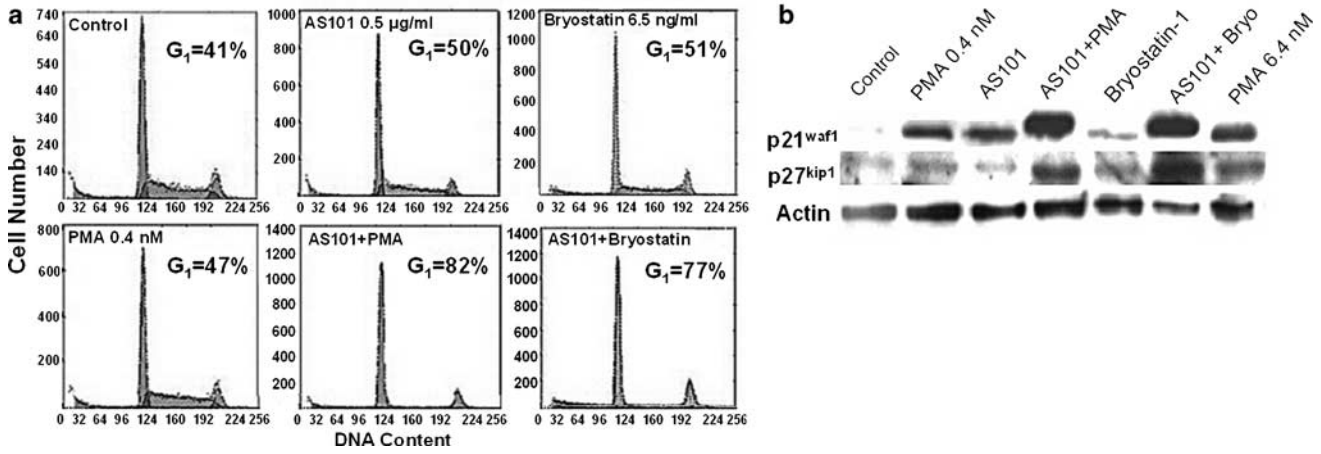

Figure 3 Synergistic effect of AS101 with Bryostatin-1/PMA results in increased accumulation of cells in G_1 phase, and in $p21^{waf1}$ and $p27^{kip1}$ upregulation, during HL-60 cell differentiation. Cell-cycle distribution was assessed in HL-60 cells exposed to 0.5 μg/ml AS101, 0.4 nM PMA, 6.5 ng/ml Bryostatin-1 for 48 h or with AS101 for 24 h with the addition of PMA/Bryostatin-1 for another 24 h. (**a**) Cells were stained with propidium iodide and analyzed by flow cytometry. (**b**) Lysates were obtained from cells treated for 48 h with 0.4 nM PMA, 6.4 nM PMA, 0.5 μg/ml AS101, 6.5 ng/ml Bryostatin-1 or with AS101 for 24 h with the addition of 0.4 nM PMA or Bryostatin-1 for another 24 h. Western blot analysis was performed by using anti-$p21^{waf1}$ or anti-$p27^{kip1}$ antibodies. Results show one representative experiment out of three performed. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PMA, phorbol-12-myristate-13-acetate.

this end, we treated the cells with a series of inhibitors of known biochemical pathways. As can be seen in Figure 5a, AS101 activity was Ras, Raf-1 and MEK1 dependent, since treatment of cells with FTI or pharmacological depletion of Raf-1 by using geldanamycin (GA) or by the pharmacological inhibition of MEK1 by PD98059, prevented the induction of cell differentiation induced by AS101 with Bryostatin-1 (or PMA, data not shown.) In addition, the ability of the compounds alone or the combination of AS101 + Bryostatin-1/PMA to activate Raf-1 and MAPK ERK1/2 was analyzed. As can be seen in Figure 5b, AS101 alone, PMA (0.4 nM) alone or Bryostatin-1 alone had only a slight effect on the activation of these kinases. In contrast, marked increases in Raf-1 phosphorylation and in MAPK ERK1/2 activation were observed with the combined treatments of AS101 + Bryostatin-1/PMA. Similar effects were obtained with an active concentration of PMA (6.4 nM). No involvement of p38 MAPK in HL-60 cell differentiation was found, since inhibition of this kinase by SB203580 was ineffective in preventing HL-60 cell differentiation. The involvement of JNK MAPK was not examined. To test whether the upregulation of $p21^{waf1}$ is necessary for HL-60 differentiation, and if it is Raf-1 and ERK1/2 dependent, the cells were treated with geldanamycin or with PD98059 2 h before AS101 or before PMA/Bryostatin-1 addition to HL-60 cells. These cells expressed very low levels of $p21^{waf1}$ protein compared to control culture (AS101 + PMA or AS101 plus Bryostatin-1 without inhibitors) (Figure 5c). Microscopic examination, to assess adherent cells, revealed no macrophage differentiation in the cultures with low or no $p21^{waf1}$ expression. Therefore, the inability of HL-60 cells to differentiate following Raf-1 and MEK1 inhibition (as demonstrated in Figure 5a) may be due to the low expression level of $p21^{waf1}$. Upregulation of $p21^{waf1}$ appears to be essential for HL-60 cell differentiation induced by AS101 + Bryostatin-1/PMA.

Involvement of PKCα and PKCδ isoforms in HL-60 cell differentiation induced by AS101 + Bryostatin-1/PMA

Bryostatin-1 and PMA have been previously found to activate the PKC-Raf-1 pathway.[33] The induction of $p21^{waf1}$ expression in myeloid cells is also activated by the PKC pathway.[34] We sought to identify the PKC isoforms involved in the synergistic activity of AS101 + Bryostatin-1/PMA by using a specific PKCα

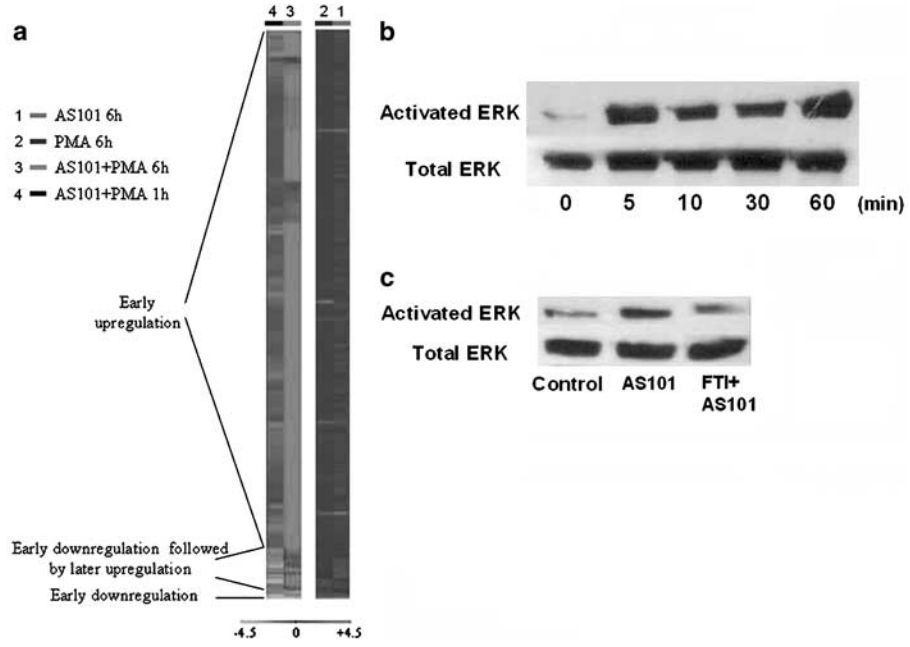

Figure 4 Gene array analysis of HL-60 cells treated with AS101, PMA or AS101 plus PMA. RNA was extracted from 3×10^6 HL-60 cells treated with either AS101 (1 μg/ml) or PMA (0.4 nM) alone, or in combination of both for 1 or 6 h, labeled and hybridized to 6.5 K OncoChip gene array (described in Materials and methods). Genes with altered expression were analyzed using the KEGG pathways database. (**a**) Gene clustering was analyzed using the GEPAS gene expression pattern analysis suite v3.0. (**b**) HL-60 cells were incubated with AS101 for the indicated times or (**c**) pretreated with FTI (50 μM) 2 h before cells were exposed to AS101 for 5 min. Western blot analysis was performed by using anti-active MAPK ERK1/2 or ERK2 antibodies. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; ERK, extracellular signal-regulated kinase; PMA, phorbol-12-myristate-13-acetate.

inhibitor (RO 31-8220) and a specific PKCδ inhibitor (Rottlerin). To test the role of PKC in mediating the effects of AS101, RO 31-8220 or Rottlerin were added to the culture 2 h before exposure to AS101; 24 h later, cells were washed and Bryostatin-1 or PMA were added for another 24 h. Figure 6a and b, shows that addition of the PKC inhibitors to cultured cells before treatment with AS101 was ineffective in preventing HL-60 differentiation. To test the role of PKC on the action of Bryostatin-1 or PMA, cells were incubated with AS101 for 24 h, and then washed and RO 31-8220 or Rottlerin were added to the relevant cultures. After 2 h, Bryostatin-1 or PMA were added for an additional 24 h. As can be seen in Figure 6a and b, the addition of the inhibitors before Bryostatin-1 or PMA, prevented HL-60 cell differentiation. Thus, both PKCα and PKCδ isoforms participate in HL-60 cell differentiation induced by AS101 + Bryostatin-1/PMA. The inhibitors had to be present when the PKC activator (Bryostatin-1/PMA) was added to block macrophage differentiation. Thus, only Bryostatin-1 and PMA, but not AS101 are involved in reactivating PKC.

Synergistic antitumor effect of AS101 + Bryostatin-1 in SCID mice transplanted with HL-60 cells

The synergistic effect of AS101 with an inactive concentration of PMA or with Bryostatin-1 on HL-60 differentiation *in vitro* led us to test whether the combination of these two agents may have an antitumor effect *in vivo*. We performed the *in vivo* experiments combining AS101 and Bryostatin-1, rather than with the tumor-promoting PMA. For this purpose, HL-60 AML cells were transplanted i.v. into SCID mice. In this model, injection of cells reproducibly induces a disease process similar to acute leukemia in humans, with an early infiltration of hematopoietic tissues. Mice were systemically treated every second day with i.p. injections of PBS, AS101 alone, Bryostatin-1 alone, or combined AS101 and Bryostatin-1 injected on alternate days for 65 days. A group of control mice was not injected with HL-60 cells and received PBS only. Splenocytes and peritoneal cells were harvested from leukemic mice 30 days after transplantation of leukemic cells, and labeled with FITC anti-human CD45 monoclonal antibody to distinguish between HL-60 leukemia cells ($CD45^+$) and normal murine splenocytes and peritoneal cells ($CD45^-$). The infiltration of leukemic cells into the spleen and the peritoneum was detected by flow cytometry. Significant numbers of labeled cells were detected in splenocytes and peritoneal cells harvested from leukemic mice treated with PBS, AS101 or with Bryostatin-1 (Figure 7a). However, the number of $CD45^+$ cells harvested from mice treated with a combination of AS101 + Bryostatin-1 was significantly reduced (twofold to threefold lower) vs that detected in mice treated with each agent alone, or with PBS.

The effect of combined treatment with AS101 + Bryostatin-1 on leukemia colony-forming cells in soft-agar was analyzed by colony assay. Peritoneal cells were harvested from leukemic mice treated with PBS, AS101, Bryostatin-1 or with a combination of AS101 + Bryostatin-1. The harvested cells were seeded on soft agar. Figure 7b shows that HL-60 colony-forming cells derived from the peritoneum of tumor-bearing mice treated with AS101 + Bryostatin-1 were significantly reduced in number ($P<0.005$) compared with the number of colony-forming cells noted after treatment with AS101, Bryostatin-1 or PBS alone.

Finally, the effect of AS101 + Bryostatin-1 on the survival of leukemic SCID mice was examined. As shown in the survival curve (Figure 8a), all mice treated with AS101 + Bryostatin-1 on

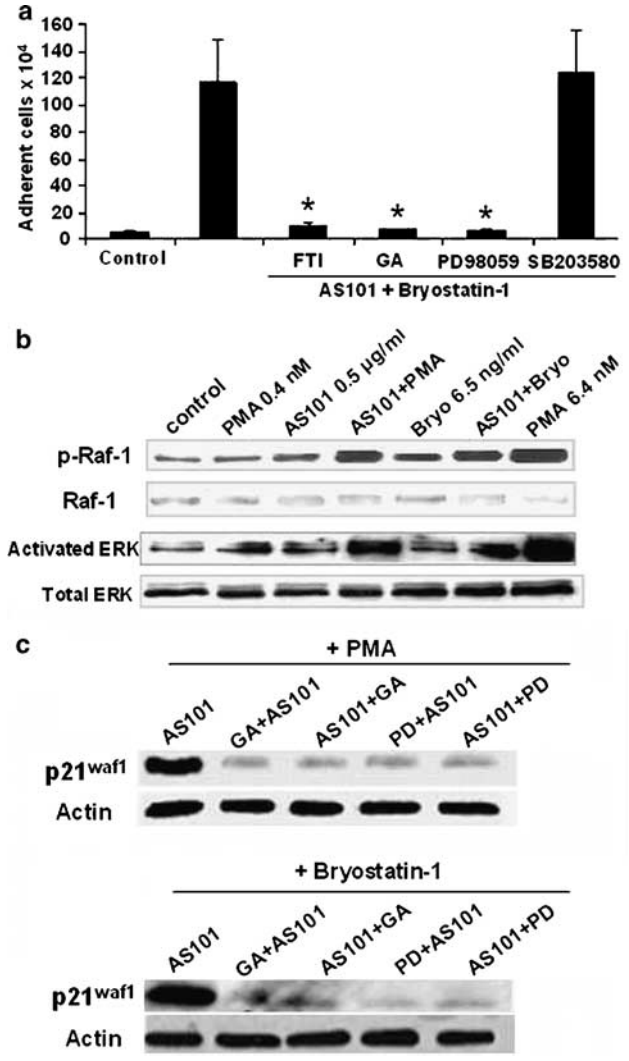

Figure 5 Induction of the Ras/MAPK signaling pathway is necessary for HL-60 cell differentiation induced by AS101 + Bryostatin-1/PMA. The inhibitors FTI (50 μM), geldanamycin (2 μM), PD98059 (20 μM) and SB203580 (10 μM) were added to the culture 2 h before the cells were exposed to AS101. (**a**) After 24 h, cells were washed two times with RPMI and 6.5 ng/ml Bryostatin-1 was added for another 24 h. Adherent cells were scraped from the tissue plate, washed and counted. Results represent mean ± s.e. of six different experiments. *$P<0.05$, decrease vs AS101 plus Bryostatin-1. (**b**) Lysates were obtained from cells treated for 48 h with 0.4 nM PMA, 6.4 nM PMA, 0.5 μg/ml AS101, 6.5 ng/ml Bryostatin-1 or with AS101 for 24 h with the addition of 0.4 nM PMA or Bryostatin-1 for another 24 h. (**c**) Cultures cells were treated as described in (**a**) plus addition of geldanamycin or PD98059 (for 2 h) after AS101 treatment and before the addition of 0.4 nM PMA or 6.5 ng/ml Bryostatin-1. Western blot analysis was performed by using anti-phospho-Raf-1, anti-active MAPK ERK1/2 or anti-p21^{waf1} antibodies. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; ERK, extracellular signal-regulated kinase; PMA, phorbol-12-myristate-13-acetate.

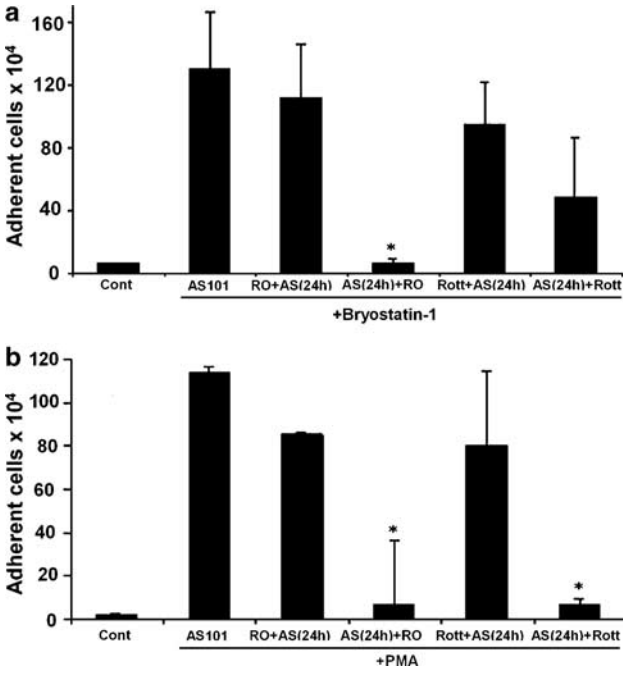

Figure 6 Involvement of PKCα and PKCδ isoforms in HL-60 cell differentiation induced by AS101 plus Bryostatin-1/PMA. To test the role of PKC on AS101 effects, RO 31-8220 (0.5 μm) or Rottlerin (3 μm) were added to the culture 2 h before exposure to 0.5 μg/ml AS101. After 24 h, cells were washed twice with RPMI and 6.5 ng/ml Bryostatin-1 (a) or 0.4 nm PMA (b) were added for another 24 h. To test the role of PKC on the effect of PMA/Bryostatin-1, cells were incubated with AS101 for 24 h, then washed two times with RPMI and RO 31-8220 or Rottlerin were added to the relevant cultures; 2 h later, Bryostatin-1 (**a**) or PMA (**b**) were added for another 24 h. Adherent cells were scraped from the tissue plate, washed and counted. Results represent mean ± s.e. of three different experiments. *$P<0.05$, decrease vs AS101 plus Bryostatin-1 or vs AS101 plus PMA. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PMA, phorbol-12-myristate-13-acetate.

alternate days survived for 125 days (100% of the mice). The PBS-treated control group, as well as AS101 or Bryostatin-1 mice groups, mainly died between 21 and 32 days after tumor implantation. In addition, mice from these groups developed solid tumors generally involving lymph nodes (as presented in Figure 8b). However, in the combination of AS101 + Bryostatin-1, no solid tumors were detected when the experiment was terminated (as shown in Figure 8c).

Discussion

In the present study, we demonstrated synergistic activity of AS101 with PMA or Bryostatin-1 in inducing differentiation of an acute myeloblastic leukemia cell line *in vitro*, and examined its mechanism of action. We demonstrated further synergistic effects of AS101 with Bryostatin-1 in a SCID mouse xenograft model of human AML.

We tested the effect of AS101, PMA or Bryostatin-1, or a combination of AS101 with PMA/Bryostatin-1 on three human myelocytic leukemia cell lines (HL-60, U937 and THP-1) and found synergistic activity of AS101 treatment with the PKC activators in induction of HL-60 differentiation. The promyeloblastic HL-60 cells were significantly affected by a combined treatment using AS101 and an inactive concentration of PMA or with Bryostatin-1, which resulted in the appearance of differentiated cells with the characteristics of macrophages. Furthermore, the differentiated HL-60 cells failed to form colonies in soft- agar.

Treatment of leukemic mice with AS101 alone or with Bryostatin-1 alone was found to be ineffective, whereas combination of both compounds had antileukemic properties *in vivo*. The synergistic antileukemic activity of AS101 and Bryostatin-1 is reflected in the 100% survival of the leukemia-bearing mice at 125 days and also in the marked decrease of

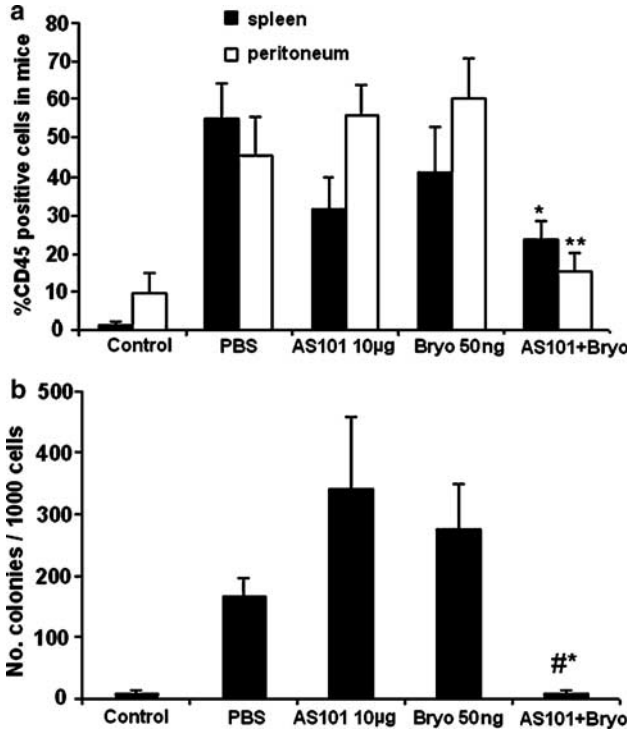

Figure 7 *In vivo* treatment with AS101, Bryostatin-1, or with combination of AS101 plus Bryostatin-1. (**a**) Detection of human CD45-positive cells by flow cytometry in spleen and peritoneum of SCID mice transplanted with HL-60 cells. Mice injected i.v. with 2×10^6 cells were treated with i.p. injections of PBS, AS101 (10 μg), Bryostatin-1 (50 ng), or a combination of AS101 plus Bryostatin-1, starting 2 days after HL-60 transplantation. After 30 days, splenocytes and peritoneal cells were harvested from leukemic mice, and stained with FITC-labeled anti-human CD45 antibody for FACS analysis (data shown are average of six mice per group). $^*P<0.01$, decrease vs PBS; $^{**}P<0.05$, decrease vs PBS. (**b**) Number of HL-60 colony forming cells. Cells (1×10^4) obtained from the peritoneum of the leukemic SCID mice were seeded on soft agar. Colonies were scored after 7 days of incubation (data shown are average from six mice per group). $^*P<0.005$, decrease vs PBS; $^{\#}P<0.0005$, decrease vs AS101 or Bryostatin-1. Control mice are normal SCID mice not inoculated with tumor cells that received PBS. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PBS, phosphate-buffered saline.

leukemic cell infiltration into the spleen and the peritoneum. This treatment was also found to inhibit clonogenicity on soft agar of HL-60 cells extracted from these organs. We therefore suggest that the combined treatment of AS101 with Bryostatin-1 *in vivo* acts similarly to its activity *in vitro* by inducing growth arrest of HL-60 cells when inoculated into SCID mice, apparently leading to their differentiation.

We performed a series of experiments in an attempt to elucidate the mechanism of the synergistic activation of AS101 with Bryostatin-1 or with an inactive concentration of PMA (0.4 nM). Cell-cycle analysis of HL-60 cells treated with AS101 + Bryostatin-1/PMA revealed a significant accumulation of cells in the G_1 phase, which is essential for their terminal differentiation. These data correlate with the ability of PMA (at an active concentration) to induce myeloid cell differentiation through a G_1 cell-cycle block.[31] However, reversing the order of incubation by treating HL-60 cells with Bryostatin-1 or PMA for 24 h followed by AS101 treatment, did not induce HL-60 cell differentiation (data not shown). These findings raise the possibility that the failure of inactive concentrations of Bryostatin-1 or PMA alone to initiate a differentiation program, stems from their inability to induce cell-cycle arrest. Thus, it is possible that AS101 acts in early G_1, thereby allowing the later addition of Bryostatin-1 or PMA to trigger pre-commitment events in G_1 required for cellular maturation.

On the basis of the results of the gene array analysis, we examined the involvement of the Ras/MAPK pathway in the differentiation process induced by AS101 + Bryostatin-1/PMA. Indeed, treatment of HL-60 cells with AS101 + Bryostatin-1/PMA resulted in the activation of the Ras signaling pathway: AS101 induced Ras activation, since FTI (Ras inhibitor) prevented the activation of ERKs induced by AS101 (Figure 4b and c) and furthermore, HL-60 cell differentiation (Figure 5a and b), whereas cell differentiation was not prevented when FTI was added after AS101 treatment (data not shown). In this differentiation process, Bryostatin-1 and PMA share the same signaling pathway through PKC. Our results are consistent with those of Park *et al*.[35] and Schwaller *et al*.,[34] who suggested that PKCδ and particularly PKCα isoforms are involved in HL-60 cell differentiation, and also in the upregulation of $p21^{waf1}$ expression. The PKC inhibitors were effective in the combined treatment (AS101 plus PMA/Bryostatin-1) only when added to the cultures before PMA/Bryostatin-1. PKC inhibitors were not

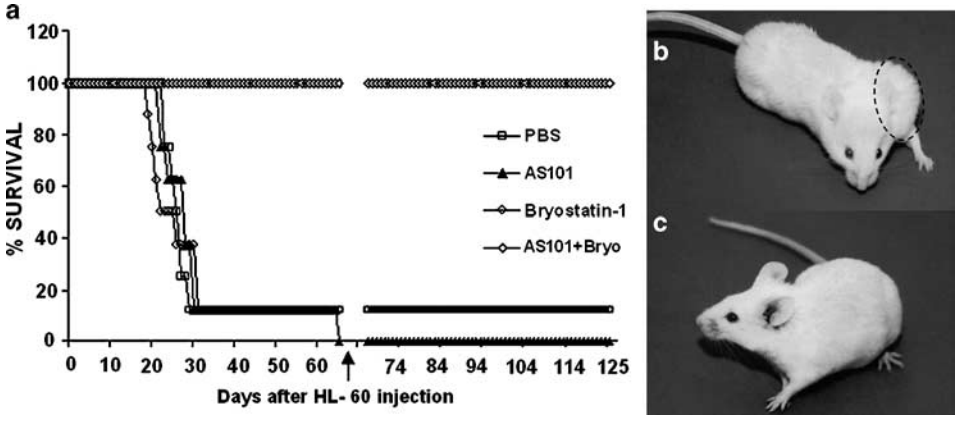

Figure 8 Synergic antileukemic effect of AS101 plus Bryostatin-1 in leukemia-bearing SCID mice model. HL-60 cells (2×10^6) were transplanted i.v. into SCID mice. Treatment with i.p. injection of PBS, 10 μg AS101, 50 ng Bryostatin-1 or with combination of AS101 + Bryostatin-1 was started 2 days after HL-60 transplantation, for 65 days. Survival of mice was monitored for 125 days (**a**). Representative photograph of untreated mouse with solid tumor in the lymph node (**b**) and ↑mouse treated with AS101 + Bryostatin-1 (**c**). Eight mice were included in each group. ↑ Treatment termination. AS101, ammonium trichloro-(dioxoethylene-0-0′)-tellurate; PBS, phosphate-buffered saline.

effective when added before AS101, subsequently washed, and PMA/Bryostatin-1 were then added to the culture (Figure 6a and b). These results suggest that PKC is activated only by PMA/Bryostatin-1 in the synergistic activity of AS101 plus PMA/Bryostatin-1. AS101 alone is ineffective in inducing differentiation.

The signal-transduction pathways of AS101 and of the PKC activators is shared with both AS101 and Bryostatin-1/PMA at Raf-1 protein, as reflected by the inhibition of HL-60 cell differentiation by specific inhibitors of Raf-1 and also for MEK1 kinases, and in the key role of the phosphorylation of Raf-1, as well as the activation of MAPK ERK1/2 (Figure 5b). We assume that the ability of AS101 to potentiate Bryostatin-1 or PMA differentiation-inducing action could result from alterations in expression levels of phosphorylated Raf-1, needed to transduce the downstream signals. This assumption may be supported by the studies of Kharbanda *et al*.,[36] who demonstrated the necessity of Raf-1 and MAPK ERK1/2 activation during HL-60 cell differentiation. However, the exact role of Bryostatin-1/PMA and AS101 is still elusive.

$p21^{waf1}$ plays an important role in G_1/S transition and its expression might require the participation of p53.[37] Treatment of HL-60 cells with AS101 plus Bryostatin-1/PMA synergistically increased $p21^{waf1}$ expression levels, independently from p53. We show that the upregulation of the $p21^{waf1}$ protein is necessary for inducing differentiation of HL-60 cells, which was found to be both c-raf-1 and MEK-dependent but not p38 dependent.

The early events in the synergistic effect of AS101 and PMA were examined using a gene array approach. We identified several differentiation- and actin-related genes that play a critical role in cells shape and deformability. Identification of these genes suggests that the synergistic effect involves actin cytoskeleton changes during HL-60 cell differentiation.

In summary, we present evidence that the immunomodulator AS101 and Bryostatin-1 or a low, inactive concentration of PMA exert a synergistic antileukemic activity in HL-60 cells and induce their differentiation from transformed cells into macrophage-like cells *in vitro*. AS101 + Bryostatin-1 also exhibit antileukemic activity in an HL-60 mouse xenograft model. These findings provide evidence that HL-60 cells may be induced to differentiate *in vivo*. Our study suggests that combined treatment with AS101 plus Bryostatin-1, each of which has been separately tested in clinical trials, has promising therapeutic implications for inducing cell differentiation in myeloid malignancies.

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