Redox modulation of adjacent thiols in VLA-4 by AS101 converts myeloid leukemia cells from a drug-resistant to drug-sensitive state

Adi Layani-Bazar1*, Itai Skornik1*, Alain Berrebi2, Maor H. Pauker1, Elad Noy1, Alon Silberman1, Michael Albeck3, Dan L. Longo4, Yona Kalechman1, Benjamin Sredni1

1C.A.I.R. Institute, The Safdie AIDS and Immunology Research Center, The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 5290002, Israel.
2Department of Hematology, Kaplan Medical Center, POB 1, Rehovot, 76100, Israel.
3Chemistry Department, Bar-Ilan University, Ramat-Gan 5290002, Israel.
4Laboratory of Molecular Biology and Immunology, Biomedical Research Center, National Institute on Aging, 04C228, Baltimore, MD 21224-6825, USA.
*These authors contributed equally to this work

Correspondence should be addressed to: Prof. Benjamin Sredni
Bar Ilan University, Max & Anna Webb St, Ramat Gan, 5290002, Israel.
Phone: 972-3-531-8250, Fax: 972-3-738-4060
E-mail: srednib@mail.biu.ac.il

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Abbreviations: AS101: Ammonium trichloro(dioxyethylene-O-O')tellurate; VLA-4: very late antigen-4; AML: acute myeloid leukemia; MRD: minimal residual disease; VCAM-1: Vascular cell adhesion protein 1; FRET: Fluorescence Resonance Energy Transfer

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Abstract:

Interactions between the integrin VLA-4 on acute myelogenous leukemia (AML) cells with stromal fibronectin is a decisive factor in chemotherapeutic resistance. In this study, we provide a rationale for a drug repositioning strategy to blunt integrin activation in AML cells and restore their sensitivity to chemotherapy. Specifically, we demonstrate that the non-toxic tellurium compound AS101, currently being evaluated in clinical trials, can abrogate the acquired resistance of AML. Mechanistic investigations revealed that AS101 caused redox inactivation of adjacent thiols in the exofacial domain of VLA-4 after its ligation to stromal fibronectin. This effect triggered cytoskeletal conformational changes that decreased PI3K/Akt/Bcl2 signaling, an obligatory step in chemosensitization by AS101. In a mouse xenograft of AML derived from patient leukemic cells with high VLA-4 expression and activity, we demonstrated that AS101 abrogated drug resistance and prolonged survival in mice receiving chemotherapy. Decreased integrin activity was confirmed on AML cells in vivo. The chemosensitizing activity of AS101 persisted in hosts with defective adaptive and innate immunity, consistent with evidence that integrin deactivation was not mediated by heightening immune attack. Our findings provide a mechanistic rationale to reposition the experimental clinical agent AS101 to degrade VLA-4-mediated chemoresistance and improve clinical responses in AML patients.
Introduction

A major problem in the treatment of acute myeloid leukemia (AML) remains the recurrence of the pathology following chemotherapy. This common and severe complication is due to resistant leukemic cells which are localized in the bone marrow. In the hematopoietic niche, specific components of the microenvironment play a crucial role in the pathogenesis of AML by promoting tumor cell growth and survival as well as drug resistance(1). Evidence supporting this concept includes the finding that AML cells bind to bone marrow stromal cells through combined β, and β integrin mechanisms(2). Furthermore, β integrins on AML cells interact with fibronectin on stromal cells, thereby mediating anti-apoptotic and proliferative signals. Recent data suggest that the interaction between the VLA-4 integrin on leukemic blasts and fibronectin on stromal cells activates phosphatidylinositol 3-kinase (PI3K)/Akt/Bcl-2 signaling, which contributes to drug resistance in AML(3). Thus, activation of the signaling cascades downstream of the VLA-4 integrin engagement may play a critical role in the chemoresistance of bone marrow–resident AML cells. Hence, VLA-4 has emerged as a promising therapeutic target in AML. Still, its role as a clinical target to augment remission induction, lower disease recurrence, or improved prognosis remains controversial.

Because relapses following chemotherapy are a major contributor to poor survival in acute myeloid leukemia(4, 5), elucidation of the VLA-4 activation/deactivation process, and eventually control and manipulation of its activation is important.

Although a wealth of evidence shows that inside-out signaling can control integrin activation(6, 7), it has been postulated that at least specific integrin function may be directly affected by redox rearrangements within the cysteine-rich domain of the extracellular integrin regions(8-11). Thus, a disulfide bond reshuffling mechanism is proposed in which resting and active integrins differ in the number and position of unpaired cysteine residues(12). Nevertheless, the physiological significance of this mode of integrin activation is not known.
AS101, an organotellurium(IV) compound previously(13, 14) used in Phase II clinical trials in cancer patients, is a potent immunomodulator with a variety of potential therapeutic applications(15-17). The compound has been shown to have beneficial effects in diverse preclinical and clinical studies(13, 14, 18).

Accumulated evidence suggests that much of the biological activity of AS101 is directly related to its specific chemical interactions with cysteine thiol residues. The TeIV-thiol chemical bond may lead to conformational change or disulfide bond formation in a specific protein, possibly resulting in the loss of its biological activity, if the thiol residue is essential for that function(19, 20). Indeed we demonstrated that the specific redox-modulating activities of AS101 result in a variety of beneficial biological effects: inhibition of IL-10(20) resulting in tumor sensitization(18); neuroprotection in both Parkinson's disease models(20) and ischemic stroke(21), all mediated by the Te(IV) redox chemistry of the compound. Likewise, the protective mechanism of AS101 against homocysteine toxicity was shown to be directly mediated by its chemical reactivity, whereby AS101 reacted with homocysteine to form homocystine, the less toxic disulfide form of homocysteine(22).

Vicinal thiols are closely spaced protein thiols in native proteins that undergo reversible conversions with disulfide bonds. Vicinal thiols do not necessarily have to be in close proximity to the primary amino acid sequence of a protein to be brought into close opposition by protein folding(23). Vicinal thiol-containing proteins regulate a variety of receptors and transcription regulatory proteins(24, 25). These thiols are also found on cell surfaces(26). Vicinal thiols in VLA-4 and, in particular on VLA on AML cells have not been yet described.

Based on the thiol-Te interaction of AS101, the present study reveals a unique approach in regulating cellular VLA-4 activity, via redox modulation of vicinal thiols on VLA-4 by AS101, and explores its role in the conversion of resistant to chemotherapy-sensitive human myeloid leukemic cells both in vitro and in vivo.
Materials and Methods

Reagents, plasmids construction and PCR analysis are described in supplementary materials:

**Cells:** The human AML cells (U937 and HL-60) and the mouse myelomonocytic leukemia cells (Wehi-3B) were obtained from the American Type Culture Collection. The DNA profiles characteristic of cells was authenticated by Short tandem repeat (STR) analysis. Cells were cultured in RPMI 1640 containing 10% FCS at 37°C with 5% CO₂ and 95% air. Patients’ leukemic cells were obtained from 14 newly diagnosed AML patients before chemotherapy, following approval by the institutional Ethics Committee upon receipt of patients informed consent. Six-well plates (Costar) were coated with either 5μg/ml Fibronectin, 1μg/ml rVCAM-1 or 2%BSA.

**FACS analysis:** VLA-4 or VLA-5 expression on AML cells was determined by FACS after incubation with primary (mouse anti human Cd49d and anti cd49e) and secondary antibodies (FITC-conjugated Goat Anti Mouse IgG) and finally with PE-conjugated CD45 antibodies. Blast cells were first identified by CD45/SSC gating in all cases of AML patients as described by Lacombe at al(27). Expression of active β₁ on U937 leukemic cells was determined with anti-human HUTS-21 mAb which reacts with an activation-dependent epitope on the VLA-β1 chain. For cell cycle distribution studies, cells were treated with RNase and stained with propidium Iodide. DNA content was measured using a FACStar plus (Becton Dickinson, San Jose, CA) flow cytometer using Cell Quest software.

**Cell surface labeling, immunoprecipitation and Western blotting**

Cells were pretreated with or without Fibronectin, PAO or AS101. After 60min cells were washed and incubated with DMPS or 2-mercaptoethanol for further 30min. Washed cells were incubated with biotin-BMCC, the sulphydryl group modification reagent, to tag unpaired cysteines, in serum-free RPMI 1640 medium at room temperature for 1 h with rotation. After removal of unbound biotin-BMCC by centrifugation, cells were washed and lysed. Cell lysates were subjected to immunoprecipitation with anti α₄ or anti β₁ antibodies. For detection of biotin-BMCC, blots were incubated with HRP-conjugated
monoclonal anti-biotin antibody. Immunoprecipitation and Western Blot analysis was performed as described(20).

Attachment assay: 96 wells plates were coated with 80 μl of FN or VCAM-1 or BSA. Cells with or without AS101 were incubated in the wells for one hour, Thereafter, cells were washed three times. The attached cells were tested by the XTT assay at 450nm.

Enrichment of human leukemic CD45+ cells. Human CD45+ cells were enriched from total spleen cells using the MACS magnetic cell sorter (Miltenyi Biotec, Germany) according to the manufacturer’s instructions, obtaining purity of about 95%. Purified cells were used freshly for attachment assays.

Actin shape index. A quantitative estimate of the actin shape changes was obtained as previously described(28). Briefly, actin shape index was calculated as \( \frac{P^2}{4\pi S} \), where P and S are the perimeter and the surface of the cell, respectively. These values were obtained by thresholding images of phalloidin staining to outline the polymerized actin. A perfectly circular shape of the polymerized actin yields a shape index of 1, and departure from a circle yields a shape index larger than 1. Actin reorganization was determined following 30 min of activation, which is the best time for observing this process in these cells.

Animals: 7-8 week-old male SCID mice were obtained from Harlan Laboratories (Jerusalem, Israel). NOD.CgPrkdc<scid> Il2rg<tm1Wjl>/SzJ (NSG) mice were purchased from The Jackson Laboratory, ME US. Animal experiments were performed in accordance with approved Institutional protocols. Mice were irradiated at 2 Gy and were then administered patients’ leukemic cells \( (1 \times 10^7) \), intravenously. SCID mice transplanted with patients’ leukemic cells were treated with AS101 (0.5mk/kg) or PBS, I.P. 3 times weekly as specified in figure legends. ARA-C was injected I.P. at days 3 or 4 (40mg).

FRET analysis. Fluorescence resonance energy transfer (FRET) was measured by the donor-sensitized acceptor fluorescence technique as previously described(29). Three sets of filters were used: one
optimized for donor fluorescence (excitation, 468 nm, and emission, 475 to 505 nm), a second for acceptor fluorescence (excitation, 514 nm, and emission, 530 nm longpass [LP]), and a third for FRET (excitation, 468 nm; emission, 530 nm LP). FRET was corrected, and the FRET efficiency was determined (suppl methods).

Statistics. Results are expressed as mean ± S.E. Differences in cells viability between groups in response to chemotherapy 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 leukemic cells to chemotherapy-induced death via inhibition of VLA-4 activity. In the present study, we show that AS101 increases the sensitivity of human AML cells to chemotherapy and prevents the minimal remaining of resistant cells in a xenograft mouse model. Furthermore, the mechanism of action of this phenomenon is defined.

Fig. 1 shows that AS101 significantly sensitizes the human AML leukemic cells U937 (Fig. 1a) and HL-60 (Fig. 1b) to chemotherapy-induced death in a dose-dependent manner only when cells are cultured on Fibronectin (i) but not on VCAM-1(ii) nor on BSA (iii). The decrease in cell viability has apoptotic features (Fig. S1) as reflected by increased annexin binding (Fig. S1a) and increased caspase 3,7 activity (Fig. S1b). U937 cells responded well to chemotherapy when cultured on BSA or VCAM-1 but were poorly responsive on FN (Fig.1 and Fig. S1). Thus, AS101 disrupts the interaction between fibronectin and leukemic cells, resulting in enhanced sensitivity to drug-induced apoptosis.

Since fibronectin is a ligand for α4β1 (VLA-4), abundantly expressed on both leukemic cell lines examined, we studied the potential effect of AS101 on the activity of this integrin. For this purpose we used the FRET technique to investigate the spatial proximity of α4 and β1 cytoplasmic domains in living cells in the presence or absence of fibronectin and AS101. Fig. 2a shows that in the presence of fibronectin, U937 AML cell cultures exerted a significant decrease in FRET efficiency compared to its absence (21.8±5.9 vs 44.7±3.9; p<0.05). Furthermore, treatment of AML cells with AS101 in the presence of fibronectin significantly increased FRET efficiency compared to fibronectin alone (43±3.9 vs 21.8±5.9; p<0.005) (Controls presented in Fig. S1c). Moreover, treatment of AML cells with AS101 in the presence of fibronectin reduced the expression of the active β1 form in a dose-dependent manner (Fig. 2b). Collectively, these results suggest that in the presence of fibronectin, AS101 inhibits the activation of α4β1. Moreover, treatment of U937 cells, cultured on fibronectin-coated plates, with neutralizing anti VLA-4 antibody, significantly enhanced leukemic cells sensitivity to chemotherapy-induced death (Fig. 2c) while addition of AS101 did not further enhance this sensitivity. Furthermore,
neutralizing anti VLA-5 antibodies did not affect cells sensitivity to chemotherapy (Fig. 2c). These results suggest that U937 cells acquire drug-resistance via $\alpha_4\beta_1$, but not $\alpha_5\beta_1$, and VLA-4 is an important target for AS101’s sensitization of leukemic cells to chemotherapy-induced death. As further proof for integrin deactivation by AS101, Fig. 2d shows that AS101 prevents attachment of AML cells to both VCAM-1(a VLA-4 ligand) and Fibronectin (ligand to VLA-4 and VLA-5) (Fig. 2d and e). With respect to cells cytoskeletal behavior in the presence of fibronectin, U937 cells exhibited normal actin rearrangement as shown in figure 2f. Cells were well spread and demonstrated radial morphology. However, cells preincubated with AS101 for 15min displayed significantly aberrant spreading behavior ($p \leq 0.0001$; Fig. 2f), as indicated by the actin shape index and found to be similar to cells seeded over Poly-L-Lysine (PLL) only. These findings demonstrate that AS101 impairs normal cytoskeletal behavior. This is in line with the results shown in Fig. 2g showing decreased expression of pFAK in treated cells.

Furthermore, using the highly VLA-4 expressing mouse myelomonocytic leukemia cells, Wehi-3B, co-cultured on syngeneic BM stromal cells, we show that treatment with AS101 abrogates leukemic cells resistance to ARA-C (Fig. S2a) suggesting a potential sensitizing effect of myeloid leukemic cells in vivo. The VLA-4 integrin activity on AML cells can be directly affected by AS101 by redox rearrangements within vicinal thiols in the extracellular integrin domain resulting in physiologic consequences. Many of the beneficial effects of AS101 were shown to be attributed to redox modulation by the compound (20-22). The following experiments show that the VLA-4 integrin activity on AML cells can be regulated by vicinal thiols redox rearrangements and that this mechanism of action exerted by AS101 has physiologic consequences both in vitro and in vivo. Fig. S2b shows that treatment of U937 cells with AS101 or with the sulfhydryl blocker 5,5'-dithiobis(2-nitrobenzoic acid (DTNB), which is membrane impermeable, significantly increased leukemic cells sensitivity to chemotherapy-induced death. AS101 did not potentiate DTNB activity suggesting that the membrane target of AS101 and DTNB responsible for increased drug-sensitivity in the presence of the VLA-4 ligand may be similar. Importantly, when U937 cells were cultured in the presence of fibronectin, and labeled with the sulfhydryl-binding chemical BMCC, the immunoprecipitated $\alpha_4$ chain, but not the $\beta_1$ chain, contained...
exposed sulfhydryl groups. These thiol groups were diminished following treatment with AS101. Furthermore, biotin-BMCC labeling was found on both α4 and β1 peptides when the cells were pre-reduced with dithiothreitol (DTT). AS101 significantly lowered this thiol labeling on both chains (Fig.S3a). Importantly, the thiol residues on the α4 chain were diminished in AML cells treated with the vicinal dithiol-binding agent, phenylarsine oxide (PAO) either when thiols were labeled with the membrane impermeable sulfhydryl-binding chemical 3-N-maleimidylpropionyl-Biotin (MBP) (Fig. 3a) or with BMCC (Fig. S3b). The addition of the vicinal thiol containing reagent 2,3-Dimercapto-1-propanesulfonic acid (DMPS) after incubation with PAO or with AS101 reversed the inhibition of both compounds labeling (Fig. 2f, lane 5 vs. lane 4 and lane 8 vs lane 7). In contrast, the monothiol β-mercaptoethanol had minimal effect on reversing the inhibition of labeling. Overall, these data thus showed that ligand binding conditions alter the sulfhydryl group exposure on the α4 but not the β1 chain and that the exposure is different from nonspecific reduction by DTT. Furthermore, Fibronectin binding results in exposure of vicinal thiols on the extracellular integrin regions of VLA-4 and these are diminished following treatment with AS101. Notably, the ability of AS101 to alter vicinal thiols on the VLA-4 chain had physiological implications, since as shown in Fig. 3b, alteration of vicinal thiols, but not that of monothiols by AS101, resulted in the sensitization of AML cells to chemotherapy. Interestingly, no exposed sulfhydryl groups were found on the immunoprecipitated α5 chain of U937 cells cultured in the presence of fibronectin (Fig. 3c).

**The resistance of AML patients leukemic cells expressing high VLA-4 to chemotherapy may be converted by redox modulation of the VLA-4 integrin.** We then asked whether the sensitizing effect of AS101 in the presence of FN, as shown in two human AML cell lines, is also exerted in leukemic cells from untreated patients with AML. For this purpose leukemic cells from 12 patients were analyzed for VLA-4 and VLA-5 expression. While leukemic cells from all patients expressed high levels of VLA-5 (≥80%), 8 patients exhibited high expression of VLA-4 (≥75% of cells) while 4 patients exhibited very low expression of VLA-4 (≤15% of cells) (not shown). Fig. 3(d-g) shows that leukemic cells from AML
patients, that express high VLA-4 slightly respond to ARA-C (10⁻⁶M) in vitro when plated on fibronectin (d) as opposed to BSA (e). Treatment of AML cultures in the presence of FN, with AS101 significantly increased their sensitivity to drug-induced apoptosis in a dose-dependent manner (a). On the other hand, leukemic cells from AML patients that express very low VLA-4 responded well to chemotherapy when plated on either FN (f) or BSA (g). AS101 did not further increase this drug-sensitivity. Similarly to the leukemic cell lines, leukemic cells from AML patients expressing high VLA-4 were sensitized to chemotherapy-induced death by either DTNB or AS101 when plated on FN, while the sensitivity of leukemic cells from AML patients expressing low VLA-4 was not further enhanced by either one of them (Fig. S4a and b). These data imply that the sensitivity of AML leukemic cells expressing high VLA-4 to chemotherapy may be enhanced by redox modulation of the VLA-4 integrin. In order to validate AS101’s target on AML cells, VLA-4 from AML patient leukemic cells expressing high VLA-4, or VLA from mouse AML cells, was knocked out by shRNA. As seen in Fig. S5, knocking-out VLA-4 rendered these cells sensitive to chemotherapy while AS101 did not further potentiate this activity.

**Signaling alterations involved in enhanced drug-sensitivity induced by AS101.** Inhibition of VLA-4 activity by AS101 was associated with decreased PI3K/Akt/Bcl-2 signalling and this effect was related to the enhanced drug-sensitivity induced by AS101. Fig. 4a shows that AS101 decreases the expression of pAkt in U937 cells plated on fibronectin in a dose-dependent manner. Pharmacological inhibition of PI3K with LY294002 significantly increased drug sensitivity of leukemic cells when cells were plated on fibronectin, while AS101 did not further enhance this sensitivity (Fig. 4b). Moreover, overexpression of Akt in leukemic cells (Fig. 4c) abolished the sensitizing effect of AS101 to drug-induced death when leukemic cells were plated on fibronectin as reflected by both cell viability (Fig. 4d) and subG1 accumulation assays (FigS6). These data collectively suggest that inhibition of VLA-4 activity by AS101 decreases the expression of pAkt and this property, at least in part, mediates AS101’s sensitizing activity. Similarly, Bcl-2, a downstream substrate of Akt, was also shown to mediate AS101’s sensitizing activity.
Overexpression of Bcl-2 abolished AS101’s sensitizing effect in the presence of fibronectin (Fig. 5a and b). Moreover transient transfection of Bcl-2 antisense oligonucleotides (Fig. 5c) partially but significantly restored leukemic cells sensitivity to chemotherapy, while addition of AS101 slightly but insignificantly further enhanced this sensitivity (Fig. 5d), suggesting Bcl-2 as a mediator of AS101’s activity.

Conversion of resistant to chemotherapy-sensitive human myeloid leukemic cells in vivo by AS101

We then asked if the VLA-4 inactivation by AS101 translates into enhanced therapeutic effects in vivo. For this purpose we inoculated leukemic cells from either U937 cell lines or from AML patients i.v. to SCID mice, and monitored their organ distribution by PCR using the human α satellite sequence. 8 days after U937 inoculation (Fig. S7a) or 4 days after inoculation of leukemic cells from an AML patient expressing high VLA-4 (Fig. 6a), leukemic cells resided only within the BM. While they persisted in the BM of both AS101 or ARA-C-treated mice, they were totally eliminated from the BM of mice subjected to combined treatment with AS101+ARA-C (Fig. S7a and Fig. 6a). This therapeutic effect persisted at 60 days after U937 cells implantation (Fig. S7a). At 14 days after implantation, leukemic cells from both origins were detected in all organs examined (Fig. S7a and Fig. 6b). Nevertheless, no detectable human AML cells were found in organs of AS101+ARA-C-treated mice (Fig. 6b). Importantly, the improved sensitivity of leukemic cells to chemotherapy following treatment with AS101 was reflected by the increased survival of mice implanted with U937 cells (Fig. S7b). Moreover, the combined treatment of mice inoculated with leukemic cells from an AML patient expressing high VLA-4 significantly increased mice survival (Fig. 6c) as opposed to the relative insensitivity to ARA-C alone, while mice inoculated with leukemic cells from an AML patient expressing low VLA-4, responded well to chemotherapy alone, yielding 80% survival (Fig. 6d). Co-treatment with AS101+ARA-C further but insignificantly increased mice survival. In order to preclude the possibility that AS101 may potentially alter the innate immune response to the leukemia cells, which can be integrin α4β1 driven, we used the NSG (NOD SCID GAMMA) mice with profound immunological multidysfunction in both adaptive and innate immunologic function. Fig. 7a shows that at 14 days after implantation, leukemic cells were detected in all organs
examined. Nevertheless, no detectable human AML cells were found in organs of AS101+ARA-C-treated mice (Fig. 7a). Furthermore, AS101 is shown to inhibit integrin activity in these mice in vivo. Fig. 7b shows that treatment with AS101 inhibits attachment of leukemic cells, enriched from mice spleens, to both VCAM-1 and Fibronectin. These data collectively suggest that high VLA-4 expression and activity in AML leukemic cells confers resistance to chemotherapy and VLA-4 is a target for AS101 in sensitization of AML cells in vivo.
Discussion

The novelty of this study resides within the concept that redox modulation of vicinal thiols on cellular VLA-4 on AML leukemic cells by agents such as AS101, alters VLA-activity, converting resistant to drug-sensitive cells, enabling the eradication of residual leukemic cells. In a xenograft model of AML, the combination of chemotherapy (cytarabine) with AS101 produced improved survival. This is the first time in which this mechanism of VLA-4 regulation is demonstrated to have physiological meanings in AML both in vitro and in vivo. Furthermore, this is the first report in which AS101's activity is shown to be mediated by alteration of vicinal thiols.

Some of our data are consistent with the data previously described by Matsunaga et al(3) showing that adhesion through the VLA-4 integrin engagement triggers chemoresistance of AML blasts to drug-induced apoptosis. The novelty of the present study resides within the mechanism of VLA inactivation. We show here that both established AML cell lines and leukemic cells obtained from AML patients expressing high VLA-4, acquire resistance to chemotherapy-induced death as a result of the interaction of leukemic VLA-4, but not VLA-5, with fibronectin. This resistance is significantly reduced upon treatment with the tellurium small molecule AS101 via redox modulation of vicinal thiols on VLA-4.

Although a wealth of evidence shows that inside-out signaling via factors in the cytoplasm can control integrin activation state(30-32), an alternative concept now suggests that at least some integrin’s activation could be controlled directly by a redox site in the extracellular domain, independent of factors in the cytoplasm(33-36). Thus, integrin disulfide exchange may be involved in aspects of integrin activation, altering integrin conformation and increasing the ligand binding affinity of the integrin(10, 37, 38). The two pathways to integrin activation, inside-out and redox modulation, may serve different purposes. The redox switch could regulate rapid and transient changes in activation state that require no "filtering" through the cytoplasm. Inside-out signaling may then play a larger role in persistent control of activation, a process that may benefit from filters provided by the interconnections among intracellular signaling paths.
Using a proteomic approach, Laragione et al identified the $\alpha_4$ integrin as a molecular target susceptible to redox regulation (39). Furthermore, Liu et al (40) found that ligand binding of VLA-4 induced exposure of sulfhydryl groups on the $\alpha_4$ peptide.

The Te(IV)-thiol chemical bond formed between AS101 and the $\alpha_4$ chain leads to conformational change in the VLA-4 integrin as seen in this study. Our study suggests that the inactivation of VLA-4 is due to binding of AS101 to the thiol groups of vicinal cysteines on the $\alpha_4$ chain. At least some tellurium derivatives are known to interact with specific vicinal thiols within certain proteins (41). We thus assume that these dithiols reversibly interconvert to a disulfide bond under physiologic conditions, providing sites where changes in the redox environment can regulate protein function.

We cannot exclude the possibility that AS101 also modifies sulfhydryls on other membrane cell proteins (including other integrins) that could be involved in chemoresistance. Nevertheless, the consistency between the results obtained with $\alpha_4\beta_1$ expressed on cell surface leukemic cultures and on patients’ leukemic cells, with regulation of chemosensitivity, and the lack of free thiols on the exofolial region of $\alpha_5$ strongly suggests a mechanistic link between the decrease in free cysteine residues in $\alpha_4\beta_1$ and the decrease in chemotherapy-induced cell resistance following treatment with AS101. Yet, a differential reactivity of various cysteines toward AS101 may exist, possibly reflecting their variable access to AS101. Indeed, $\alpha_{IIb}\beta_3$ is subject to S-nitrosylation at specific critical cysteines residues located within specific motifs resulting in the integrin loss of function (42). This agent does not however deactivate all other types of integrins. In general some indications suggest that AS101 is relatively specific: A. As shown, the inactivation of VLA-4 is due to binding of AS101 to the thiol groups of vicinal cysteines on the $\alpha_4$ chain. These thiols do not necessarily provide redox sensitive sites for regulation of other integrins. Therefore, this property of AS101 may afford specificity to the compound. B. The small tellurium molecule, AS101, has been shown to have an excellent safety profile in patients treated with the compound for several years. This suggests that the compound is probably relatively specific.
VLA-4 has emerged as a promising therapeutic target in AML. Still, its role as a clinical marker to define remission induction, disease recurrence, or prognosis remains controversial. Initially, anti–VLA-4 antibodies were shown to chemosensitize human AML cells and to eradicate MRD in experimental mice when combined with chemotherapy, implicating VLA-4 in acquired chemotherapy resistance and MRD(3). These findings suggested that high VLA-4 expression might reduce chemosensitivity, resulting in poor remission induction, MRD, disease recurrence, and short survival(3). Another study supported this prediction. Tavernier-Tardy et al(43) showed that high VLA-4 expression was associated with shorter survival of AML patients. Furthermore, although nonsignificant (p=0.058), there was a trend for lower VLA-4 expression in samples from patients in remission as compared to refractory patients. Moreover, they found a high significant correlation between the expression of CXCR4 and VLA-4. Over expression of CXCR4 on AML cells has been previously described to predict adverse overall survival(44-46). Our results are in line with those of Matsunaga et al. Nevertheless, a recent study (47) failed to confirm the prognostic role of VLA in AML as suggested by Matsunaga, and reported that functional VLA-4, but not merely VLA-4 expression, is associated with longer overall survival of adult AML patients. This apparently controversy may be hypothetically explained by the fact that high VLA-4 expression and function may merely be a surrogate for certain favorable factors that affect prognosis, and they do not contradict the principle that inhibition of VLA-4 activity on AML leukemic cells is advantageous with respect to increased chemosensitivity and improved outcome.

The ability of AS101 to sensitize leukemic cells from patients expressing high VLA-4 to chemotherapy-induced death was associated with the inhibition of Focal adhesion kinase (FAK) phosphorylation at Tyr-397, a residue that is critical for its function. FAK is a downstream signal that follows α4β1 integrin engagement. Recently Recher et al(48) have shown that FAK is frequently expressed and activated in AML cells, and that FAK expression correlates with enhanced migratory properties, drug resistance, high leukocytosis, and reduced survival.

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 that eradication of residual leukemic cells in a xenograft model of AML can be achieved by a unique alternative approach to existing strategies of controlling the VLA-4 integrin activation using AS101, currently being tested in clinical trials. In light of these results clinical studies involving AML patients refractory to chemotherapy treated with AS101 and chemotherapy have been approved and will be soon initiated. Such treatment might be particularly beneficial for patients with high functional VLA-4 expression.
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Figure Legends

Fig. 1: Effect of FN or VCAM-1 on chemosensitivity of AML cell lines. U937 (a) or HL-60 cells (b) were incubated with ARA-C on FN (i), VCAM-1 (ii) or BSA (iii), in the presence of various concentrations of AS101, with or without ARA-C at 10^{-6}M, 10^{-5}M, or DNR at 0.005\mu g/ml. After 24h the percentage of cell viability was assessed by the XTT assay. Data are shown as mean± S.E of three independent experiments.

Fig. 2: The VLA-4 integrin activity on AML cells can be directly affected by AS101 by redox rearrangements within vicinal thiols in the extracellular integrin domain resulting in physiologic consequences. Inactivation of VLA-4 conformational structure was detected by FRET(a) and FACS analysis (b). VLA-4 activation involves the separation of the fluorescently tagged cytoplasmic ends of the alpha and beta subunits of the integrin, resulting in a reduction in the FRET signal. U937 cells were transfected with \alpha 4-mCFP and \beta 1-mYFP. After 48h cells were activated and fixed. FRET of the molecular interaction between the cytoplasmic domains of \alpha 4 and \beta 1 was measured as described in Methods section. Expression of active \beta 1 on U937 leukemic cells was determined with anti-human HUTS-21 mAb which reacts with an activation-dependent epitope on the VLA-\beta 1 chain (b) Experiments were repeated three times (a and b). Chemotherapy-induced death was assessed by the XTT assay following incubation of U937 cells plated on FN in the presence of ARA-C (10^{-6}M), AS101, or neutralizing antibodies to VLA-4 (1\mu g/ml) or VLA-5 (5\mu g/ml). Data are shown as mean± S.E of three independent experiments. *p<0.001; #<0.003 vs PBS+ARA-C (c). Cells were cultured in VCAM-1(d), BSA(d and e) or FN(e)-coated plate in the presence or absence of AS101 for one hour and washed three times. The attached were then subjected to XTT assay. Data are shown as mean± S.E of four independent experiments. Cells were plated on Poly-L-Lysine (PLL), FN or on FN and AS101, fixed, and stained with phalloidin. Confocal images were collected after 30 min of activation (n=128). In the upper panel, a representative image of each group is presented. Data are shown as mean±S.E. *p<0.0001 vs PLL and FN+AS101(f). U937 cells were cultured on FN-coated plates in the presence of various doses of AS101.
pFAK protein expression was assessed by western blot analysis. Results represent one of two experiments performed (g).

**Fig. 3: Vicinal thiols on VLA-4, but not on VLA-5 are oxidized by AS101 and play an important role in AS101’s sensitization of leukemic cells to chemotherapy.** U937 cells were cultured on FN in the presence or absence of AS101 (1 µg/ml), PAO (5 µM), DMPS (50 µM), or βME (100 µM) for 60 min. After washing, some cultures were supplemented with DMPS or βME for further 30 min and washed and labeled with Biotin-MBP. The α4 chain was immunoprecipitated and blotted with either anti α4 or anti biotin. 1-control PBS; 2-control DMPS; 3-control βME; 4-AS101; 5-AS101+DMPS; 6-AS101+βME; 7-PAO; 8-PAO and DMPS; 9-PAO and βME. The results represent one of three experiments performed (a).

U937 cells were cultured on FN in the presence or absence of AS101 (1 µg/ml), PAO (5 µM), DMPS (50 µM), or βME (100 µM) for 60 min. After washing, some cultures were supplemented with DMPS or βME for further 30 min and thereafter ARA-C (10⁻⁶ M) or PBS. Cell viability was assessed by XTT. Data are shown as mean± SE of 3 independent experiments. *p<0.05; #p<0.001; ##p<0.0005 (b). U937 cells were cultured on FN or BSA in the presence or absence of AS101 (1 µg/ml) or DTT (10 µM) for 60 min and labeled with Biotin-MBP. The α5 chain was immunoprecipitated and blotted with either anti α5 or anti biotin. The results represent one of three experiments performed (c). **Leukemic cells from AML patients with high VLA-4 are more resistant to chemotherapy and are sensitized by AS101.** AML patients were monitored for VLA-expression by FACS. Cells from patients expressing high (n=8) or low (n=4) VLA-4 were cultured on FN (d and f) or on BSA (e and g) with or without ARA-C (10⁻⁶ M) and AS101. Cell viability was assessed by XTT. *p<0.001 vs the relevant doses of AS101 in the control group.

**Fig. 4: Inhibition of pAkt mediates AS101’s activity involving chemotherapy-induced AML cell death.** AS101 inhibits the expression of pAkt in U937 cells plated on FN, in a dose-dependent manner (a). Cells were cultured on FN-coated plates in the presence of AS101 (1 µg/ml), or ARA-C (10⁻⁶ M) or both combined, with or without the PI3K inhibitor LY294002 (LY) at 50 µM (b). *p<0.001 vs control.
U937 cells were transfected with pBabe overexpressing Akt or with an empty vector (c). Both were cultured on FN in the presence or absence of ARA-C and AS101 (d). Percentage of viable cells was assessed by XTT. Data are shown as mean± SE of 3-4 independent experiments. *p<0.001 vs ARA-C.

**Fig. 5: Inhibition of Bcl-2 mediates AS101’s activity involving chemotherapy-induced AML cell death.** U937 cells were transfected with pEGFP-C3 overexpressing BCL-2 or with an empty vector. Both were cultured on FN in the presence or absence of ARA-C and AS101 (a). % of viable cells was assessed by XTT. Data are shown as mean± SE of 3 independent experiments**p<0.005 vs ARA-C. Cells transfected as in a were cultured on FN in the presence or absence of ARA-C and AS101. Cells were tested for cell death by quantitation of subG1 accummulation (b). Data represent one representative experiment of three performed. U937 cells were transfected with Phosphothioate-modified BCL-2 antisense or mismatch control ODNs (c). Both were cultured on FN in the presence or absence of ARA-C, with or without AS101(d) *p<0.0001 vs AS101

**Fig. 6: Treatment with AS101 prevents MRD in a xenograft mouse model of AML.** 7-8 week-old male SCID mice were irradiated at 2 Gy and were then administered patient leukemic cells (1×10^7) expressing high (a, b, c) or low (d) VLA-4, intravenously. Mice were treated with AS101 (0.5mg/kg) or PBS with or without ARA-C (40mg), I.P. on days 3 and 4 or ARA-C on days 3 and 4. AS101 was administered 3 times weekly starting at day 3. At the experiment termination, PCR analysis of the human αsatelite gene and of GAPDH from different organs was assessed for assessment of leukemic mass. 10 mice/group were used in experiments a, b c and d. *p<0.05 vs PBS, ARA-C, AS101 (c). *p<0.05 vs PBS, ARA-C, AS101; **p<0.05 vs PBS, AS101 (d).

**Fig. 7: Inhibition of integrin activity in vivo by AS101 is not mediated by possible alteration of the innate immune response to the leukemia cells.** 7 week-old male NOD/SCID/GAMMA (NSG) mice were irradiated at 2 Gy and were then administered human U937 cells (5×10^6) intravenously. Mice were treated with ARA-C (20mg), I.P. on day 7 with or without AS101 (0.5mg/kg) 3 times weekly starting at day 7 until the end of experiments. 14 days after transplantation, PCR analysis of the human αsatelite
gene and of GAPDH from different organs was assessed for assessment of leukemic mass (N=4/group) (a). Human leukemic CD45+ cells were enriched from total spleen cells using the MACS magnetic cell sorter. Purified cells were used fresh for attachment assays (b). (N=5 mice/group)
Figure 1

(a) U937

FN-coated plates

Viability (% of control)

- PBS
- ARA-C 10^{-6} M
- ARA-C 10^{-5} M

(b) HL-60

FN-coated plates

Viability (% of control)

- PBS
- DRB 0.05 μg/ml

VCAM-1-coated plates

- PBS
- ARA-C 10^{-6} M
- ARA-C 10^{-5} M

BSA-coated plates

- PBS
- ARA-C 10^{-6} M
- ARA-C 10^{-5} M

Note: Symbols # and * indicate statistical significance.
Figure 7

14 Days after implantation

a

| Control  | AS101   | ARA-C   | AS101+ARA-C |
|----------|---------|---------|-------------|
| 1-BM     | 2-PBL   | 3-Spleen| 4-Liver     | 5-Kidney   |

Human $\alpha$ Satellite

GAPDH

b

![Graph showing cell attachment](image)

- PBS
- AS101

% Attachment

|        | FN  | VCAM-1 | BSA |
|--------|-----|--------|-----|
| PBS    | *   | *      |     |
| AS101  |     |        |     |
Redox modulation of adjacent thiols in VLA-4 by AS101 converts myeloid leukemia cells from a drug-resistant to drug-sensitive state

Adi Layani-Bazar, Itai Skornik, Alain Berrebi, et al.

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