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Arsenic trioxide and Ascorbic Acid Demonstrates Promising Activity against Primary Human CLL Cells in Vitro

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Abstract

The compromised antioxidant defense system in chronic lymphocytic leukemia (CLL) suggested a potential use for Reactive Oxygen Species (ROS) generating Arsenic Trioxide (ATO) and Ascorbic Acid. While both ATO and ascorbic acid mediated cytotoxicity in CLL B cells as single agents, the efficacy of ATO is enhanced by ascorbic acid. This effect is dependent on increased ROS accumulation, as pretreatment of B-CLL cells with a glutathione reducing buthionine sulfoximine or catalase inhibiting aminotriazole, enhanced ATO/ascorbic acid mediated cytotoxicity. Petreatment with reducing agents such as catalase, or thiol anti-oxidant, N-acetyl cysteine or GSH also abrogated ATO/ascorbic acid mediated cytotoxicity. Furthermore, Hu1D10 mediated cell death was enhanced with ATO and ascorbic acid, thus justifying potential

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combination of ATO/arsenic trioxide therapy with antibodies such as Hu1D10 that also cause accumulation of ROS.

Keywords

CLL; Arsenic trioxide; ascorbic acid

Introduction

B-cell chronic lymphocytic leukemia (CLL) is one of the most common adult leukemias and has a high mortality rate. In recent years monoclonal antibody therapies, like Rituximab, have shown some promise in combating this disease; however the search for a more effective therapy is still continuing. In 1984, Farber *et al* [1] showed that B-lymphocytes from CLL patients lost viability rapidly compared to T-cells when incubated with a H_2O_2 generating system. Several reports have suggested that CLL cells have a compromised antioxidant defense system evident from the low activities of the major antioxidant enzymes superoxide dismutase and catalase and the accumulation of degradation products like malonaldialdehyde and 8-oxo-deoxyguanosine [2-4]. However there were no documented pre-clinical reports attempting to exploit this phenomenon with therapeutic agents relevant to clinical investigation.

Arsenic trioxide, a potent carcinogen and environmental toxin, has the ability to induce apoptosis in several tumors/leukemias [5-10]. It is highly active in acute promyelocytic leukemia (APL). [11-16] In APL, its cytotoxic effect is thought to be mediated via its ability to generate reactive oxygen species (ROS) [17], possibly through the membrane bound NADPH oxidases [18]; and also due to its binding affinity to thiol groups which results in a decreased glutathione pool in the cells [19,20]. The cytotoxic effect of arsenic trioxide has been shown to increase with the addition of ascorbic acid which presumably aids in its redox cycling [21,22]. Based upon the susceptibility of CLL cells to oxidative stress previously demonstrated, we sought to determine the effect of arsenic trioxide with or without ascorbic acid treatment in CLL[1].

Materials and Methods

Cells

Blood was obtained from patients with B-cell CLL with informed consent under a protocol approved by the hospital internal review board. All patients examined in this series had immunophenotypically defined CLL as outlined by the modified 96 National Cancer Institute criteria (add reference Cheson BD et al). Enriched B-lymphocyte fractions were prepared by using MACS negative selection kit (Miltenyi Biotec, Auburn, CA) or by “Rosette-Sep” kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer's instructions.

Culture conditions

Cells were incubated at 1×10^6 cells/ml with 1μM arsenic trioxide (Sigma, City/State), 1mM ascorbic acid (Sigma, St. Louis, MO) and 10μg/ml Hu1D10 (Protein Design Laboratories, Fremont, CA) with 10μg/ml goat anti-human IgG, Fcγ fragment specific (Jackson Immuonotechnology, West Grove, PA) as cross linker. CLL B cells, human B-lymphocyte cell lines Raji, 697, Ramos, Wac or MC60A were cultured at 37°C in an atmosphere of 5% CO2 in RPMI 1640 media (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco). All other additions and treatments are noted in figure legends. Z-VAD-fmk, buthionine sulfoximine, aminotrizole, catalase, N-acetyl cystein and H_2O_2 (all from Sigma) were used at indicated concentrations.

Analysis of cell viability and apoptosis

Flow cytometric analysis of cell viability was carried out by dual staining with Annexin V conjugated to FITC and Propidium iodide according to the manufacturer's instructions (BD Pharmingen, San Diego, CA). Cells excluding both FITC and PI were considered to be viable.

Measurement of ROS accumulation

ROS generated was measured by flow cytometry. After incubation 10μM dihydroethidine (Molecular Probes, City/State) was added to the incubation mixtures and the cells were further incubated for 60 minutes. The non-fluorescent dihydroethidine reacts with cellular superoxide to form ethidium which binds to nucleic acids to give a fluorescent product detectable by flow cytometry.

Statistics

The statistical analysis was performed in consultation with the Center for Biostatistics, the Ohio State University. SPSS software (version 9.0, SPSS, Chicago, IL) and SAS (version 9.2, SAS Institute, Inc., Cary, NC) softwares were used for statistic analysis. For the multiple factor experiments, ANOVA and mixed linear models were used to account for correlated responses within individual patient's cells.

Results

Arsenic trioxide and ascorbic acid mediated cytotoxicity in CLL Cells In Vitro

To systematically analyze the cytotoxic effects of arsenic trioxide (ATO) and ascorbic acid in B-CLL cells, we isolated purified $CD19^+$ B cells from B-CLL patients. The demographics of the patient samples used are shown in Table 1. Cells from 10 patients were incubated with increasing concentrations of ATO for 24 and 72 hours cell viability was analyzed by flow cytometry using propidium iodide (PI) and Annexin V conjugated FITC reagents. Populations excluding both PI and Annexin V-FITC staining were considered viable. Incubation of CLL B cells with 0.5μM, 1μM and 2μM of ATO (concentrations previously reported to be physiologically achievable [24]) resulted in dose and time dependent decreased viability (16% and 49% reduction in viability with 1μM and 2μM ATO respectively at 24

hours; $p \leq 0.001$). The effect of ATO was time dependent as >80% reduction in viability was observed at 72 hour cultures (Fig. 1a).

To determine the effect of ascorbic acid on B-CLL cells, purified $CD19^+$ B cells from B-CLL patients were treated with increasing concentrations of ascorbic acid in the form of sodium ascorbic acid at final concentrations of 0.1, 0.3, 0.5, 1 and 5mM. Ascorbic acid treatment resulted in dose and time dependent decrease in viability (Fig. 1b). While 0.1, 0.3, 0.5 and 1mM ascorbic acid treatment resulted in minimal decrease in viable cells at 24 hours post treatment, 5mM induced significant reduction in viability (23±19% vs 85% viability in control groups). A more significant dose and time dependent reduction in viability was observed in these cells by 72hrs resulting in 2.4±6.3% viability. Interestingly a dramatic delayed decrease in viability was also observed with 1mM concentration of ascorbic acid (86± 12.6% at 24 hours to 10± 14.6% at 72 hours) (n=10; $p \leq 0.001$) (Fig 1b).

Enhanced cytotoxicity by combined ATO and ascorbic acid treatment in primary CLL cells

The cytotoxic effect of ATO has been shown to increase with ascorbic acid in multiple myeloma cells. In order to determine if combined treatment will result in enhanced cytotoxicity in primary CLL cells, we used combination of ATO (1uM) and ascorbic acid (1mM), at concentrations both of which were found to mediate minimal reduction in CLL B cell viability at 24 hours when tested independently (Fig 1). As shown in Fig 2a, combination of ATO and ascorbic acid resulted in enhanced cytotoxicity in a time dependent manner with viability decreasing by 40% at 24 hours over either agent alone (n=12, p=0.0009 for the interaction test of ATO and ascorbic acid). The combinatorial effect of ATO and ascorbic acid appears to be additive as evidenced by ∼20% decrease in viability with ATO or ascorbic acid independently by 24 hours and ∼44% decrease in viability in the presence of ATO and ascorbic acid together (Fig 2b). Detailed kinetics analysis revealed that the combined cytotoxic effects of ATO and ascorbic acid were dose dependent (Fig 2b). When ATO and ascorbic acid were incubated at the same ratio but at different concentrations, the viability of CLL B cells decreased with increased concentrations of ATO/ascorbic acid. Thus 96.2% ± 0.4 viability seen with 0.5μm ATO and 0.1mM ascorbic acid reduced to 44.4% ± 5.6 with 2μM ATO and 0.1mM ascorbic acid (Fig 2b).

ATO and/or Ascorbic acid induced apoptosis in CLL Cells is dependent on activation of Caspase cascade

Apoptosis in B-CLL cells is mediated through various mechanisms involving activation of caspase dependent and/or independent pathways. In order to determine if the ATO and ascorbic acid induced cytotoxicity is medidated through caspase activation we tested the effect of pan caspase inhibitor z-VAD-fmk on ATO and/or ascorbic induced apoptosis. Concentrations of z-VAD-fmk that efficiently resuced the fludarbine induced apoptosis associated with prevention of cleavage of PAPR, a downsteam target of activated caspase 3, inhbited the ATO and/or ascorbic acid induced apoptosis (Fig 3a). Consistant with the activation of caspase cascade, ATO and ascorbic acid either alone or in combination induced PARP cleavage that is prevented by z-VAD-fmk (Fig 3b). These results indicate a critical role for caspase activaiton in ATO and ascorbic acid mediated cytotoxicity of primary CLL cells.

The cytotoxicity of ATO/ascorbic acid is dependent on the accumulation of Reactive Oxygen Species (ROS)

Arsenic trioxide exerts its cytotoxic effects in part by the accumulation of reactive oxygen species (ROS) [17,19,25,26]; and ascorbic acid is also known to undergo autooxidation in the presence of transition metals. In both cases superoxide anion (O_2^-) and subsequently hydrogen peroxide (H_2O_2) is formed. For determining whether superoxide was formed, cells were treated with ATO/ascorbic acid for 2hours, washed in PBS and resuspended in PBS containing 10μM dihydroethidine and incubated for a further 60 minutes. The DHE^+ cells were analyzed by flow cytometry. As shown in Fig. 4, the percentage of cells showing DHE fluorescence increased by 10 times when both ATO and ascorbic acid were added compared to ATO or ascorbic acid alone.

Hydrogen peroxide is the major ROS formed by ATO/ascorbic acid and it is also considerably more stable compared to the superoxide anion. Since catalase is the major enzyme involved in the detoxification of H_2O_2, we hypothesized that pretreatment of CLL patient derived B cells with catalase should protect them from the cytotoxic effects of ATO/ascorbic acid. Addition of 500 units of catalase per ml reaction mixture showed minimal effect on cell viability as detected by Annexin-V^-/PI^- populations. While ATO and ascorbic acid in combination resulted in 40.5% ± 15% viability, addition of purified catalase exhibited ∼45% reduction in the cytotoxic effect mediated by ATO/ascorbic acid resulting in 74.1% ± 13.4 in ATO/ascorbic acid + catalase treated cells $p \leq 0.001$; n=6, $p \leq 0.009$ for the interaction test of catalase and ATO/ascorbic acid)) [Fig. 5a]. The catalase mediated rescue in these studies is further confirmed to be dependent on the catalase enzyme activity, as heat inactivated catalase failed to exhibit any protective effect on ATO/ascorbic acid mediated cellular cytotoxicity (40.5% ± 15.3 viability in ATO/ascorbic acid treated cells compared to 26.7% ± 10.1 in ATO/ascorbic acid and heat inactivated catalase treated cells (n=6, p=0.143;), (Fig 5a). Further, inhibition of catalase using aminotriazole, a specific inhibitor of catalase, when its substrate H_2O_2 is present, provides further evidence of the protective effect of catalase and also shows the involvement of H_2O_2 as a result of ATO/ascorbic acid treatment. Thus, pre-treatment of CLL cells for 30 minutes with 100mM aminotriazole prior to adding ATO/ascorbic acid increased the cytotoxic effect of arsenic trioxide and ascorbic acid (Fig. 5b). Interestingly, aminotriazole alone also was cytotoxic towards CLL cells. (Fig. 5b).

Role of GSH on the cytotoxicity of arsenic/ascorbic acid mediated cytotoxicity

Reduced glutathione (GSH) is the major antioxidant present in cells. Its concentration can reach up to millimolar levels in healthy cells and it plays a major role in the detoxification of xenobiotics. Arsenic or ascorbic acid is known to bind thiol groups and reduce glutathione levels in the cells [27,28]. We hypothesized that GSH and also N-acetyl cysteine (NAC), a known thiol antioxidant, would have a protective effect against ATO/ascorbic acid induced cytotoxicity either by scavenging ROS or by directly binding ATO. The protective effect of NAC can also be due to its ability to increase the levels of cellular thiols. Treating primary CLL cells with 1mM NAC or GSH effectively abrogated the ATO/ascorbic acid mediated cytotoxicity, by increasing the viability from 41 ± 13.7% (in ATO/ascorbic acid treated cells) to 78 ± 9.4 % with 1mM NAC and 80 ± 8.5% with 1mM GSH (Fig 6a). To determine

if depletion of thiol levels would enhance the cytotoxicity of ATO/ascorbic acid, cells were grown in the presence of 200μM buthionine sulfoximine, which inhibits a key enzyme in glutathione biosynthesis and thus reducing the cellular free thiol pool. As shown in Fig 6b, depletion of GSH by buthionine sulfoximine treatment decreased the viability due to ATO/ascorbic acid by 40% ($p \leq 0.05$). Buthionine sulfoximine by itself failed to show any significant toxicity compared to the ATO/ascorbic acid control.

Hu1D10 induced cytotoxicity is enhanced by ATO/ascorbic acid in CLL B cells

Hu1D10 (apolizumab), a humanized HLA-DR beta-chain-specific antibody directed to the 1D10 antigen, has been shown to be cytotoxic towards primary B-cell chronic lymphocytic leukemia (CLL) cells and is currently in clinical trials for this disease. We previously reported that *in vitro* Hu1D10 treatment of CLL cells resulted in accumulation of reactive oxygen species (ROS)[23]. We hypothesized that CLL cells would be susceptible to damage by ROS generating agents, and that ROS generating agents would enhance the Hu1D10 mediated cytotoxicity. To test this hypothesis we investigated the effect of ATO/ascorbic acid on Hu1D10 induced cytotoxicity in B-CLL cells. Primary $CD19^+$ B cells isolated from CLL patients were treated with Hu1D10 ± ATO/ascorbic acid for 48 hours (Fig 7a.) While Hu1D10 or ATO/ascorbic acid resulted in comparable levels of cytotoxicity, the combination of HuID10 and ATO/ascorbic acid resulted in increased cytotoxicity, compared to individual agents [% viability in Hu1D10=61.9±19.5%; ATO/ascorbic acid= 64.8±21.5%; ATO/ascorbic acid +Hu1D10=38±25.7%). ($n=1 p < 0.0001$) for the interaction test of Hu1D10 and ATO/ascorbic acid). This is further confirmed by detailed dose kinetic analysis of ATO/ascorbic acid dependent enhancement in Hu1D10 cytotoxicity (Fig 7b). Hu1D10 induced dose dependent cytotoxicity at 0.1, 1 and 10μg/ml concentrations. The Hu1D10 induced cytotoxicity at each of these concentrations was enhanced by increasing concentrations (0.25, 0.5 and 1mM) ATO/ascorbic acid (Fig. 7b). Thus, the viability decreased with increasing concentrations of ATO/ascorbic acid and Hu1D10; at the highest concentrations of ATO(1μm)/ascorbic acid (1mM), the viability decreased from 44.4% ± 5.6 in the absence of Hu1D10 to 5.7% ± 0.7 in the presence of 10μg/ml of Hu1D10. Similar dose dependent enhancement by ATO/ascorbic acid was also observed at 0.1 and 1μg/ml of Hu1D10 (44.4± 5.6 in the absence of Hu1D10 reduced to 35.7 ± 9.5% and 5.7±0.7% at 0.1μg/ml and 1ug./ml of Hu1D10 respectively. (Fig 7b).

Discussion

In the present work we have shown that the susceptibility of CLL B-lymphocytes to ROS can be exploited with arsenic trioxide, a therapeutic agent currently approved for clinical use in acute promyelocytic leukemia. Furthermore, we have demonstrated that CLL is similar to multiple myeloma where the cytotoxic effect of arsenic trioxide is greatly enhanced by the addition of ascorbic acid. Diverse forms of ROS (O_2^-, OH, H_2O_2, $^1O_2$, etc.) can be formed due to ATO/ascorbic acid. We have demonstrated that O_2^- is formed when these agents are used in combination. Most ROS have a very short biological half-life but among these H_2O_2 is comparatively long-lived and has the potential to do the most damage. Catalase, the major enzyme which scavenges H_2O_2, is a major component of the cellular antioxidant defense system. The susceptibility of CLL cells to H_2O_2 has been shown previously [1] and also that

these cells have a compromised catalase activity [2,3]. The addition of exogenous catalase helped CLL cells to survive *in vitro* [4]. The importance of catalase is further borne out by our observation that aminotriazole, which inhibits catalase in the presence of H_2O_2 by itself is also cytotoxic to CLL cells. It is clear that endogenous H_2O_2 is formed at least when CLL cells are cultured *in vitro* and it contributes to and may be even accelerates their eventual mortality.

The reduced form of γ-glutamyl cysteinyl glycine (glutathione, GSH) is the major antioxidant in the cell and was able to protect against ATO/ascorbic acid cytotoxicity. The levels of GSH in CLL cells are comparable to healthy cells and sometimes even greater [2,29-31]. This could be in part due to the cell's attempt to compensate for its compromised antioxidant enzyme activity. ATO or ascorbic acid is known to deplete GSH levels in the cells and thus increase its susceptibility to various toxicants. Indeed, depletion of GSH levels by BSO, which inhibits γ-glutamyl cysteinyl synthetase, a key enzyme in GSH biosynthesis, increased the cytotoxicity mediated by ATO/ascorbic acid. N-acetyl cysteine, a thiol antioxidant in wide use also effectively reduced the cytotoxicity due to ATO/ascorbic acid. Therefore ATO and ascorbic acid is able to serve a dual purpose, on one hand they lower the cellular thiol pool and thus make them more susceptible to ROS, and on the other hand they produce ROS which can exert their cytotoxicity without interference from GSH. Karasavvas and collaborators have demonstrated that a reduced form of ascorbic acid protects against ATO induced toxicity[36]. While the present study shows the opposite effect, it should be pointed out that we have used the oxidized form of ascorbic acid. The differences of both forms of Vitamin C toward the amplification or blockage of ATO redox cycle remains to be evaluated as this may justify the use of oxidized instead of reduced form.

CLL cells by virtue of their compromised antioxidant defense system would be more susceptible than healthy cells. However it is likely that the indiscriminate nature of ROS will make healthy cells potential target for these agents albeit to a lesser extent. Monoclonal antibody therapy is attractive because of its specificity and is increasingly used in various cancers [32]. In CLL the anti CD20 antibody, rituximab, has shown remarkable success [33,34]. Another monoclonal antibody, the humanized 1D10 (Hu1D10; apolizumab) is among the antibodies that are currently under evaluation in phase II clinical trials in CLL patients. The 1D10 antibody is directed against a polymorphic epitope on the beta-chain of human leucocyte antigen (HLA) class II [35]. Our laboratory had previously demonstrated the efficacy of Hu1D10 against CLL [23], and there were indications that ROS might be involved in this cytotoxicity. However N-acetyl cysteine failed to abrogate the cytotoxicity of Hu1D10. We have shown that in both cell lines expressing the 1D10 antigen and also in primary CLL cells Hu1D10 by itself shows considerable cytotoxicity which is enhanced by the addition of ATO/ascorbic acid, indicating a promise for combining a targeted therapy such as Hu1D10 with a non-targeted therapeutic approach such as ATO and ascorbic acid or another ROS generating agent. The cytotoxic pathways followed by these agents are recently beginning to be unraveled and it appears that there are many diverse pathways including, caspase dependant or independent, apoptotic or necrotic pathways, which ultimately lead to the demise of the cancer cells. B-CLL cells are dependent on cell to cell

communication events (follicular dendritic cells or other stromal cells) to fully "activate" their anti-apoptotic machinery. The effect of ATO/Ascorbic acid on B-CLL cells in the context of stromal cells remains to be tested. Recently, the ATO has been shown to induce apoptosis preferentially in B-CLL cells of patients with unfavorable prognostic factors including del17p13. [37] Given the defects in specific cell death pathway in CLL contributing to resistance to spontaneous and treatment induced apoptosis, combinatorial therapeutic approaches aimed at both targeted and a non-targeted cell death induction strategies are very promising.

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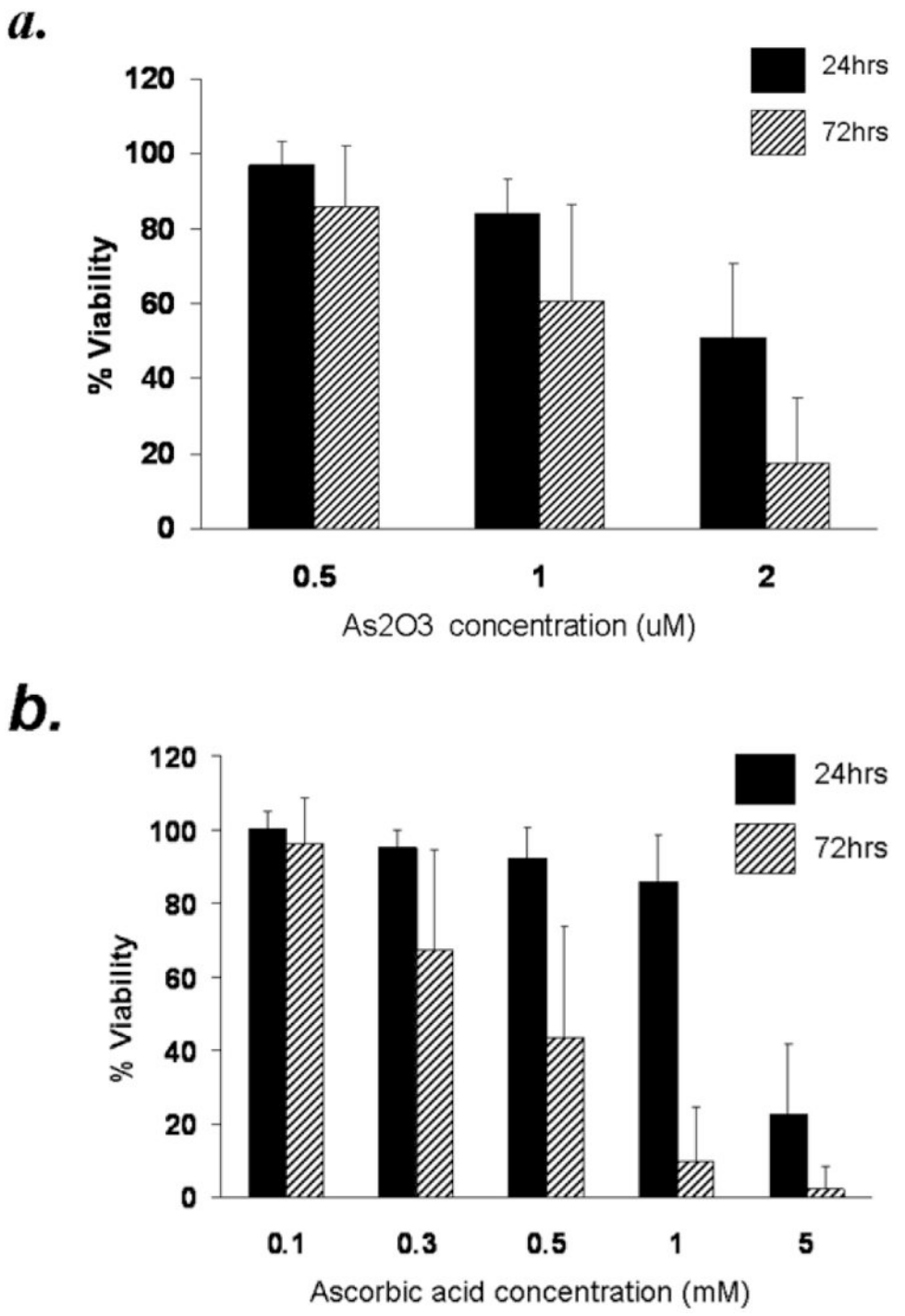

Figure 1. Arsenic trioxide or ascorbic acid mediated cytotoxicity in B cells from B-CLL patients-Dose and time kinetic analysis

Purified B-lymphocytes (1×10^6cells/ml) from B-CLL patients were incubated in the presence of indicated concentrations of arsenic trioxide (Panel A) or ascorbic acid (Panel B) for indicated time periods. The cells were stained with Annexin-V-FITC and propidium iodide as described by us previously[23]. The cells were analyzed by flow cytometry and data

collected under list mode. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. (n=10)

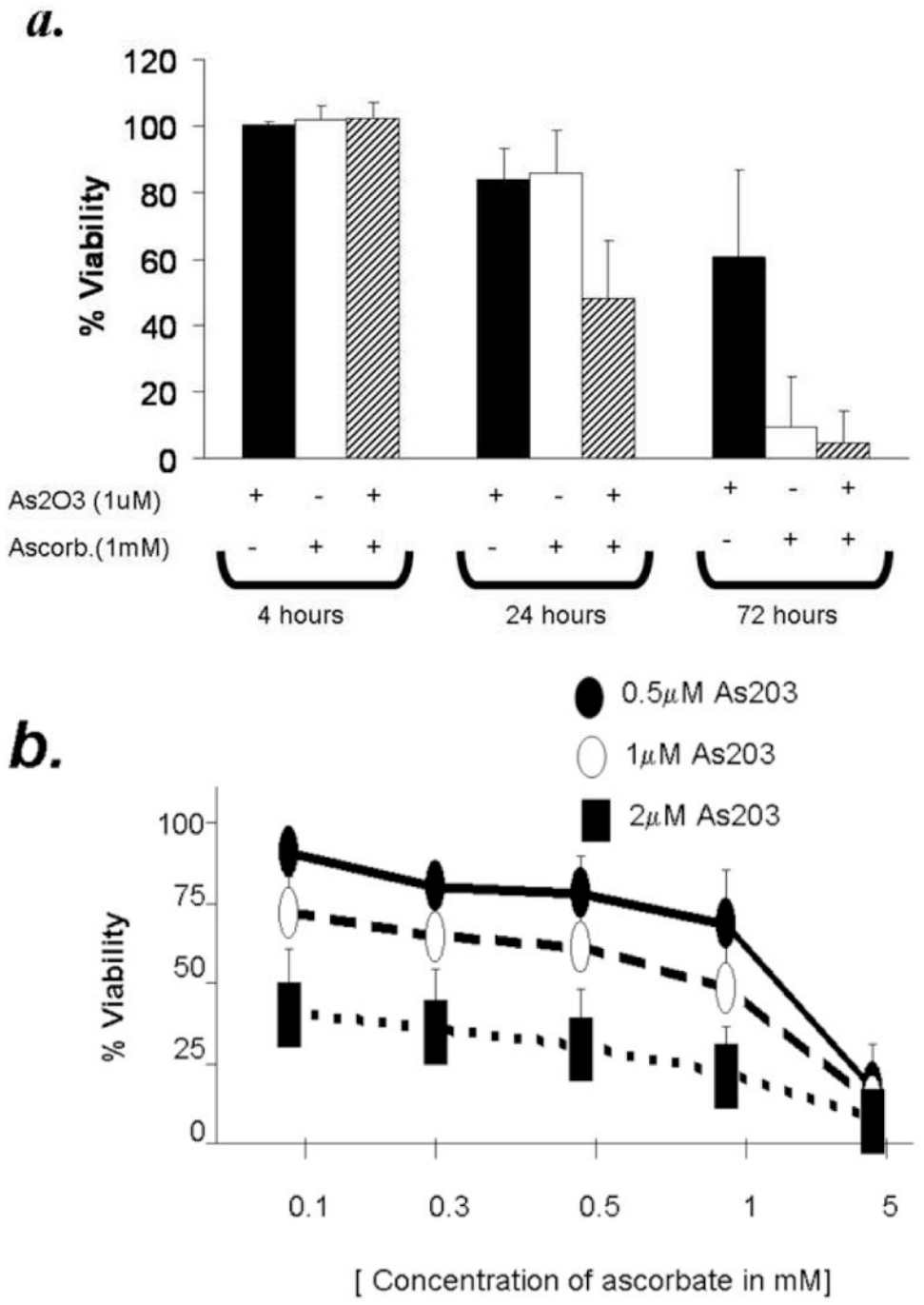

Figure 2. Combination of Arsenic trioxide and ascorbic acid enhance cytotoxicity in B cells from B-CLL patients

Panel a: Purified B-lymphocytes from CLL patients (1×10^6/ml media) were incubated with either arsenic trioxide [ATO] (1μM), ascorbic acid (1mM), or with both agents together for the indicated time periods. The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry and data collected under list mode. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. (n=12)

Panel b: Purified B-lymphocytes from CLL patients (1×10^6/ml media) were incubated with 0.5, 1 and 2μM arsenic trioxide [ATO] in conjunction with indicated concentrations of ascorbic acid for 24 hours. The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry and data collected under list mode. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. (n=12; $p<0.001$).

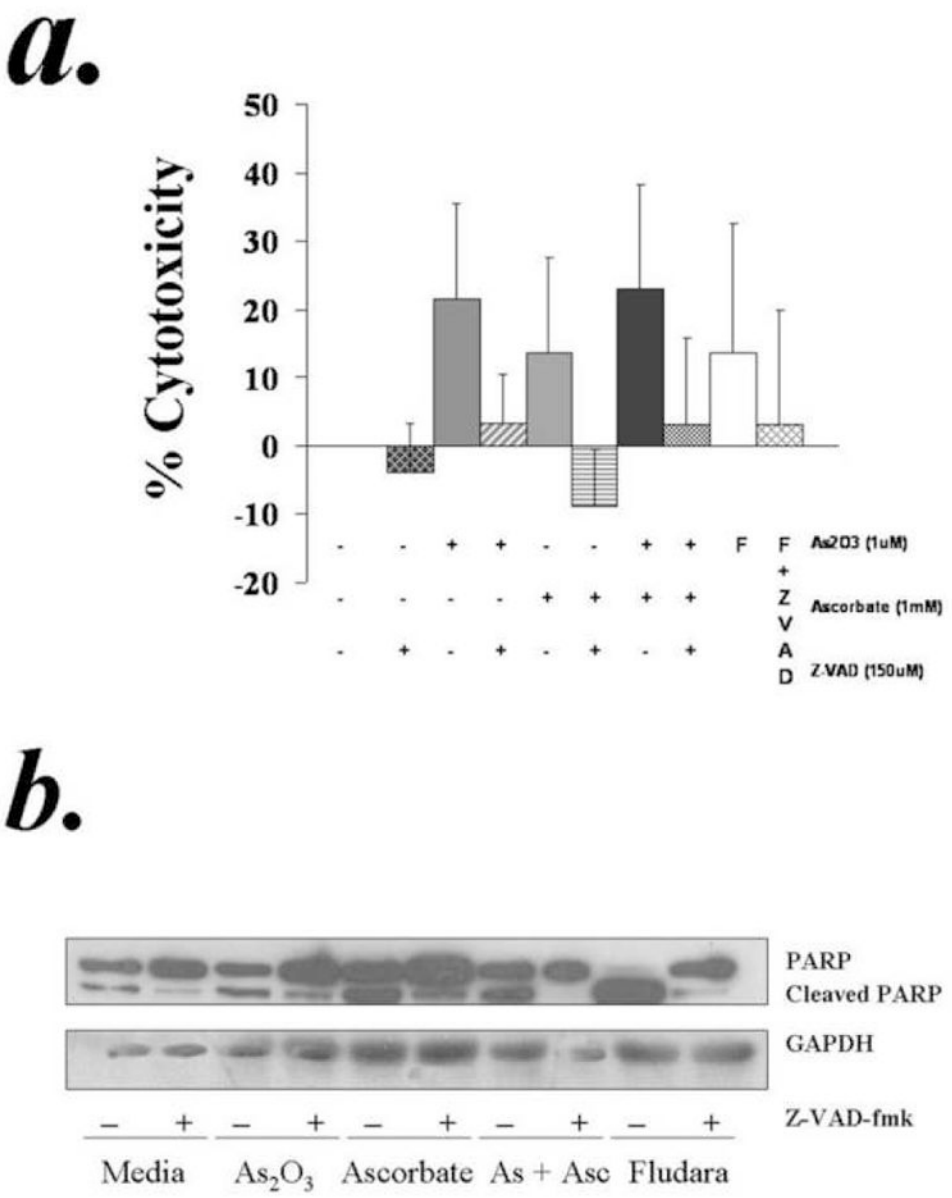

Figure 3. Arsenic trioxide and ascorbic acid induced cytotoxicity in CLL cells is dependent on activation of caspase

Purified B-lymphocytes from CLL patients (1×10^6/ml media) were incubated with 1μM arsenic and 1mM ascorbic acid either alone or in combination in the presence or absence of z-VAD-fmk (150uM) for 24 hours. The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry for apoptotic cells (panel a). The data shown represent % Annexin-V^+/PI^+ cells ± SD.(n=3). Panel B shows Westernblot analysis of the lysates from one of the above experiments was assessed for incleaved and cleaved PARP. Fludarabin is used as a control in these studies.

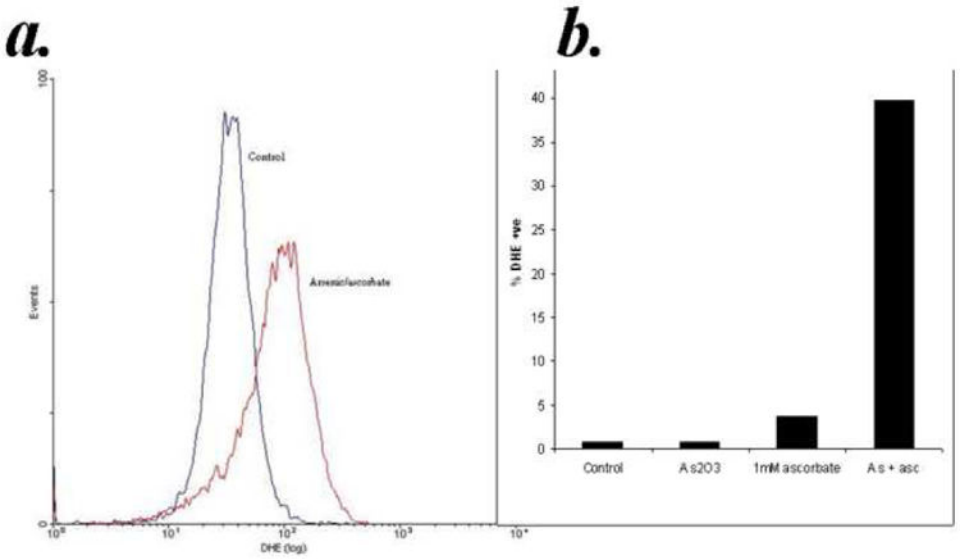

Figure 4. Accumulation of reactive oxygen species by arsenic trioxide and ascorbic acid

Purified B-lymphocytes from CLL patients (1×10^6/ml media) (1×10^6/ml) were treated with 1μM arsenic and 1mM ascorbic acid either alone or in combination. Subsequently DHE was added to 10μM final concentration and the cells were incubated for an additional 60 minutes. The cells were analyzed by flow cytometry and data collected under list mode. A representative histogram showing the vehicle control and arsenic trioxide [ATO]/ascorbic acid treated group is shown in the top panel. The bottom panel shows %DHE positive cells in media control, ATO (0.1uM), ascorbic acid (1mM) and ATO/ascorbic acid treated groups.

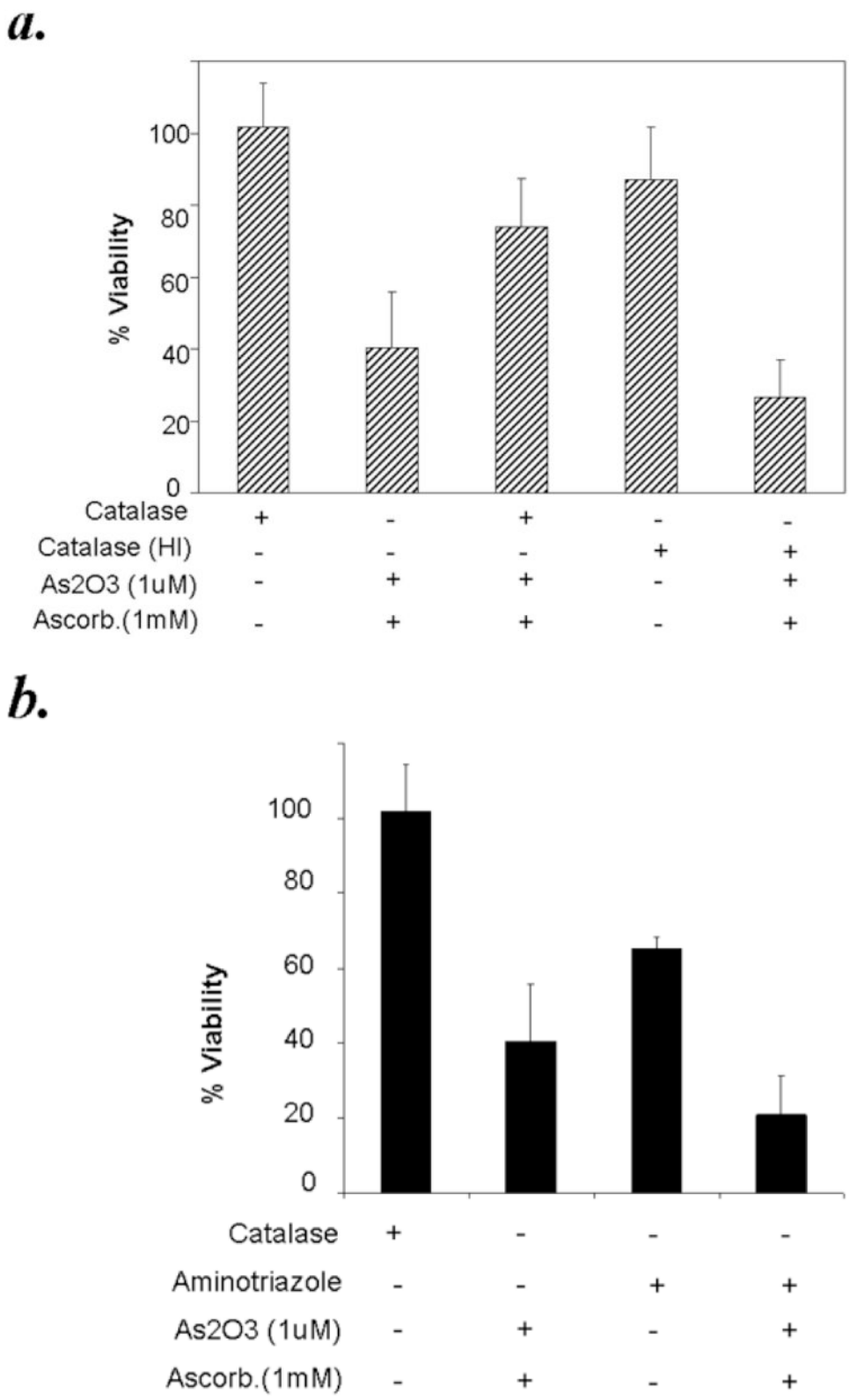

Figure 5.

Panel a: Active but not heat inactivated catalase, protects against As_2O_3 and ascorbic acid mediated cytotoxicity in primary CLL B cells. Purified B-lymphocytes from CLL patients (1×10^6/ml media) were pretreated with 500 units of active catalase or heat inactivated catalase (HI) for 30 minutes prior to addition of arsenic trioxide [ATO] (1μM) and ascorbic acid (1mM). The cells were stained with Annexin-V-FITC and propidium iodide and the cells were analyzed by flow cytometry after 24 hours. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. (n=6; $p<0.05$).

Panel b: Catalase inhibitor aminotriazole enhances the cytotoxicity of As_2O_3 and ascorbic acid towards primary CLL B cells. Purified B-lymphocytes from CLL patients (1×10^6/ml media) were pretreated with 100mM aminotriazole for 30 minutes prior to addition of arsenic trioxide [ATO] (1μM) and ascorbic acid (1mM). The cells were stained with Annexin-V-FITC and propidium iodide and the cells were analyzed by flow cytometry after 24 hours. The data shown represent % Annexin-V^-/PI^-viable cells ± SD that are normalized to media control. (n=6; $p<0.05$).

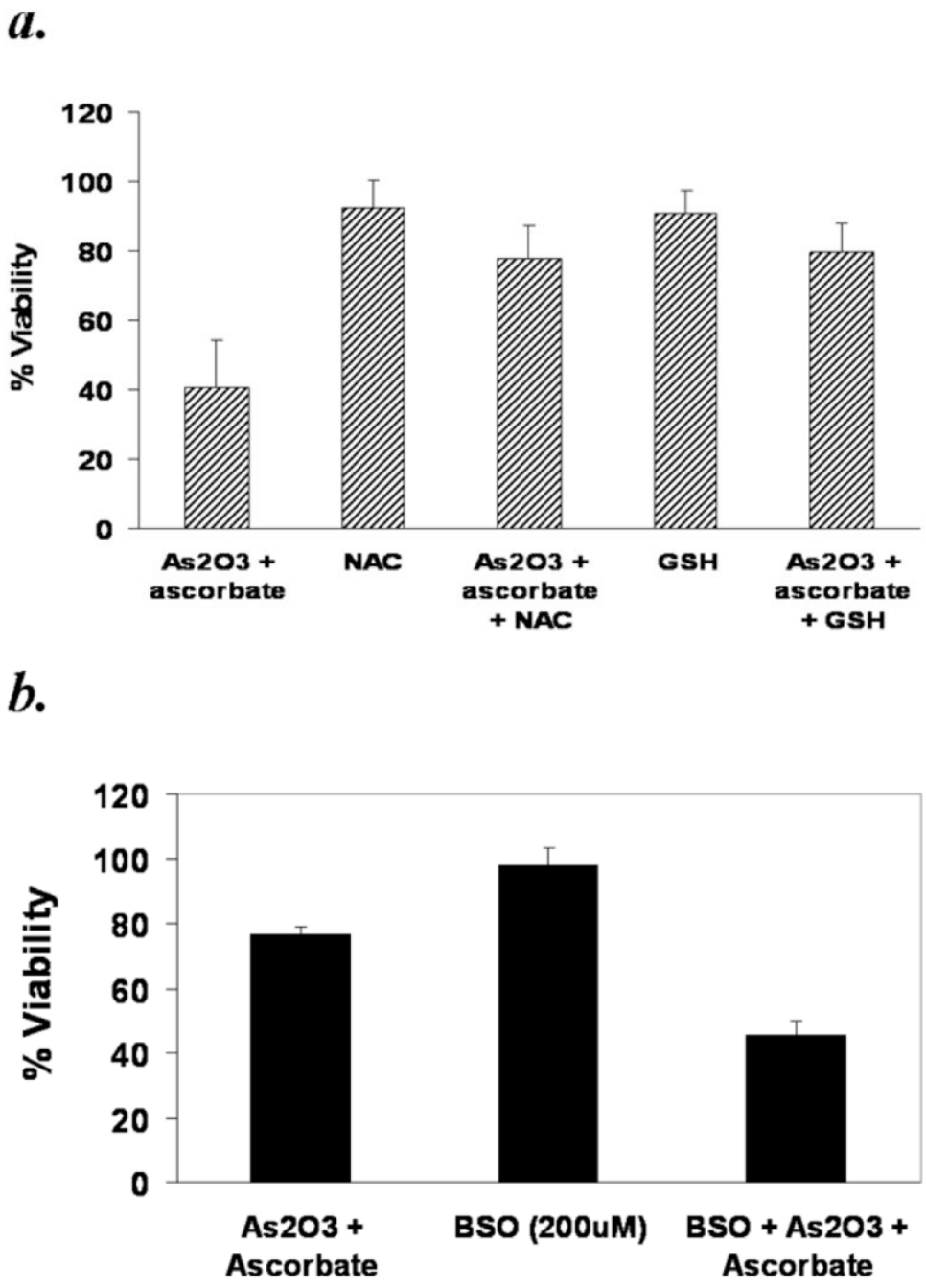

Figure 6.

Panel a: N-acetyl cysteine (NAC) or Glutathione (GSH) mediated abrogation arsenic trioxide/ascorbic acid induced cytotoxicity in primary CLL B cells. Purified B-lymphocytes from CLL patients (1×10^6/ml media) were pretreated with Glutathione [GSH (1mM)] or N-acetyl cysteine (NAC) for 30 minutes prior to addition of ATO (1μM) and ascorbic acid (1mM). The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry after 24 hours. The data shown represent % Annexin-V^-/PI^- viable cells + SD that are normalized to media control. (n=6; $p<0.05$).

Panel b: Depletion of GSH by buthionine sulfoximine (BSO) enhances the As_2O_3 and ascorbic acid mediated cytotoxicity. Purified CLL B cells (1×10^6/ml) were grown in the presence of 200μM BSO for 24 hours to deplete GSH levels. The cells were analyzed by flow-cytometry 24 hours after addition of ATO/ascorbic acid as described above.

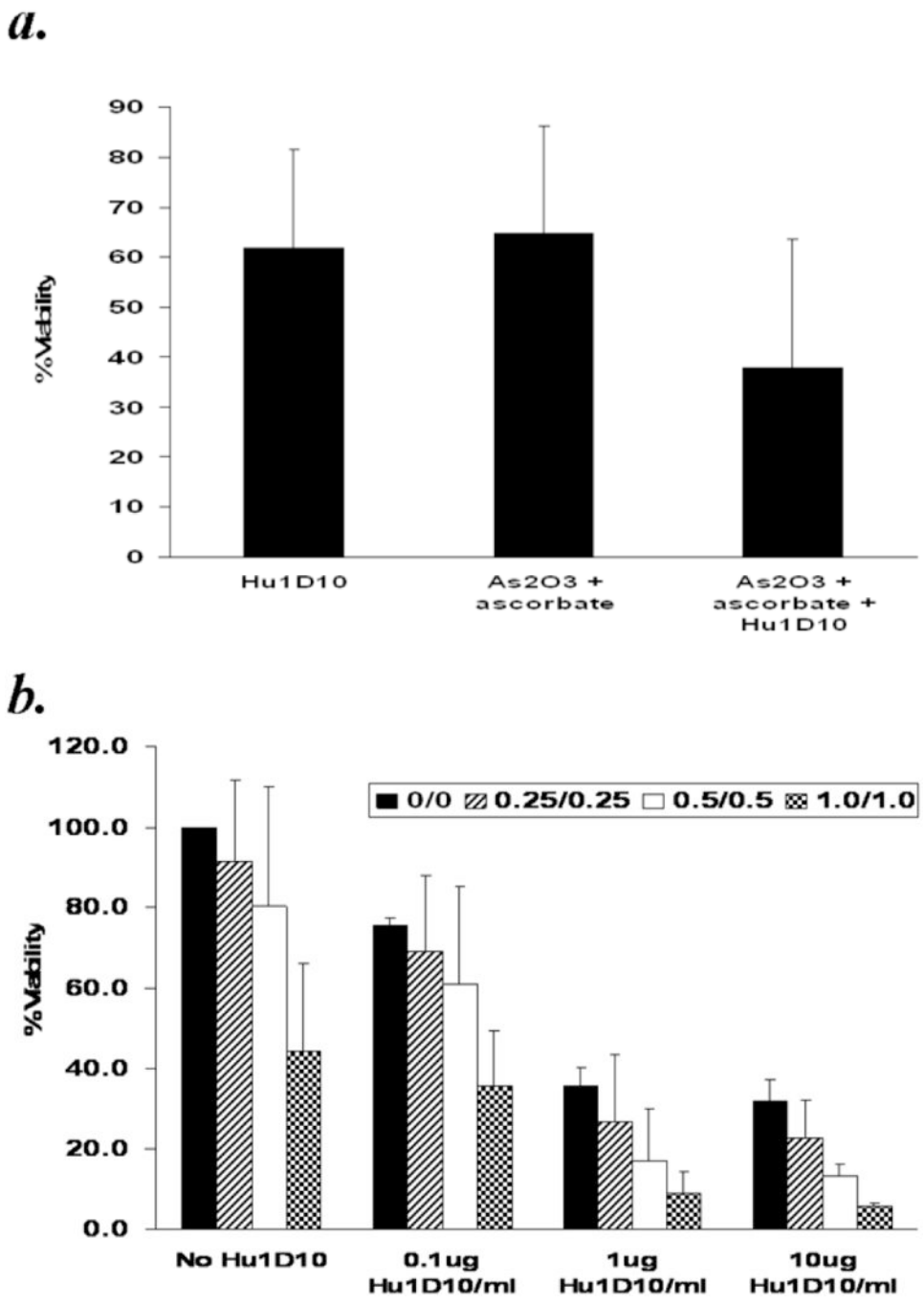

Figure 7.

Panel a: Arsenic trioxide and ascorbic acid enhance Hu1D10 mediated cytotoxicity of primary CLL B cells. Purified B-lymphocytes from CLL patients (1×10^6/ml media) were treated with Hu1D10 (10ug/ml), arsenic trioxide [ATO](1μM)/ascorbic acid (1mM) or Hu1D10 and ATO/ascorbic acid. The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry as described above. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. (n=11).

Panel b: Arsenic trioxide and ascorbic acid enhance the cytotoxicity of Hu1D10 in a dose dependent manner. Purified B-lymphocytes from CLL patients (1×10^6/ml media) were treated with indicated concentrations of Hu1D10, arsenic trioxide [ATO] and ascorbic acid. The cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry as described above. The data shown represent % Annexin-V^-/PI^- viable cells ± SD that are normalized to media control. Varying arsenic trioxide/ascorbic acid and Hu1D10 concentrations show that even if Hu1D10 concentration is lowered 10 fold, the cytotoxicity in conjunction with ATO/ascorbic acid is significantly enhanced.

Table 1

Description of Rai stage, IgVH mutational status and Zap70 status of patient samples

Patient #	Rai stage	Ig VH status (%)	Zap70 (%+ve)
1	1	0	11.3
2	2	0.4	7.5
3	0	N.D	N.D
4	2	0.4	32.9
5	0	6.3	55.9
6	2	0.7	90.7
7	0	N.D	N.D
8	0	0	79.2
9	0	0	N.D
10	2	N.D	28.5
11	2	4.8	14.7
12	1	0	32.4
13	0	7	21
14	2	0.4	0.9
15	1	2.1	N.D
16	2	0	26.7
17	1	2.8	63.2
18	3	0	79.2