Sensitizing B- and T-cell Lymphoma Cells to Paclitaxel/Abraxane-Induced Death by AS101 via Inhibition of the VLA-4–IL10–Survivin Axis

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Abstract

Cancer cell resistance to chemotherapy is a major concern in clinical oncology, resulting in increased tumor growth and decreased patient survival. Manipulation of apoptosis has emerged as a new therapeutic strategy to eliminate cancer cells. The focus of this study resides within a novel approach to target survivin, an integrator of both cell death and mitosis. This protein plays a pivotal role in the resistance of tumors to chemotherapy, especially to paclitaxel. The data herein demonstrate an indirect repression of survivin in both B- and T-cell lymphoma and human NHL by the nontoxic tellurium compound, AS101 [ammonium trichloro(dioxoethylene-o,o')tellurate], via inhibition of tumor autocrine IL10–STAT3–Survivin signaling. As a result of survivin abrogation, sensitization of lymphomas to paclitaxel or to Abraxane, the new albumin-stabilized nanoparticle formulation of paclitaxel, occurs both in vitro and in vivo. Importantly, inhibition of lymphoma cell IL10 secretion is mediated by inactivation of the VLA-4 integrin, recently shown to be an important target of AS101. This activity is followed by inhibition of the PI3K–AKT axis that mediates IL10 suppression. Because a wide variety of lymphomas and other tumor types express VLA-4 and secrete IL10 in an autocrine manner, inhibition of survivin with a small nontoxic agent has vast clinical significance in modulating chemosensitivity in many tumor types.

Implications: Combination therapy with AS101 and paclitaxel has novel therapeutic potential targeting deregulated active pathways in lymphoma, overcoming endogenous resistance to apoptosis.

Introduction

From the increasing evidence that dysregulation of apoptosis contributes to in vivo drug resistance of tumor cells comes confidence that new therapeutic approaches aimed at correcting the dysfunctional apoptotic program could improve the treatment outcome of patients with chemotherapy-refractory tumors. The possibility of modulating the chemosensitivity of various cancer cell types by targeting survivin has been actively pursued in the last few years. This protein is a structurally unique member of the inhibitor of apoptosis (IAP) family, which acts as a cell survival factor because it is involved in the control of mitotic progression and inhibition of apoptosis. Survivin significantly affects cell death and is selectively expressed in most common human tumors. The selective overexpression of survivin in cancer appears to reflect a global deregulation of survivin gene transcription in transformed cells. This is consistent with the cancer-specific transcription of the survivin promoter, and the several oncogenic pathways that converge to upregulate survivin gene expression in transformed cells. These include PI3K/Akt signaling, STAT3 activation, and loss of suppressor molecules such as p53 that was shown to transcriptionally repress survivin. Survivin expression in normal tissues is developmentally regulated and the protein was found to be absent or low in most terminally differentiated tissues. However, recent studies show that although survivin is expressed in normal tissues characterized by self-renewal and proliferation, its expression is significantly lower than in transformed cells. The differential expression in cancer and what appears to be a requirement to preserve tumor cell viability, identify survivin as a novel therapeutic target in cancer.

Growth autonomy of tumors appears as the result of multiple mechanisms by which growth regulatory pathways are deregulated. Malignant cells may produce excessive levels of autocrine growth factors, in a constitutive manner, which substitute for exogenous growth factor requirements. These factors may affect critical genes that tightly regulate cell-cycle checkpoints, cell survival, and apoptosis. IL10 is spontaneously secreted by a variety of human cancer cells, including lymphoma. High IL10 levels have been detected in sera from patients with a wide variety of solid and hematopoietic tumors. IL10 serum levels in patients with II.10-producing tumors were found to correlate with tumor progression and the presence of distant metastases. Most importantly the curative and noncurative surgery rates were correctly predicted by...
preoperative IL10 serum levels in the majority of cases, with high cytokine serum levels paralleled by low rates of curative surgery (14). Stat3 is the main target of IL10 because constitutive Stat3 activation upregulates survivin gene expression in malignant cells; it may be speculated that inhibition of IL10 in IL10-producing tumors, may result in sequential events leading to inhibition of pStat3 followed by survivin repression.

The nontoxic immunomodulator ammonium trichloro(dioxoethylene-o,o')tellururate (AS101) first developed by us, has been shown to have beneficial effects in diverse preclinical and clinical studies. A large part of its activities has been primarily attributed to the indirect inhibition of the anti-inflammatory cytokine IL10 (20), followed by the simultaneous increase of specific cytokines. (21–23). Other tellurium derivatives developed by us had no or low immunomodulating activity. These immunomodulatory properties were found to be crucial for the clinical activities of AS101, demonstrating the protective effects of AS101 in parasite and viral-infected mice models (24), in autoimmune diseases (25), in septic mice (26), and in a variety of tumor models in mice and humans where AS101 had a clear antitumor effect (21, 27). Phase I clinical trials on advanced cancer patients treated with AS101 showed increased production and secretion of a variety of cytokines, leading to a clear dominance in Th1 responses with a concurrent decrease in the Th2 responses (27). The predominance of Th1 responses was shown to be related to AS101 antitumor activity. All AS101 activities were associated with minimal toxicity. AS101 was shown to protect mice from hemopoietic damage caused by sublethal dose of chemotherapy and increased the rate of survival of mice treated with different cytokotoxic drugs acting by distinct mechanisms (22, 28, 29). In light of these results, phase II clinical trials with cancer patients treated with AS101 in combination with chemotherapy have been initiated and completed, showing that treatment with AS101, with no major toxicity, induced a significant reduction in the severity of neutropenia and thrombocytopenia that accompany chemotherapy (30). We have previously shown that inhibition of IL10 by AS101 results in decreased Stat3 activity in both solid tumor cells and rat glomerular mesangial cells (31, 32). This activity resulted in the sensitization of tumors to chemotherapy. Because of the central role of IL10 inhibition in a large part of AS101’s preclinical activities, the present study offers a new approach to indirectly target survivin in both T and B lymphoma cells and solid tumors, this study aims at examining the new approach to indirectly target survivin in both T and B lymphoma cells and elucidating the role of this activity in the sensitization of hematologic malignancies to Abraxane and paclitaxel. Abraxane is the novel albumin-stabilized nanoparticle formulation of paclitaxel, designed to overcome insolubility problems encountered with paclitaxel, and facilitating the passage of the drug to the underlying tumor tissue. Albumin has the potential to increase drug delivery to tumors by initiating albumin receptor (gp60)-mediated transcytosis across endothelial cells (33) and accumulating drug in tumors due to binding of albumin to secreted protein, acidic and rich in cysteine (SPARC).

Because resistance of many tumors to established treatment regimens still constitutes a major problem in cancer therapy, novel strategies to target tumor cell resistance are essential to improve patient outcome. Elucidating the mechanism of survivin repression by AS101 in hematologic malignant cells, and investigating the mechanism of tumor cell death ensuing from manipulation of the survivin pathway by AS101, may be relevant for novel therapeutic strategies aimed to improve the efficacy of chemotherapy-induced apoptosis in patients.

Materials and Methods

Reagents

All reagents are described in Supplementary Data.

Animals

Male BALB/c 6 to 8 weeks of age, were purchased from Harlan Laboratories. Animal experiments were performed in accordance with approved institutional protocols and approved by the Institutional Animal Care and Use Committee. A20 B lymphoma cells were injected s.c. At day 10, when tumors were palpable, mice were treated as follows: control mice treated with PBS on alternate days. AS101 was injected on alternate days i.p. at 10 μg/mouse. Abraxane was injected at 0.5 mg/mouse on alternate days (×4). Survival of mice was monitored for 80 days.

Cell cultures

The mouse T (YAC) and B (A20) cell lymphoma cells and the NHL cells were obtained from the American Type Culture Collection. Cells were cultured in RPMI-1640 containing 10% fetal calf serum.

Attachment assay

Ninety-six-well plates were coated with 80-μL VCAM-1 (1 μg/mL). Cells with or without AS101 were incubated in the wells for 1 hour. Thereafter, cells were washed three times. The attached cells were tested by the XTT at 450 nm.

Detection of apoptosis

The percentage of cells undergoing apoptosis was quantitatively determined using an apoptosis detection kit (R&D Systems) by virtue of their ability to bind Annexin V and exclude propidium iodide (PI).

Evaluation of caspase activity

Determination of apoptosis was assessed by double staining for Annexin V/PI using an apoptosis detection kit (Bender Med Systems Inc.). Activity of caspases was analyzed by FACS using FLICA caspase activity kits (Alexis Biochemicals).

Cell-cycle distribution studies

Cells were treated with RNase and stained with PI. DNA content was measured using a FACStar plus (Becton Dickinson) flow cytometer using Cell Quest software.

Quantification of IL10

Cells were cultured at various doses for different time points with or without AS101. Supernatants were collected and evaluated by ELISA kits (R&D Systems).
Western blot analysis
Total cell extracts were prepared by suspension in ice-cold lysis buffer containing 50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, 5 mmol/L EDTA, 0.5% deoxycholate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 200 μmol/L sodium vanadate, 5 μg/mL aprotinin, and 5 μg/mL leupeptin. Cell lysates were boiled and electrophoresed on 10% SDS–PAGE and were then blotted with specific antibodies. Blots were developed using horseradish peroxidase–conjugated secondary antibodies and the ECL detection system (Amersham Pharmacia Biotech).

Cell viability assays were performed by the XTT test as described in Ref. 34.

Plasmids
pBabe-Akt (NM_005163) and the empty vector were a gift from Dr. Ranan Berger (Sheba, Ramat-Gan, Israel), pECE-Stat3 (GenBank U06922), and the empty vector were a gift from Prof H. Brody (Bar Ilan University, Ramat-Gan, Israel). PSM2c encoding IL10shRNA (NM_010548) and the empty vector (GE Healthcare). The tetracycline (or doxycycline) inducible (Tet-On) pSUPERIOR.retro.puro (OligoEngine) was used for survivin shRNA expression. These vectors are tetracycline-regulated expression vectors that use regulatory elements from the Escherichia coli Tn10-encoded tetracycline (Tet) resistance operon. Tetracycline regulation in pSUPERIOR vectors is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest. The TetR-expressing vector pCDNA6/TR was used for regulation of gene transcription. shSurvivin DNA inserts (NM_009689) were used. For transfection of vectors, poly-cationic transfection reagent (Lipofectamine;Invitrogen) was used to facilitate uptake, according to the protocol recommended by the manufacturer.

Statistical analysis
Results are expressed as mean ± SE. For in vitro studies, differences between groups were analyzed using two-way ANOVA. Differences in survival curves between groups were calculated by the Kaplan–Meier method. P < 0.05 was considered statistically significant.

Results

AS101 sensitizes lymphoma cells to Paclitaxel/Abraxane

Figure 1 shows that both A20 (Fig. 1A; B-cell lymphoma) and YAC (Fig. 1B; T-cell lymphoma) murine cells constitutively secrete high levels of IL10. The B lymphoma cells secreted higher levels of IL10 (2,733 ± 169 pg/mL) compared with T lymphoma cells (945 ± 238 pg/mL) following 48 hours of incubation. Addition of AS101 to cell cultures significantly decreased IL10 secretion by both cell types in a dose-dependent manner amounting to 86.1% inhibition at 2.5 μg/mL AS101 in A20 cells (P < 0.01; Fig. 1A) and 73.8% inhibition at 1 μg/mL AS101 in YAC cells (P < 0.01; Fig. 1B).

Figure 1.
AS101 inhibits spontaneous IL10 secretion by A20 and YAC lymphoma cells. A20 (A) and YAC (B) lymphoma cells were cultured at 0.5 × 10^6/mL at 37°C in the presence or absence of various AS101 concentrations. Supernatants were collected after 48 hours and tested by ELISA for IL10 content. Results represent mean ± SE of three different experiments. *, P < 0.05 decrease versus control. **, P < 0.01 decrease versus control. Accumulation of A20 (A) and YAC (B) cells in the sub-G1 fraction of the cell cycle following treatment with AS101 and paclitaxel. A20 and YAC lymphoma cells were cultured at 0.5 × 10^6/L with or without various concentrations of paclitaxel in the presence or absence of AS101 at 0.5 μg/mL (C and D) or with different concentrations of AS101 in the presence of 30 mmol/L paclitaxel (E and F). Cell-cycle distribution was assessed after 24 hours of incubation and the accumulation of cells in sub-G1 was determined. Results represent mean ± SE of four different experiments. *, P < 0.05 and **, P < 0.01 increase versus paclitaxel alone (C and D) and versus AS101 alone (E and F).
to 74.5% in YAC cells (P < 0.01; Fig. 1B). The concentrations of AS101 used did not affect cell viability (Fig. 1E and F). Similar results of IL10 inhibition by AS101 were obtained at 24 hours of incubation with control IL10 levels being lower than those at 48 hours (data not shown).

AS101 sensitizes B- and T-cell lymphoma cells to paclitaxel-induced cell death

Figure 1C and D show that AS101 may increase the responsiveness of cells from hematologic malignancies to the damaging effect of paclitaxel. Figure 1 shows the response of both B (Fig. 1C) and T (Fig. 1D) lymphoma cells to paclitaxel in which a dose response accumulation of the cells in the sub-G1 fraction occurs. Although AS101 alone did not induce cell death, its coincubation with paclitaxel (at 10⁻⁶ to 60 nmol/L) resulted in a significant shift toward the sub-G1 fraction. The sensitization of B and T lymphoma cells to paclitaxel-induced death and cycle alterations was dependent on AS101 doses (Fig. 1E and F). In A20 cells (Fig. 1E), the dose of 2.5 μg/mL AS101 induced the highest paclitaxel-induced death. YAC cells were more sensitive to AS101’s effects. The low AS101 dose (0.5 μg/mL) affected maximally both parameters (Fig. 1F).

The death induced by AS101 and paclitaxel in lymphoma cells has apoptotic features

This was expressed by increased rate of three characteristics of apoptotic cell death: annexin binding, PARP cleavage, and caspase-3 activity. Figure 2A shows negligible amounts of annexin-binding (non-PI-stained) YAC cells following treatment with either AS101 or paclitaxel. However, cotreatment of YAC cells

Figure 2.
AS101 induces paclitaxel-induced apoptotic death. YAC cells were incubated in the presence of paclitaxel (30 nmol/L), AS101 (2.5 μg/mL), or the combination of both for 24 hours. Apoptosis was assessed by AnnexinV binding (A) or by quantitation of caspase 3 activity (B and C) by FACS using FLICA caspase activity kits. A20 cells were incubated with various AS101 concentrations with or without 30 nmol/L paclitaxel. Caspase-3 activity was assessed (D). PARP cleavage was assessed in A20 cells subjected to AS101 and paclitaxel. The results of A, B, and E represent one of four independent experiments performed. The data in C and D represent mean ± SE of four different experiments (E). #, P < 0.01 increase versus AS101 alone; *, P < 0.01 increase versus paclitaxel alone; **, P < 0.001 increase versus paclitaxel alone; ##, P < 0.001 increase versus AS101 alone.
with AS101 and paclitaxel considerably elevated the proportion of annexin-positive, PI-negative cells. Figure 2B shows the sensitizing activity of AS101 to paclitaxel-induced apoptosis as reflected by increased caspase-3 activity in treated cells. Although 0.14% and 1.23% of control and AS101-treated YAC cells expressed active caspase-3, 20.77% of paclitaxel-treated cells expressed this active cysteine protease. Nevertheless, coinubation with AS101 resulted in 86% of active caspase-expressing cells. This effect was dose-dependent in both T and B lymphoma cells (Fig. 2C and D). Analysis of A20 cells revealed that treatment with paclitaxel considerably increased the expression of cleaved PARP. This increase was further amplified when cells were treated with AS101 and paclitaxel (Fig. 2E). These results collectively imply that AS101 sensitizes B and T lymphoma cells to paclitaxel-induced apoptotic death.

AS101 sensitizes B- and T-cell lymphoma cells to Abraxane-induced cell death

Abraxane, the new albumin-stabilized nanoparticle formulation of paclitaxel, was designed to overcome insolubility problems encountered with paclitaxel, facilitating the passage of the drug to the underlying tumor tissue. This drug appears to represent an improvement in paclitaxel formulation in that it can be administered safely without the risk of hypersensitivity reactions. Furthermore, the increased MTD and favorable toxicity profile of Abraxane may ultimately prove advantageous in term of rate and quality of response. Thus, we tested the sensitizing effect of AS101 on Abraxane-induced lymphoma cell death.

Supplementary Fig. S1 shows that AS101 sensitizes T (A) and B (B) lymphoma cells to Abraxane-induced death in a dose-dependent manner. Similarly to the results involving paclitaxel, presented in Fig. 2, YAC cells were more sensitive to the combined treatment of Abraxane+AS101, as compared with A20 cells. The combined treatment was effective starting with 5 nmol/L Abraxane (Supplementary Fig. S1C). In these cells, the accumulation of cells in the sub-G1 fraction increased in Abraxane dose-dependent manner (Supplementary Fig. S1). In A20 cells, the magnitude of the synergistic effect was lower. Treatment of YAC cells with AS101 combined with 60 nmol/L Abraxane resulted approximately 60% death when combined with AS101, compared with 9.2% death in Abraxane-treated cells, while in A20 cells the maximal response amounted to approximately 21% at the dose of Abraxane 70 nmol/L+AS101, compared with 6.3% in Abraxane-treated cells. Both types of lymphoma cells responded in an AS101 dose-dependent manner (Supplementary Fig. S1A and S1B).

AS101 sensitizes human breast carcinoma cells in vitro to Abraxane-induced death

Abraxane is now approved for the treatment of recurrent breast cancer after combination chemotherapy or relapse within 6 months of adjuvant chemotherapy (33). We therefore wished to assess if AS101 may sensitize breast carcinoma cells to Abraxane. For this purpose, we used human cultured MCF-7 breast carcinoma cells, known to produce IL10. It can be clearly seen that while AS101 does not affect cell viability, it significantly sensitizes cells to Abraxane in a dose-dependent manner (Supplementary Fig. S2A). Furthermore, AS101 significantly downregulated IL10 secretion (Supplementary Fig. S2B), pstat3 and srvivib expression (Supplementary Fig. S2C).

AS101 sensitizes T and B lymphoma cells to paclitaxel via IL10 inhibition

Inhibition of IL10 has been previously shown to mediate many of AS101’s activities. Here, we show that inhibition of IL10 is indispensable for sensitizing the lymphoma cells studied to paclitaxel. Figure 3A and B shows that treatment of lymphoma cells with paclitaxel combined with anti-IL10-neutralizing antibodies resulted in a similar sensitizing effect as AS101+paclitaxel–induced death. Furthermore, this sensitization was abrogated in the presence of rIL10, suggesting that sensitization of lymphoma cells to paclitaxel-induced death by AS101 may be attributable to the inhibition of IL10. Moreover, T leukemic cells that do not secrete IL10 (data not shown; T127 and Jurkat) were not sensitized by AS101 to paclitaxel-induced death (Supplementary Fig. S3A and S3B). To further ascertain the role of IL10 inhibition in AS101-sensitizing effect, the IL10 gene was silenced by transfection with a plasmid expressing IL10shRNA. Figure 3C shows the significant decrease in IL10 production by lymphoma cells transfected with the silencing plasmid (from 630.67 ± 30.18 to 158.9 ± 5.55 pg/mL; P < 0.001). AS101 significantly decreased IL10 secretion by control but not by silenced cells (from 630.67 ± 30 to 144 ± 19.3 pg/mL; P < 0.001). Figure 3D shows that treatment of lymphoma cells transfected with the control plasmid, with either paclitaxel or Abraxane, resulted in a significant accumulation of cells in the G2-M fraction. Addition of AS101 combined with chemotherapeutic induced considerable apoptosis in treated cells. Nonetheless, treatment of IL10-silenced cells with paclitaxel or Abraxane alone, induced extensive sub-G1 cell accumulation that was not further augmented following addition of AS101 (Fig. 3D). These results collectively suggest that AS101 sensitizes lymphoma cells to Abraxane and paclitaxel via inhibition of IL10 and probably not by additional targets.

Inhibition of the VLA-4 integrin activity on lymphoma cells mediates IL10 inhibition by AS101

We have recently shown that AS101 inactivates the VLA-4 integrin by redox modulation of vicinal thiols within the exofacial membranal side (34). This activity accounted for the conversion of resistant to chemotherapy-sensitive acute myelogenous leukemia cells in vivo (34) and to favorable activities in murine models of autoimmune diseases (35, 36). Figure 4A shows that AS101 inhibits the attachment of lymphoma cells to the VLA-4 ligand VCAM-1 in a dose-dependent manner, suggesting inhibition of VLA-4 activity in these cells. Neutralization of VLA-4 by neutralizing anti-VLA-4 antibodies inhibited IL10 secretion by lymphoma cells (Fig. 4B) while combination with AS101 did not further decrease this cytokine production, suggesting that AS101 and anti-VLA-4 may act on a similar target. Inhibition of VLA-4 activity by AS101 was followed by dose-dependent decreased pAkt expression (Fig. 4C) and this effect was associated to the inhibition of IL10 production by AS101. Figure 4D shows that the PI3K inhibitor LY294002 inhibits IL10 secretion and this inhibition is not further increased by addition of AS101, implying a common target for both compounds. To further substantiate this point, we show that overexpression of Akt (Fig. 4E) abrogates the IL10 inhibitory effect of AS101 on lymphoma cells (Fig. 4F). These results collectively imply that regulation of IL10 in lymphoma cells by AS101 is mediated by VLA-4 inactivation and the resulting pAkt decrease.
Mechanism of IL10-associated lymphoma cells sensitization by AS101

Involvement of Stat3 in AS101’s activity. To gain further insight into the mechanism of IL10-induced resistance to chemotherapy of lymphoma cells, and the consequences of its inhibition by AS101, we tested the role of Stat3 in these effects. The best characterized IL10 signaling pathway is the Jak–Stat system. The IL10–Jak10R interaction engages the Jak family tyrosine kinases Jak1 and Tyk2 and induces tyrosine phosphorylation and activation of the latent transcription factors Stat3, Stat1, and Stat5. However, Stat3 is used primarily by IL10 to carry out its signaling effect on these cells. Because we showed that inhibition of Stat3 by AS101 may account for its sensitizing effect on these cells, we asked whether overexpression of Stat3 in lymphoma cells significantly abrogates the sensitizing effect of AS101 to paclitaxel-induced death (Fig. 5D). Accumulation of cells in sub-G1 fraction decreased from 18.95% to 7.6% (0.49% to 0.75%) in cells transfected with either piceatanol or AG490 in combination with paclitaxel, similarly to cells treated with paclitaxel combined with AS101. Collectively, these results suggest that the decrease in pStat3 by AS101 may account for its sensitizing effect on these cells. Because we showed that inhibition of lymphoma cells IL10 by AS101 accounts for both its inhibition of Stat3 and its sensitizing effects to paclitaxel, we assumed that indeed AS101 sensitizes lymphoma cells to paclitaxel via inhibition of the IL10–pStat3 axis. To demonstrate this fact, we showed that overexpression of Stat3 in lymphoma cells significantly abrogates the sensitizing effect of AS101 to paclitaxel-induced death (Fig. 5D). Accumulation of cells in the sub-G1 fraction decreased from 18.95% ± 1.45% in cells transfected with the control vector and treated with AS101 (0.5 μg/mL) and paclitaxel to 7.6% ± 0.75% in cells overexpressing Stat3 (P < 0.05). At the higher dose of AS101 (2.5 μg/mL), the decrease was more pronounced (from 42.11% ± 0.49% to 15.04% ± 0.33%; P < 0.001).

The ability of AS101 to inhibit the expression of the above mentioned proteins led us to assess their role in the resistance of lymphoma cells to paclitaxel. We first used two known pStat3 inhibitors: the JAK1 inhibitor piceatanol and the JAK2 inhibitor AG490. Figure 5B shows that both inhibitors are active at a dose-dependent manner. As can be seen in Fig. 5C, treatment of cells with either piceatanol or AG490 in combination with paclitaxel significantly increased the sensitivity of lymphoma cells to paclitaxel, similarly to cells treated with paclitaxel combined with AS101. Collectively, these results suggest that the decrease in pStat3 by AS101 may account for its sensitizing effect on these cells. Because we showed that inhibition of lymphoma cells IL10 by AS101 accounts for both its inhibition of Stat3 and its sensitizing effects to paclitaxel, we assumed that indeed AS101 sensitizes lymphoma cells to paclitaxel via inhibition of the IL10–pStat3 axis. To demonstrate this fact, we showed that overexpression of Stat3 in lymphoma cells significantly abrogates the sensitizing effect of AS101 to paclitaxel-induced death (Fig. 5D). Accumulation of cells in the sub-G1 fraction decreased from 18.95% ± 1.45% in cells transfected with the control vector and treated with AS101 (0.5 μg/mL) and paclitaxel to 7.6% ± 0.75% in cells overexpressing Stat3 (P < 0.05). At the higher dose of AS101 (2.5 μg/mL), the decrease was more pronounced (from 42.11% ± 0.49% to 15.04% ± 0.33%; P < 0.001).
AS101 sensitizes T and B lymphoma cells to paclitaxel via inhibition of survivin expression

The antiapoptotic protein Survivin has been previously shown to afford resistance to chemotherapy-induced death. As seen in Fig. 5A, treatment of lymphoma cells with AS101 results in downregulation of pStat3 and Survivin. pStat3 is one of the main mediators of survivin upregulation. Because inhibition of pStat3 by AS101 has been shown to mediate AS101’s sensitizing effect on lymphoma cells (Fig. 5D), we wished to explore the role of survivin inhibition in AS101’s ability to sensitize lymphoma cells to chemotherapy. For this purpose, we used an inducible Tet-On expression vector system to control the silencing of survivin by survivin-shRNA—expressing plasmid transfected into lymphoma cells. The inducible system was chosen because survivin is crucial for tumor cell survival, which precludes the option of permanently silencing the protein in a stable transfection. Figure 6A shows that both the protein expression was quantitated (C). A20 cells were incubated with 1 μg/mL AS101, 50 μmol/L LY294002, or both combined for 48 hours. IL10 in supernatants was quantitated (D). A20 cells were transfected with either an empty vector or with a plasmid encoding Akt (pBabe-Akt; E). Each type of cells was incubated with AS101 for 48 hours for quantitation of IL10 (F). C and E are one representative experiments of three performed. Results in A, B, D, and F represent mean ± SE of three different experiments. *, P < 0.01 versus control; **, P < 0.05 versus control.

Inhibition of lymphoma VLA-4 activity mediates IL10 inhibition by AS101. A20 cells were cultured with various AS101 concentrations on VCAM-1–coated plates for 1 hour. The Percentage of attached cells was determined (A). A20 cells were cultured with 1 μg/mL AS101 or 1 μg/mL neutralizing anti-VLA-4 or control antibodies, or the combination of AS101+α-VLA for 48 hours. IL10 was quantitated in supernatants (B). A20 cells were incubated with AS101 for 24 hours, pAkt protein expression was quantitated (C). A20 cells were incubated with 1 μg/mL AS101, 50 μmol/L LY294002, or both combined for 48 hours. IL10 in supernatants was quantitated (D). A20 cells were transfected with either an empty vector or with a plasmid encoding Akt (pBabe-Akt; E). Each type of cells was incubated with AS101 for 48 hours for quantitation of IL10 (F). C and E are one representative experiments of three performed. Results in A, B, D, and F represent mean ± SE of three different experiments. *, P < 0.01 versus control; **, P < 0.05 versus control.

Collectively, the results show that AS101 sensitizes B and T lymphoma cells to paclitaxel or Abraxane-induced apoptotic death by inhibition of the VLA-4–IL10–Stat3–survivin axis. These in vitro results led us to evaluate whether AS101 may sensitize lymphoma cells to chemotherapy via inhibition of the IL10–Survivin axis also in vivo. For this purpose, mice were inoculated with B lymphoma cells in which IL10 was silenced by stable transfection of IL10shRNA. Control cells were transfected with scrambled shRNA. As can be seen in Fig. 7B, only 20% of control mice survived 80 days after lymphoma cells inoculation. Although treatment with Abraxane increased survival rates, cotreatment with AS101 and Abraxane resulted in 90% survival. It can be clearly seen that silencing IL10 in B lymphoma cells sensitizes them to Abraxane in vivo (Fig. 7C). Eighty percent of mice inoculated with IL10-silenced cells survived when treated...
with Abraxane alone, compared with only 50% of mice inoculated with control B lymphoma cells (Fig. 7B; \( P < 0.05 \)). Furthermore, no statistical difference was observed between the survival of mice treated with Abraxane alone and that of mice treated with AS101 and Abraxane. These results strengthen the in vitro data implicating the IL10 axis as a major target of AS101 in sensitizing B lymphoma tumors secreting IL10 to chemotherapy-induced death. To provide clear relevance of the data to disease pathogenesis and therapy, we analyzed the mechanism of sensitization in two human subtypes of NHL. Ramos and OCI-Ly19 cells differed in the level of IL10 secreted. Diffuse large B-cell lymphomas (OCI-Ly19) secreted significantly higher levels of IL10. Nevertheless, the secretion of IL10 by both subtypes of NHL was significantly inhibited by AS101 (Fig. 7D). The protein expression of pStat3 and survivin also differed between the two NHL subtypes. The human diffuse large B-cell lymphoma cells expressed higher levels of both proteins. As seen, AS101 clearly downregulated the expression of both proteins (Fig. 7E). This was correlated with the cell response to paclitaxel. Although the OCI-Ly19 cells, expressing higher pStat3, surviving, and IL10, were resistant to 30 nmol/L paclitaxel, the Ramos cells responded to chemotherapy. Nevertheless, AS101 significantly enhanced the responsiveness of both cell subtypes to chemotherapy (Fig. 7F).

Coculture of both NHL cell subtypes with human BM stromal cells rendered them resistant to paclitaxel as opposed to the significant response of Ramos cells to chemotherapy. Treatment with AS101 sensitizes both the resistant and the responsive cells to chemotherapy in a dose-dependent manner. Importantly, because the survival of cells was performed on the mixture of both stromal and NHL cells together, Supplementary Fig. S4B shows that stromal cells are not affected by the dose of paclitaxel used, implying the decrease in cell viability reflects that of NHL and not that of stromal cells. Furthermore, stromal cells were not sensitized to paclitaxel even in the presence of AS101 (Supplementary Fig. S4B). These data imply that AS101 may sensitize NHL cells to paclitaxel at physiologic conditions.

**Discussion**

This study proposes a novel approach to target survivin in B- and T-cell lymphomas, a protein that plays a pivotal role in the resistance of tumors to chemotherapy, especially to paclitaxel.
Our approach is based on the indirect repression of survivin by the nontoxic tellurium compound, AS101, via inhibition of tumor autocrine IL10–Stat3–Survivin signaling. The inhibition of this axis is mediated by inactivation of the VLA-4 integrin. The study presents an attractive approach for anticancer therapy, having a vast significance in modulating chemosensitivity of various tumor cell types not only lymphomas, for the reasons presented below:

a. The differential expression of survivin in cancer.
b. The wide variety of tumors, of both solid and hematologic origin, that secrete IL10 in an autocrine manner, expressing constitutively active Stat3.
c. The variety of tumors that highly express VLA-4.
d. The use of a small molecule that has proved excellent safety profile in humans.

In recent years, considerable efforts have been made to validate survivin as a new target in cancer therapy (37). Results from studies exploiting different strategies to interfere with survivin expression and function provided direct and convincing evidence that targeting the survivin network inhibits tumor growth potential in vitro and in vivo and increases spontaneous and treatment-induced apoptosis of cancer cells, thus indicating survivin as a promising molecular target for cancer therapy (37).

Three independent approaches have been used for this aim in preclinical testing:

b. Use of molecular antagonists, including antisense, ribozymes, siRNA, and dominant-negative survivin mutants results in suppression of tumor growth (39–41).
c. Mutation of Thr34 in survivin that abolishes a phosphorylation site for the mitotic kinase p34cdc2, and results in a dominant-negative phenotype, with induction of apoptosis and anticancer activity in vivo (40).

The present study introduces an alternative and novel mode to indirectly target survivin in IL10 secreting B and T lymphoma.

Figure 6.
Role of targeting survivin by AS101 in the sensitization of lymphoma cells to paclitaxel-induced death. A20 cells were transfected with the tetracycline (or doxycycline) inducible (Tet-On) pSUPERIOR.retro.puro vector expressing survivin shRNA or scrambled shRNA (A). Cells transfected with scrambled (B and C) or survivin (D and E) shRNA were incubated with AS101, paclitaxel, or the combination of both for 24 hours and were either induced (B and D) or not induced (C and E) by doxycycline. Viability of the cells was assessed by the XTT assay. The data represent mean ± SE of three different experiments.

\*P < 0.05 decrease versus control; **P < 0.01 decrease versus control; ##P < 0.01 decrease versus paclitaxel.
Role of targeting survivin by AS101 in the sensitization of lymphoma cells to Abraxane-induced death and the role of IL10 inhibition in sensitization of lymphomas to Abraxane in vivo. A20 cells were transfected with the tetracycline (or doxycycline) inducible (Tet-On) pSUPERIOR.retro.puro vector expressing survivin shRNA and incubated as described in A. Cells were either induced or not induced by Doxocycline. Cell viability was assessed after 24 hours by the XTT assay. *P < 0.05 versus control DOX., #P < 0.05 decrease versus Abraxane DOX. The data represent mean ± SE of three different experiments (A). A20 cells expressing control (B) or IL10 shRNA (C) were implanted into mice. At day 10, when tumors were palpable, mice were treated as follows: control mice treated with PBS on alternate days. AS101 was injected on alternate days i.p. at 10 μg/mouse. Abraxane was injected at 0.5 mg/mouse on alternate days (+). Survival of mice was monitored for 80 days. The data represent a total of 10 mice/group. †, P < 0.05 increase versus PBS or AS101 or Abraxane; ‡, P < 0.05 increase versus PBS or AS101; *, P < 0.05 increase versus PBS. Ramos and OCI-Ly19 NHL cells were cultured at 0.5 × 10^6/mL at 37°C in the presence or absence of various AS101 concentrations. Supernatants were collected after 48 hours and tested by ELISA for IL10 content. Results represent mean ± SE of three different experiments. †, P < 0.05 decrease versus control; ‡, P < 0.01 decrease versus Ramos cells. D, Ramos and OCI-Ly19 NHL cells incubated with AS101 for 24 hours. pStat3 and survivin protein expression was quantitated (E). Ramos and OCI-Ly19 NHL cells were cultured at 0.5 × 10^6/mL with or without 30 nmol/L paclitaxel in the presence or absence of AS101 (F). Cell viability was assessed after 24 hours of incubation by the XTT assay. Results represent mean ± SE of four different experiments. †, P < 0.05 versus “without paclitaxel” and **, P < 0.01 versus control.

The study provides sound evidence on the sequential events occurring following treatment of lymphoma cells with AS101. These events initiate by interaction of AS101 with the VLA-4 integrin and terminate with the inhibition of survivin expression by lymphoma cells resulting in increased sensitivity of lymphoma cells to paclitaxel or Abraxane. We have recently shown that AS101 inactivates the VLA-4 integrin by redox modulation of vicinal thiols within the exofacial membranal side (34). This activity accounted for the conversion of resistant to chemotherapy-sensitive acute myelogenous leukemia cells in vivo (34) and to...
decrease in pAkt. The essential role of PI3K inhibitory effect of AS101 on lymphoma IL10 production specifically, our data imply that regulation of IL10 in lymphoma cells by AS101 is mediated by VLA-4 inactivation and the resulting decrease in pAkt. The essential role of PI3K–Akt in IL10 production may be explained by deactivation of GSK3β (43). GSK3β has been reported to decrease the nuclear translocation of transcription factors that are crucial for IL10 expression including CREB, C/EBPβ, C/EBPγ, and NF-κB (44).

The study focuses on the mechanism of survivin repression by AS101 and its impact on paclitaxel or Abraxane-induced lymphoma cell death. Our drug choice consisted on the fact that paclitaxel is one of the most effective cancer chemotherapeutic agents to date. The dual role of survivin in apoptosis inhibition and in regulation of spindle dynamics and microtubule integrity may facilitate evasion from checkpoint mechanisms of cell-cycle arrest and promote resistance to microtubules interfering chemotherapeutic drugs such as paclitaxel (45).

Part of our data is in line with other studies. Numerous studies have detected constitutively active Stat3 in diverse human tumor specimens and established persistent Stat3 activity as essential for malignant transformation of cultured cells by many oncogenic signaling pathways (46). Activated Stat3 has been previously shown to regulate survivin expression (47). Inhibition of IL10 by AS101 has been previously shown to sensitize tumors to chemotherapy (33). Nevertheless, this is the first report in which the sequential events starting by VLA-4 inactivation resulting in IL10 repression, inducing decreased pStat3 expression ensuing survivin repression, are demonstrated to be interdependent in the sensitization of mice and human lymphoma cells to paclitaxel or Abraxane. Other targets of Stat3 and Akt, such as BCl2, P21waf, or p53, might be theoretically also modulated.

Besides our prototype tellurium compound AS101, the investigation of therapeutic activities of other tellurium(IV) compounds is scarce in the literature, although tellurium is the fourth most abundant trace element in the human body. Our integrated results show sensitization of IL10-secreting lymphoma cells to paclitaxel or Abraxane-induced death can be achieved by a unique alternative approach to existing strategies of controlling survivin, a pivotal molecule at the junction of cancer cell survival and division networks. This regulation may be achieved using AS101, currently being tested in clinical trials and might be beneficial in the treatment of patients with lymphoma with high functional VLA-4 expression and high level of IL10.

Unlike most conventional chemotherapies, taxanes, such as docetaxel and Abraxane, have been shown to upregulate survivin expression levels, which is attributed to mitotic arrest at the G2–M stage and the augmented stability of survivin protein (48). In clinical settings, docetaxel and paclitaxel treatment is effective against several types of human cancers. Despite their clinical benefits, the long-term treatment of these drugs is often limited because of development of drug resistance and cumulative side effects, primarily neuropathy and myeloid toxicity. Therefore, drugs that circumvent paclitaxel or docetaxel resistance without overlapping side effects represent ideal candidates for developing novel combinatorial therapeutic regimen for lymphomas. Our study suggests that survivin is an important factor that counteracts paclitaxel/Abraxane–induced apoptosis in lymphoma cells and this protein can be distinctively counteracted by AS101.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Danoch, Y. Kalechman, M. Albeck, D.L. Longo, B. Sredni
Development of methodology: H. Danoch, M. Albeck
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Danoch, D.L. Longo, B. Sredni
Writing, review, and/or revision of the manuscript: Y. Kalechman, D.L. Longo, B. Sredni
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Danoch
Study supervision: B. Sredni

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References

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