Auranofin induces lethal oxidative and endoplasmic reticulum stress and exerts potent preclinical activity against chronic lymphocytic leukemia

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Running Title: Auranofin induces oxidative/ER stress in CLL cells

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**Precis:** This study provides a rationale to immediately reposition the approved drug Auranofin for clinical evaluation in the therapy of relapsed forms of chronic lymphocytic leukemia.

**Abstract:** Chronic lymphocytic leukemia (CLL) exhibits high remission rates after initial chemo-immunotherapy, but relapses to treatment-refractory disease is the most common outcome, especially in CLL with the deletion of chromosome 11q or 17p. In addressing the need of treatments for relapsed disease, we report the identification of an existing FDA-approved small molecule drug to repurpose for CLL treatment. Auranofin (AF) (Ridaura®) is approved for use in treating rheumatoid arthritis but it exhibited preclinical efficacy in CLL cells. By inhibiting thioredoxin reductase activity and increasing intracellular ROS levels, AF induced a lethal endoplasmic reticulum stress response in cultured and primary CLL cells. Additionally, AF displayed synergistic lethality with heme oxygenase-1 and glutamate-cysteine ligase inhibitors against CLL cells. AF overcame apoptosis resistance mediated by protective stromal cells, and it also killed primary CLL cells with deletion of chromosome 11q or 17p. In TCL-1 transgenic mice, an in vivo model of CLL, AF treatment markedly reduced tumor cell burden and improved mouse survival. Our results provide a rationale to reposition the approved drug Auranofin for clinical evaluation in the therapy of CLL.
**Introduction:**

Accelerated expansion of CLL cells with bulky lymphadenopathy and organomegaly, with or without compromised hematopoiesis, is treated with myelotoxic chemo-immunotherapy (1,2). In CLL, the un-mutated immunoglobulin heavy chain variable region genes (IGHV), acquired chromosomal abnormalities including deletion 17p13 and deletion 11q22, increased expression of ZAP70 (zeta-associated protein) or of CD38 are features associated with poor outcome (3). Notwithstanding high remission rates due to initial chemo-immunotherapy, eventual relapse with treatment-refractory disease is the typical outcome, except in a minority of patients who successfully receive allogeneic stem cell transplantation (2,3). Therefore, novel effective and safe treatments need to be tested and developed. To this end, repurposing of an existing and FDA-approved small molecule drug in the treatment of CLL is a worthy goal (4).

Compared to normal lymphocytes, CLL cells have intrinsically higher levels of reactive oxygen species (ROS) and are under oxidative stress due to an imbalanced redox status (5-8). ROS-mediated oxidation of the sulfur-containing amino acids in proteins such as phosphatases and transcription factors, e.g., NFκB, p53, HIF1α and nuclear factor erythroid 2-related factor 2 (Nrf2), regulates their function and role in modifying cellular growth and survival (9). Elevated ROS levels also render CLL cells more sensitive to agents that further increase ROS and oxidative stress (6). Nrf2 activates genes involved in the response to oxidative stress, including heme oxygenase-1 (HMOX-1) and glutamate cysteine ligase modifier (GCLM), which is involved in glutathione (GSH) synthesis (10,11). Elevated levels of ROS may overcome antioxidant mechanisms and induce protein oxidation, which leads to intracellular accumulation of potentially toxic, misfolded and polyubiquitylated (poly-Ub) proteins (12). This accumulation triggers an HDAC6-mediated, adaptive and protective heat shock and proteotoxic stress response (13,14). During this, HDAC6 binds to the poly-Ub-misfolded proteins and shuttles these into a protective aggresome, concomitantly causing the dissipation of the p97/HDAC6/hsp90/HSF1 (heat shock factor 1) complex, followed by induction of transcriptional activity of HSF1 and heat shock proteins (15,16). The dissociation of HDAC6 from this complex also causes hyperacetylation and inhibition of the chaperone function of hsp90 (17), with resulting depletion of CLL-relevant, pro-growth and pro-survival hsp90 client proteins such as ZAP70, c-RAF, AKT, as well as of HDAC6 itself (18-21). Thus, ROS-induced oxidative stress can lead to
proteotoxic and unfolded protein response (UPR), which in turn also triggers ER stress, with activation of the mediators of the ER stress response (22-24). Normally, ER stress is designed to be protective by mediating the shutdown of general protein synthesis and by increasing the production of molecular chaperones, including the ER resident hsp70 homologue, glucose regulated protein 78 (GRP78) (22,23). However, if ER stress is protracted, lethal ER stress ensues through prolonged activation of the pro-death ER stress pathways mediated by CHOP (CAAT/enhancer-binding protein homologous protein) and IRE1 (Inositol requiring protein 1) (23-25). Countering this, CLL cells receive numerous pro-survival signals from the stroma microenvironment in the bone marrow and lymph nodes through multiple mechanisms that activate B-Cell receptor (BCR) and the chemokine receptor CXCR4 signaling (26-29). Recently, stromal cells were also shown to protect CLL cells against increased intracellular levels of ROS, by providing cysteine and bolstering the intracellular levels of GSH in CLL cells (30).

Auranofin (AF), an oral gold-containing triethylphosphine used in the treatment of rheumatoid arthritis (RA), has been previously reported to inhibit cytosolic and mitochondrial thioredoxin reductase (TrxR) and induce ROS levels (31). Based on the preliminary results of an in vitro high-throughput screen (HTS) to gauge the activity against primary CLL cells, and toward the ultimate goal of repurposing AF for the treatment of CLL, we determined the in vitro and in vivo activity of AF, and its mechanism of action, against CLL cells. Our findings demonstrate for the first time that, AF induces lethal oxidative, proteotoxic and ER stress response in cultured and patient-derived primary CLL cells, including those with the biologic and genetic features that are associated with poor clinical outcome in CLL.

**Methods**

**Reagents and antibodies.** Auranofin (AF) \((C_{20}H_{35}AuO_9PS)\) was suspended in DMSO at 5 mM concentration and stored in volumes of 500 μL at −80°C, then aliquoted in volumes of 10 μL and stored at −20°C to be used once without refreezing. Additional reagents and detailed antibody information is provided in the Supplemental Methods. Polyclonal acetylated K69 hsp90 antibody was generated by Alpha Diagnostics as previously described (17).
Cell lines and primary CLL cells. Human chronic B-cell leukemia, MEC-1 cells were obtained from the DSMZ (Braunschweig, Germany). All experiments with cell lines were conducted within 6 months after thawing or obtaining from DSMZ. Cell line authentication was done by DSMZ. The DSMZ uses short tandem repeat (STR) profiling for characterization and authentication of cell lines. Cells were cultured in IMDM with 10% heat-inactivated FBS, and 1% penicillin/streptomycin. Cells were passaged 2-3 times per week and frozen in aliquots in liquid nitrogen. HK cells were kindly provided and characterized by Jianguo Tao (Moffitt Cancer Center, Tampa, FL), cultured in RPMI with 10% FBS and maintained as previously described (32). Exponentially growing cells were used for all described experiments. Primary CLL cells (Supplemental Table 1) were obtained with informed consent (in accordance with the Declaration of Helsinki) under a research protocol approved by the Institutional Review Board of Kansas University Medical Center (Protocol #12392) or the National Institutes of Health (7,30). Primary CLL cells were isolated from the peripheral blood and CD19+ B cells were purified, utilizing a positive selection immunomagnetic separation kit (Stem Cell Technologies). Normal human CD34+ cells were obtained from de-linked, de-identified human cord blood samples.

Assessment of mitochondrial transmembrane potential (ΔΨm) and reactive oxygen species (ROS). CLL cells (0.5 x 10^6 cells/tube) were incubated with 10 nM 3,3-dihexyloxacarbocyanine iodide (DiOC6), 2.5 µM dihydroethidium (DHE), and 10 µL of CD19-APC, in 400 µL AIM-V in a humidified atmosphere of 5% CO2 at 37°C for 30 minutes, then analyzed by flow cytometry.

Thioredoxin reductase assay. CLL cells were treated with AF for 8 hours. The effects of AF treatment on thioredoxin reductase activity were determined with a Thioredoxin Reductase Assay Kit (Cayman Chemicals) according to the manufacturer’s protocol.

Evaluation of cell viability. Primary CLL PBMCs (0.5 x 10^6 cells per well) were plated in triplicate in a final volume of 100 µL, in the presence of serial dilutions of AF (0.125-4.0 µM) for 24 hours. Cell viability was determined using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, WI). The % of viable cells following AF treatment is reported relative to the untreated control CLL cells.
Viability analysis in NLC co-cultures. Primary CLL cells were co-cultured in the presence or absence of Nurse-Like Cells (NLC) (33), with or without AF, for 24 hours. Cell viability was determined by flow cytometry using DiOC6 and CD19 stain.

Assessment of apoptosis. Untreated or drug-treated MEC-1 or primary CD19+ CLL cells were stained with Annexin-V, and the % of annexin V-positive, apoptotic cells were determined by flow cytometry, as previously described (25). To analyze synergism between auranofin (AF) and ZnBG or BSO, cells were treated with AF (200-1000 nM) and ZnBG (10-20 µM), or BSO (1-10 µM) for 48 hours. The combination index (CI) for each drug combination was calculated utilizing CalcuSyn (Biosoft, Ferguson, MO). CI values of less than 1.0 indicate a synergistic interaction of the two agents.

Confocal microscopy. Primary CD19+ CLL cells were incubated with indicated doses of AF for 16 hours and cytospun onto glass slides. Cells were fixed and permeabilized as previously described (25). Then, cells were stained with Nrf-2 and HSF-1 antibodies, counterstained with phalloidin (which binds F-actin) and DAPI and coverslips were mounted. The images were visualized using a Carl Zeiss LSM-5 PASCAL confocal microscope with a 63×/1.2 NA oil objective. Images were processed with LSM Image browser software (Zeiss).

RNA isolation and qPCR analyses. Total RNA was extracted from untreated and AF treated MEC-1 or primary CD19+ CLL cells using an RNAqueous 4PCR kit from Ambion (Valencia, CA) and reverse transcribed. Quantitative real-time PCR analysis and TaqMan probes were used to determine the expression levels of GRP78, CHOP, HMOX-1, and GCLM. The relative expression of each mRNA was normalized to GAPDH.

Gene expression analysis. Total RNA from primary CLL PBMCs treated with AF for 4, 8, or 10 hours or left untreated for 10 hours was purified using the RNeasy Mini Kit (Qiagen, Louisville, KY), and converted to biotin-labeled cRNA. This was hybridized to a Human Genome U133 Plus 2.0 array (Affymetrix). Signal intensity and fold changes in gene expression were analyzed by the microarray analysis, using Affymetrix GeneChip Operating Software, as detailed in the Supplemental Methods. All microarray data used in this manuscript are deposited in the Gene Expression Omnibus.
**Short hairpin RNA.** Lentiviral short hairpin RNAs targeting HMOX1 and GADD153/CHOP or non-targeting shRNA (sh-NT) were transduced into MEC-1 cells. Forty-eight hours post transduction, the cells were washed with complete media and plated with or without AF for 8 hours for immunoblot analysis or 48 hours for assessing apoptosis. MEC-1 cells with stable knockdown of CHOP were obtained by culturing the transduced cells in 0.5-1.0 µg/mL of puromycin. Stable knockdown cells were treated for 48 hours with AF to determine the effects of CHOP knockdown on AF-induced apoptosis.

**Immunoprecipitation of hsp90 from CLL cells.** Primary CD19+ CLL cells were treated with AF for 8 hours. Following treatment, cells were harvested, lysed and hsp90 was immunoprecipitated from total cell lysates as previously described (25). HDAC6, Hsf-1 and p97/VCP immunoblots were performed on the hsp90 immunoprecipitates. Blots were stripped and re-probed for detection of hsp90.

**Immunoblot analyses.** Immunoblot analyses were conducted as described in the Supplemental Methods.

**In vivo animal studies.** All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at University of Texas M.D. Anderson Cancer Center. TCL-1 transgenic mice genotype: (Tcl1-tg:p53-/-) have been previously described (34,35). TCL-1 mice (n=5) (age 2 months) were treated with auranofin by i.p. injection at the dosage of 10 mg/kg daily, 5 days per week (Monday-Friday) for 2 weeks and treatment was stopped. The total leukemia cell burden was measured in peritoneal fluid from pre-treated and AF-treated animals, as previously described (30). Survival of TCL-1 mice treated with AF compared to untreated mice is represented by a Kaplan-Meier plot.

**Statistical analysis.** Significant differences between values obtained in a population of MEC-1 or primary CD19+ CLL cells treated with different experimental conditions were determined using a two-tailed, paired t-test or a one way ANOVA analysis within Microsoft Excel 2010 software or using GraphPad Prism 5.0 (GraphPad Software, Inc., CA). P-values < 0.05 were assigned significance. For the in vivo studies, differences in the mean leukemia burden between pretreatment and post-AF treatment were determined by a one-way ANOVA test. P-values of < 0.05 were assigned significance. Differences in the survival of TCL-1 mice treated with...
auranofin were calculated by a Mantel-Cox log rank test. P-values < 0.05 were assigned significance.

Results:

Auranofin causes loss of viability of CD19+ B CLL cells. Our findings in Figures 1A demonstrate that exposure to AF for 24 hours dose-dependently induced loss of viability of primary CLL cells. The clinical and prognostic molecular-cytogenetic features of each of the 50 primary CLL samples are described in Supplemental Table 1. The table also provides the IC$_{50}$ values of auranofin against each of the primary CLL samples. Treatment with AF also significantly induced apoptosis of primary CLL, but not of normal CD34+ HPCs (Figure 1B). AF treatment increased the % of annexin V positive, CD19+ primary CLL cells, with relative sparing of the normal CD3+ T lymphocytes (Figure 1C-E). As shown in Figure 2A, treatment with AF was equally active against CLL with un-mutated as compared to the mutated IGHV genes. In contrast, treatment with AF induced significantly greater loss of cell viability of ZAP70+ versus ZAP70- CLL cells (Figure 2B). This was especially so following exposure to the lower versus the higher concentrations of AF (Figure 2C). Exposure to AF dose-dependently induced apoptosis in the cultured CLL MEC-1 cells, which carry the del17p chromosome. This was associated with an increase in the % of Sub-G1 cells and cleavage of caspase 3 and PARP (Figure 2D, 2E and Supplemental Figure 2). At lower concentrations, there was a significant difference in AF-induced apoptosis in the primary CLL cells containing del17p compared to those containing del13q or del11q (p< 0.05) (Figure 2D). On the other hand, at higher concentrations, there was no significant difference in AF-induced apoptosis in the primary CLL cells containing del17p, del13q or del11q (Figure 2D). Overall, AF treatment was as effective in inducing apoptosis of the favorable as compared to CLL cells with the unfavorable cytogenetic predictors of clinical outcome (Figure 2F).

Auranofin overcomes protection due to stroma associated cells and exerts lethal in vivo activity against CLL cells. We next determined the in vitro effect of stroma, represented in our studies by co-culture with the HK cells or the nurse like cells (NLCs), on the lethal effects of AF on CLL cells (32,33). As shown in Figure 2G, AF treatment dose-dependently induced loss of
cell viability of MEC-1 cells, which was significantly attenuated by co-culture with HK cells. Co-culture with NLCs also improved the viability of primary CLL samples in vitro (Figure 2H). However, our findings also show that AF treatment mediated loss of viability of primary CLL cells, regardless of the co-culture in vitro with NLCs (Figure 2H). In contrast, treatment with AF did not reduce the viability of NLCs (Supplemental Figure 3A-C). We next tested the in vivo activity of AF against CLL cells in the TCL-1 transgenic mice (34,35). Because the TCL-1 gene is among the top genes up regulated in CLL cells by co-culture with the stromal cells, this mouse model is also particularly relevant to ascertain the in vivo protective role of the stromal microenvironment for CLL cells (36). As shown in Figure 2I and Supplemental Table 2, treatment with AF caused a reduction in the leukemia cell burden in each of the five TCL-1 mice tested. The number of CLL cells in the peritoneal cavity was reduced by >90% during the two weeks of AF treatment (p= 0.00019). These findings demonstrate that AF is able to exert in vitro and in vivo lethal effects against CLL cells despite the protective effects of stroma. Treatment with AF also significantly improved the survival of the TCL-1 mice compared to mice that received no treatment (p=0.001) (Figure 2J).

**Auranofin inhibits TrxR and induces oxidative stress and apoptosis in CLL cells.** We next determined whether exposure to AF inhibits TrxR activity, as well as induces ROS levels and apoptosis of the MEC-1 and primary CLL cells. Treatment with AF inhibited the total intracellular TrxR activity determined by a TrxR activity assay (Cayman Chemical Company, Ann Arbor, MI), and concomitantly increased ROS levels in MEC-1 and primary CLL cells (Figure 3A). Co-treatment with the anti-oxidant N-acetyl cysteine (NAC) inhibited AF-mediated ROS induction in MEC-1 and primary CLL cells (Figure 3B and Supplemental Figure 1). Because they exhibit higher levels of ROS and activity of Nrf2, CLL cells have concomitant overexpression of the downstream Nrf2 targets such as heme oxygenase-1 (HMOX-1) (10,11). Therefore, here, we determined the effect of AF-induced ROS on the levels of Nrf2 and its target genes in CLL cells. The confocal immunofluorescence analyses in Figure 3C demonstrate that AF treatment not only increased the levels of Nrf2 protein but also augmented its nuclear localization in primary CLL cells. Moreover, immunoblot analyses of the cell lysates of MEC-1 and primary CLL cells confirmed that AF treatment increased Nrf2 levels as well as increased the levels of Nrf2 activated genes, including HMOX-1, TxNIP and GCLM (Figure 3D). Additionally, co-treatment with NAC attenuated AF-mediated increase in the levels of Nrf2 and
its targeted gene expressions, especially of HMOX-1 and GCLM, confirming their linkage to AF-induced oxidative stress (Figure 3E). In primary CLL cells, AF-induced ROS levels were associated with increases in the mitochondrial permeability transition ($\Delta\psi_m$) and % of annexin V-positive cells (Figure 3F). Co-treatment with NAC inhibited AF-induced apoptosis of primary CLL cells (Figure 3G). In cultured MEC-1 cells, co-treatment with NAC and AF inhibited AF-induced apoptosis as demonstrated by significant reduction in annexin V-positive cells and decreased sub-G1 fraction, as well as inhibition of PARP cleavage (Figure 3H).

**Auranofin perturbs biologic networks of genes in CLL cells.** We also determined the effects of AF treatment on gene expression microarray profile in primary CLL cells. Figure 4A shows a heat map of gene expression changes. AF treatment for 4 or 10 hours significantly up-regulated or down-regulated the mRNA expression of a large number of genes, and the fold changes in the 39 most altered mRNA gene expressions are shown in Figure 4B and Supplemental Table 3. The Figure shows that mRNA expression levels of the Nrf2 target genes HMOX-1 and GCLM, as well as of CHOP and the key heat shock proteins were markedly induced by AF treatment in the primary CLL cells. To confirm the induction of CHOP and Nrf2 target genes, total RNA from untreated and AF-treated cells was also reverse transcribed and the resulting cDNA was used for quantitative PCR with the specific TaqMan Real-time PCR probes. This confirmed that AF treatment markedly increased the mRNA expression of the HMOX-1, GCLM1, and CHOP genes in primary CLL cells with or without deletion of 17p (Figure 4C and 4D). Data sets of genes with altered expression profile derived from microarray analyses were imported into the Ingenuity Pathway Analysis (IPA) Tool (Ingenuity H Systems, Redwood City, CA). Within the gene list, IPA identified the top five most perturbed networks in Primary CLL cells following treatment with AF and assigned a score for these associated network functions (Supplemental Table 4). The score (i.e. a score of 41) assigned by IPA indicates the probability ($1 \times 10^{-41}$) that the focus genes in the dataset are grouped together in a perturbed network due to random chance alone. The differentially expressed genes and the five most perturbed biological networks identified by IPA revealed significant connectivity between genes (nodes) within different biological networks (Supplemental Figure 4). Among these, free radical scavenging, cellular compromise and protein degradation networks were the highest rated networks with high significance scores.
Inhibition of HMOX-1 and GCLM augments AF-induced apoptosis of CLL cells. We next determined the mechanistic relevance of AF-induced, oxidative stress response which is represented by Nrf2 induced levels of HMOX-1 and GCLM. For this, we knocked down HMOX-1 gene expression by shRNA or inhibited HMOX-1 activity by treatment with its selective inhibitor zinc deuterophyrin IX 2,4-bis-ethylene glycol (ZnBG) (37). The effect of this was determined on AF-induced apoptosis. As shown in Figure 5A, treatment with shRNA to HMOX-1 depleted HMOX-1 protein levels but did not affect the levels of Nrf2, GCLM1 or induce the levels of cleaved caspase-3. In contrast, co-treatment with shRNA to HMOX-1, but not with the non-targeted shRNA, significantly increased AF-induced apoptosis (Figure 5B). In addition, co-treatment with ZnBG and AF synergistically induced apoptosis of MEC-1 and primary CLL cells. This was assessed by the median dose effect isobologram analyses where the combination indices (CI) were noted to be < 1.0 (Figure 5C and Supplemental Figure 5) (38). GCLM is the modifier subunit which heterodimerizes with the catalytic subunit (GCLC) of glutamate cysteine ligase (GCL)-an enzyme catalyzing the initial rate-limiting step in the GSH synthesis (39). By inhibiting GCL activity, treatment with buthionine sulfoximine (BSO) inhibits GSH synthesis (40). We also determined the apoptotic effects of co-treatment with (BSO) on AF-induced apoptosis of MEC-1 and primary CLL cells. Co-treatment with BSO synergistically induced AF-induced apoptosis (Figure 5D). Again, the CI values were < 1.0. Collectively, these findings demonstrate that abrogation of Nrf2-induced target gene expressions, which represent the adaptive oxidative stress response sensitizes CLL cells to AF.

AF-induced ROS increases ER stress which contributes to AF lethality in CLL cells. Increased ROS levels and perturbation in the intracellular redox status increases the levels of unfolded proteins in the ER and induce ER stress response (UPR) (12, 22, 23). UPR induces PERK-mediated phosphorylation of eukaryotic initiation factor 2α (eIF2α), which blocks cap-dependent protein translation but allows preferential translation of ATF4 (22). While up-regulating chaperone proteins, e.g., GRP78, required in restoring the ER function, ATF4 also induces the pro-death transcriptional regulator CHOP (22, 23). Treatment with AF induced GRP78 levels and increased the mRNA and protein levels of CHOP (Figure 6A and 6B). Importantly, AF treatment, also dose-dependently, increased the ratio of CHOP to GRP78 (Figure 6B). Co-treatment with the anti-oxidant NAC attenuated the expression of ATF4 and CHOP (Figure 6C and 6D), and significantly inhibited apoptosis of CLL cells, as shown above in
Figure 3G-3H. We also determined the effects of treatment with AF on the induction of ER stress based UPR. In MEC-1 cells, treatment with AF induced the expression levels of p-PERK, the spliced form of XBP1 (XBP1-s) and CHOP (Figure 6E). AF treatment also induced the expression of the ER chaperones GRP78 and Calreticulin but not ERp57 (Figure 6E). Additionally, the AF induced effects on these protein expressions were attenuated by co-treatment with NAC. We also determined whether treatment with hydrogen peroxide, would also induce the expression of GRP78 and CHOP in CLL cells. Treatment with hydrogen peroxide also induced Nrf2, GRP78 and CHOP levels, which was associated with an increase in the levels of cleaved caspase 3 (Supplemental Figure 6). These results suggest that the effects of AF-induced oxidative response are mimicked by treatment with hydrogen peroxide. We next determined the effects of shRNA-mediated depletion of CHOP in CLL cells. Knockdown of CHOP by two separate lentivirus-transduced CHOP shRNAs, markedly attenuated CHOP expression in MEC-1 cells (Figure 6F). This was associated with an appreciable reduction in AF-induced apoptosis in MEC-1 cells (Figure 6G). These findings demonstrate that AF-induced ROS also leads to induction of a lethal ER stress, associated with increase in the ratio of CHOP to GRP78 in CLL cells, while abrogation of AF-induced CHOP levels partially undermines anti-CLL activity of AF.

**AF treatment induces proteotoxic stress with depletion of HDAC6, ZAP70 and pro-survival hsp90 client proteins in CLL cells.** Next, we determined whether AF treatment leads to proteotoxic stress and the disassociation of HDAC6 from the hsp90-HSF1-p97 complex and induction of the heat shock response (13, 14, 41). Data presented in Figure 7A are representative of the results obtained in 3 similarly treated separate samples of primary CLL cells which shows that treatment with AF appreciably increased poly-Ub proteins in primary CLL cells. Co-treatment with NAC inhibited this accumulation. AF-induced accumulation of poly-Ub proteins was associated with decreased binding of hsp90 to HDAC6, HSF1 and p97 in CLL cells (Figure 7B). AF treatment led to a marked depletion of HDAC6 levels through degradation by the proteasome, which was associated with the hyperacetylation of hsp90, detected by immunoblotting with an anti-acetylated-K69 hsp90 antibody (Figure 7C) (17). Confocal immunofluorescence analysis showed that AF treatment increased the nuclear localization of HSF1, consistent with the disruption of its binding to hsp90 (Figure 7D). This was also associated with the induction of the levels of the HSPs hsp27, hsp40, and hsp70 (Figure 7E). By
inducing hyper-acetylation and inhibition of chaperone function of hsp90, treatment with AF also dose-dependently depleted the intracellular levels of the CLL relevant, pro-growth and pro-survival hsp90 client proteins ZAP70, c-RAF and AKT (Figure 7F) (18, 19, 25). Their decline was due to degradation by the proteasome, since co-treatment with the proteasome inhibitor carfilzomib restored the levels of these client proteins (Figure 7G).

**Discussion:**

Recently, treatment with AF was discovered to inhibit TrxR and induce ROS in *E. histolytica* trophozoites, which was associated with high in vitro and in vivo potency of AF against *E. histolytica* and AF was granted orphan drug status by the FDA (42). With the goal of potential repurposing of AF for the treatment of CLL, our pre-clinical studies presented here demonstrate that AF also inhibits TrxR and induces ROS in the cultured and primary CLL cells. Here, we also show for the first time that treatment with AF induces lethal oxidative, and linked to it; proteotoxic and ER stress-based UPR responses in CLL cells. Additionally, AF treatment is selectively lethal to primary CLL cells, while sparing the CD34+ normal progenitor and B cells. This may be due to previously documented higher levels of ROS and Nrf2 levels/activity in the primary CLL versus normal B cells, which renders CLL cells more sensitive to augmented oxidative stress due to AF-induced ROS levels (11). Further, co-treatment with NAC abrogated the lethal activity of AF. Previous reports indicated that redox-active compounds, including those containing α-β unsaturated carbonyls, isothiocyanates, arsenic trioxide and ON01910.Na, that induce ROS and Nrf2 activity are also lethal against CLL cells (5, 11, 43).

AF treatment was also effective in inducing apoptosis in CLL cells exhibiting the biologic and genetic features associated with poor clinical outcome, including high expression of ZAP70, un-mutated IGHV genes, and acquired chromosomal abnormalities such as deletion 17p13 and deletion 11q22. Our findings also demonstrate that the in vitro co-culture with stromal cells reduces AF-induced apoptosis in MEC-1 cells, which has been attributed to reduced ‘apoptotic priming’, defined as the proximity of a cell to the apoptotic threshold measured by a functional assay that assesses mitochondrial depolarization in response to BH3-only peptides (30, 44). However, AF treatment retained the ability to dose-dependently exert lethal activity against CLL cells despite co-culture with NLCs. Furthermore, the capacity to markedly reduce the in vivo
CLL cell burden in the TCL-1 mouse model supports the conclusion that AF treatment overcomes the protective effects of stroma not only in vitro but also in the in vivo setting.

IPA analysis of genes differentially expressed in response to AF identified five significantly perturbed biologic networks with high significance scores, including free radical scavenging, cellular compromise and protein degradation. These findings underscore that AF-induced oxidative stress and transcriptional activity of Nrf2 play a key role in eliciting the stress-adaptive responses in AF-treated CLL cells. Nrf2 activity led to increased expression levels of HMOX-1 and GCLM, as well as increase in the ER stress-based UPR-associated CHOP levels. It is noteworthy that auranofin strikingly induced GCLM, HMOX-1 and CHOP levels in primary CLL cells with or without the deletion of chromosome 17p. As described in AML cells (45), we found that knockdown of the levels and/or activity of HMOX-1 synergistically enhanced AF-induced apoptosis of CLL cells. Co-treatment with BSO also synergistically increased AF-induced apoptosis due to upregulation of GCLM levels and GCL activity, thereby abrogating the protective effects mediated by AF-induced GSH. Co-treatment with AF and BSO is likely to have even greater efficacy in overcoming the pro-survival effects of stromal microenvironment in vitro and in vivo, given that stromal cells mediate this effect by helping elevate the intracellular levels of GSH in CLL cells (30). Previous reports have demonstrated that loss of TrxR activity renders transformed cells especially susceptible to GSH deprivation (46). Increased oxidative stress caused by simultaneous inhibition of GSH and thioredoxin metabolism has also been shown to sensitize cancer cells to other therapeutic agents (47, 48).

By inducing ROS levels and oxidative stress, AF treatment concomitantly induced ER stress response, highlighted by elevated levels of p-PERK, XBP1-s and CHOP as well as increase in the levels of GRP78 and Calreticulin. Although AF-mediated increase in GRP78 levels would be protective, a protracted greater elevation in CHOP relative to GRP78 levels could have lethal consequences (24). Protracted CHOP induction is known to mediate apoptosis, mainly attributed to induction of BIM with simultaneous inhibition of Bcl-2 and Mcl-1 levels (22). Findings presented here demonstrating that the shRNA-mediated knockdown of CHOP modestly inhibits AF-induced apoptosis suggest that auranofin-induced ER stress is a secondary response to auranofin-induced ROS that can amplify the cell death pathway, rather than being a mediator of it in CLL cells. AF-induced increase in the levels of poly-Ub proteins led to hyperacetylation and
inhibition of chaperone function of hsp90. The resulting depletion in the levels of HDAC6, ZAP70, AKT and c-RAF would also increase the apoptotic priming as well as attenuate growth and reduce the survival of CLL cells. Collectively, our findings underscore that auranofin–induced oxidative stress is also linked to proteotoxic and ER stress-based UPR which could amplify the lethal effects of auranofin in CLL cells.

Research on phosphine gold compounds for elucidating their full range of biologic activity and therapeutic potential is ongoing (31,42). Findings presented here point toward a repurposing potential for AF in the treatment of CLL. To this end, under the umbrella of an FDA approved IND, a clinical trial of AF in treatment-refractory CLL is underway (4).

Authorship: W.F., N.S., M.S., S.D.G., L.C., and R.R. performed experiments in cultured and primary CLL cells and analyzed the data. S.G. performed the microarray expression analyses and assisted with Ingenuity Pathway Analysis. K.S. provided a critical new reagent for the studies. C.P.A. provided intellectual input for the studies. K.M., J.B. provided primary CLL cells for the studies. J.L. and P.H. performed the in vivo studies with auranofin in the TCL-1 mice. A.M. provided intellectual input for the studies. A.W. and K.N.B. conceptualized, planned the experiments, supervised the studies and analyzed the data. K.N.B. prepared the manuscript.

References


Figure Legends

Figure 1. Treatment with AF induces apoptosis of primary CLL CD19+ but not CD3+ cells or normal CD19+ or CD34+ cells. A. Cell viability of primary CLL (n=50) following treatment with AF for 24 hours. Columns, mean of loss of viability; Bars, S.E.M. * indicates cell viability values significantly less in AF treated cells than untreated cells (p < 0.05). B. Percentage of apoptotic normal CD34+ cord blood cells (n=3) and primary CLL cells (n=5) following exposure to 1.0 µM of AF for 48 hours. Columns, mean apoptosis from the CD34+ cord blood cells or primary CLL cells; Bars, S.E.M. C. Schematic of the flow cytometry gating method used to determine the percentage of apoptotic CD3+ or CD19+ cells following treatment with 1.0 µM of AF for 24 hours. D. Individual reduction in viability (relative to untreated control cells) of CD3+ and CD19+ cells from CLL patients treated with 1.0 µM of AF for 24 hours. E. The % reduction in viability induced by 24 hour treatment with 1 µM of AF in CD3+ and CD19+ cells from CLL patients (n=8). The horizontal black bar indicates the median reduction in cell viability ± standard error. * indicates significantly greater reduction in viability of AF-treated CD19+ cells compared to CD3+ cells (p < 0.05).

Figure 2. Treatment with AF selectively induces apoptosis of cultured and primary CLL CD19+ cells independent of cytogenetics or IGHV mutation status, despite co-culture with NLCs and reduces in vivo leukemia burden. A. IC50 values for the primary CLL patient cells treated with AF for 24 hours sub-grouped by IGHV mutational (U= unmutated; M= mutated) status. Black line, median IC50 value. B. IC50 values for primary CLL patient cells treated with AF for 24 hours sub-grouped by ZAP70 status. Black line, median IC50 value. C. Cell viability of primary CLL cells from patients sub-grouped by ZAP70 status (ZAP70+ n=12; ZAP70- n=15) following treatment with the indicated concentrations of AF for 24 hours. Each point on the line represents the mean viability of the CLL cells at each concentration of AF; Bars represent SEM. * indicates cell viability values significantly less in ZAP70+ AF-treated cells than ZAP70- AF-treated cells (p < 0.05). D. Percent apoptosis of MEC-1 cells and CD19+ primary CLL cells (11q deleted, 13q deleted and 17p deleted), exposed to the indicated-doses of AF for 48 hours. * indicates apoptosis values significantly greater in 17p deleted CLL, than in 11q and 13q deleted CLL cells at 0.25 µM of AF (p<0.05). ** indicates apoptosis values significantly greater in 17p deleted CLL, than in 11q and 13q deleted CLL cells at 0.5 µM of AF (p< 0.05). E. The % of sub-G1 cells and % annexin V-positive, apoptotic MEC-1 cells following 24 hour exposure to AF. Immunoblot analyses were also conducted as indicated. F. IC50 values for the primary CLL patient cells treated with AF for 24 hours sub-grouped by adverse (ADV) or favorable (FAV) cytogenetics. Black line, median IC50 value. G. Percent apoptosis of MEC-1 cells with and without co-culture on HK cells following treatment with AF for 48 hours. * indicates values significantly less in Mec-1 cells co-cultured with HK cells compared to MEC-1 without co-culture (p< 0.05). H. Primary CLL cells (n=4) were cultured with or without nurse-like cells (NLC) and exposed to 2X the IC50 of AF for 24 hours. At the end of treatment, the % non-viable cells were determined by flow cytometry. The absolute viability of the CLL cells in each
condition is shown. I. Treatment of TCL-1 mice (n=5) with AF (10 mg/kg, 5 days per week for 2 weeks) significantly reduced the leukemia cell burden. Columns represent the mean ± SEM of the leukemia cell burden in all 5 mice tested. J. Kaplan-Meier survival plot of TCL-1 mice treated with AF compared to untreated mice. (p = 0.001, log-rank (Mantel-Cox) test.

**Figure 3. Treatment with AF induces an oxidative stress response in cultured and primary CLL cells:** A. The % induction of ROS and the % inhibition of TRR activity in MEC-1 and CD19+ primary CLL cells exposed to 1.0 µM of AF for 1 hour or 8 hours, respectively. B. Percentage increase of ROS in MEC-1 cells exposed to AF with or without 1 mM of N-acetyl cysteine (NAC) for one hour. Columns, mean of three independent experiments; Bars, SEM. C. Localization of Nrf2 in primary CLL cells treated with AF for 6 hours. Cells were also stained with Phalloidin and DAPI. D. Immunoblot analyses of MEC-1 cells and CD19+ primary CLL cells treated with AF for 16 hours. E. Immunoblot analyses of CD19+ primary CLL cells treated with AF and NAC for 24 hours. F. Induction of ROS, loss of mitochondrial membrane potential and apoptosis of primary CLL cells (n=8) treated with AF as indicated. Values represent the % induction corrected for the untreated, control cells. G. Percent apoptosis of CD19+ primary CLL cells following treatment with AF and/or NAC for 48 hours. * indicates apoptosis values significantly less in cells treated with AF and NAC than those treated with AF alone (p=0.00062). H. Percentage of sub-G1 and annexin V-positive MEC-1 cells following treatment with AF and/or NAC for 24 hours. * indicates values significantly less in cells treated with AF and NAC than those treated with AF alone (p < 0.001). Immunoblot analyses were also conducted as indicated.

**Figure 4. mRNA expression profiling of AF treated primary CLL cells.** A. Heatmap representing the relative expression of 98 probe sets (81 genes) in CLL cells from 2 patients treated with 1.0 µM of AF for 4 and 10 hours in vitro compared to untreated cells (>2-fold change; P <.01 by ANOVA). Patient samples are arranged in columns. Gene symbols highlight select genes. Gene expression is median-centered and scaled as indicated. B. Graphical representation of the top thirty-nine up- and down-regulated genes in primary CLL cells following treatment with 1.0 µM of AF for 8 hours compared to untreated control cells. C. Relative mRNA expression of HMOX-1, GCLM and CHOP in primary CLL cells treated with 1.0 µM of AF for 8 hours compared to untreated control cells. D. Relative mRNA expression of HMOX-1, GCLM and CHOP in 17p-deleted primary CLL cells treated with 1.0 µM of AF for 8 hours compared to untreated control cells.

**Figure 5. Inhibition of HMOX-1 by shRNA or co-treatment with AF and ZnBG or BSO synergistically induces apoptosis of cultured and primary CLL cells.** A. Immunoblot analyses of MEC-1 cells transduced with non-targeted shRNA (sh-NT) or sh-HMOX1 for 48 hours, then treated with AF for 8 hours. B. Percent apoptosis of sh-NT and sh-HMOX1-transduced MEC-1 cells following treatment with AF for 48 hours. Columns, mean of three independent experiments; Bars, SEM. * indicates apoptosis values significantly greater in sh-HMOX1-transduced cells compared to sh-NT-transduced cells (p < 0.001). C. MEC-1 cells
(black line) and primary CLL cells (gray line) were exposed to of AF (100-500 nM) and ZnBG (10-20 µM) for 48 hours. After treatment, the % of apoptotic cells was determined by flow cytometry. Median dose effect and isobologram analyses were performed utilizing CalcuSyn software. Combination index values (CI) less than 1.0 indicate a synergistic interaction between the two agents. D. MEC-1 cells (black line) and primary CLL cells (gray line) were treated with AF (10-250 nM) and BSO (1-10 µM) for 48 hours. Following this, the % of apoptotic cells was determined by flow cytometry. Isobologram analyses were performed as described above.

**Figure 6. Treatment with AF induces ER stress in CLL cells:** A. Fold induction of GRP78 and CHOP mRNA in primary CLL (n=4) treated with 1.0 µM of AF for 8 hours is presented. B. Western blot analyses of CHOP and GRP78 in primary CLL cells treated with AF for 24 hours (upper panel). Ratio of induction of CHOP to GRP78 in the primary CLL cells (lower panel). C. Relative mRNA expression of ATF4 and CHOP in MEC-1 and primary CLL cells following treatment AF and/or NAC for 8 hours. D. Immunoblot analyses of GRP78, CHOP and β-actin in the cell lysates from CD19+ primary CLL cells treated with AF and/or NAC for 24 hours. E. Immunoblot analyses of MEC-1 cells following treatment with AF and/or NAC for 24 hours. F. Relative CHOP mRNA expression 48 hours post transduction in non-targeted (sh-NT) or CHOP shRNA transduced MEC-1 cells (Top panel). Western blot of CHOP and β-actin in MEC-1 cells prior to AF treatment (Lower panel). G. Percent apoptosis of stably transfected sh-NT or sh-CHOP#2 MEC-1 cells treated with 0.25 µM of AF for 48 hours. * indicates apoptosis values significantly less in MEC-1 sh-CHOP cells treated with AF, compared to sh-NT cells (p < 0.02).

**Figure 7. Treatment with AF inhibits hsp90 chaperone function and induces a heat shock response in CLL cells.** A. Representative immunoblot analyses of polyubiquitinated proteins (poly Ub) and β-actin in the cell lysates from three primary CLL samples treated with AF alone (1.0 µM) or AF and NAC (2.5 mM) for 24 hours. B. Immunoblot analysis of HDAC6, HSF1 and p97 expression in hsp90 immunoprecipitates following treatment with 1.0 µM of AF for 8 hours. The blot was stripped and re-probed for hsp90. C. Representative immunoblot of HDAC6 expression from primary CLL cells (n=10) exposed to 1.0 µM of AF for 16 hours. The values in the graph underneath the blot represent quantitation of the levels of HDAC6 (mean ± standard error) relative to the untreated control (arbitrarily set as 1.0) as determined by densitometry analysis. The right panel shows the levels of acetylated hsp90 using anti-acetyl-K69 hsp90 antibody, following exposure of a representative primary CLL sample to 1.0 µM of AF for 16 hours. D. Localization of HSF1 in primary CLL cells exposed to AF for 6 hours. A representative image is shown. E. Immunoblot analyses of hsp40, hsp27, hsp70, and β-actin in the cell lysates from primary CLL cells exposed to AF for 16 hours. F. Immunoblot analyses of ZAP70, c-RAF, AKT and β-actin in the cell lysates from primary CLL cells exposed to AF for 16 hours. G. Immunoblot analyses of c-RAF, ZAP7, HDAC6, and β-actin in the lysates from primary CLL cells treated with 1.0 µM of AF and/or 20 nM of carfilzomib (CZ) for 8 hours. The numbers underneath the bands represent densitometry analysis performed on representative immunoblots.
Figure 1

A n=50

0 100 200 300 400 500 600 700 800

Mean viability (%) vs μM AF, 24 hrs

B

0 10 20 30 40

% apoptosis vs μM AF, 48 hrs

CD34+ cord blood n=3
Primary CLL n=5

C

Control

CD3

CD19

FSC

CD3

Annexin V

11%

CD19

12%

D

1.0 μM AF, 24 hrs

% Reduction in viability

CD3

CD19

E

p=0.0002

% Reduction in viability

CD3+ CD19+

1.0 μM AF, 24 hrs
Figure 2

A. IC_{50} µM vs. IGVH-U and IGVH-M

B. IC_{50} µM vs. ZAP70-

C. Viability (%) vs. µM, AF, 24 hrs

D. % apoptosis vs. µM, AF, 48 hrs

E. % of cells (24 hrs)

F. IC_{50} µM

G. % apoptosis vs. nM, AF, 48 hrs

H. Viability (%) vs. AF

I. Leukemia burden (x10^6 cells)

J. Percent survival vs. Months
Figure 3

A. % increase in ROS

B. % decrease in TRR activity

C. Primary CLL cells

D. Primary CLL

E. Primary CLL

F. Primary CLL cells (n=8)

G. % apoptosis

H. % of cells (24 hrs)
Figure 4

A. Primary CLL

B. Fold Expression Change

C. Relative mRNA expression

D. 17p-deleted Primary CLL

- HMOX-1
- GCLM
- CHOP
Figure 6

A. Relative mRNA expression in Primary CLL (n=4) compared to control and 1 µM, AF.

B. Primary CLL with 0, 0.25, and 1.0 µM, AF, showing CHOP, GRP78, and β-Actin expression levels.

C. Relative mRNA expression in MEC-1 and Primary CLL with 0.5 µM AF, 0.5 µM AF + 2.5 mM NAC, 1.0 µM AF, and 1.0 µM AF + 2.5 mM NAC.

D. Primary CLL with 0, 1.0, and 1.0 µM, AF and 0, 0, and 2.5 mM, NAC, showing GRP78, CHOP, and β-Actin expression levels.

E. MEC1 with 0.1, 1.0, and 1.0 µM, AF and 2.5 mM NAC, 24 h, and 1.0 µM AF, 24 h, showing p-PERK, PERK, XBP1-s, ERp57, Calreticulin, CHOP, GRP78, and β-Actin expression levels.

F. Relative CHOP mRNA expression in MEC-1 with sh-NT, shCHOP#1, and shCHOP#2.

G. Percentage of apoptosis in MEC-1 with sh-NT, shCHOP#1, and shCHOP#2 with 0 and 0.25 µM, AF, 48 h.
Figure 7

A. Primary CLL

0 1.0 1.0 µM, AF
0 0 2.5 mM, NAC

Poly Ub proteins

β-Actin

B. Primary CLL

IgG 0 1.0 µM, AF, 8 hrs

IP: hsp90

IB:

hsp90
HSF1
p97
HDAC6

Expression of HDAC6

Primary CLL (n=10)

C. Primary CLL

16 hrs

0 1.0 µM, AF

HDAC6

β-Actin

D. Primary CLL

DAPI Phalloidin HSF-1 Merge

control

0.25 µM, AF

1.0 µM, AF

E. Primary CLL

0 0.25 1.0 µM, AF, 16 h

hsp40

hsp27

hsp70

β-Actin

F. Primary CLL

0 0.25 1.0 µM, AF, 16 h

ZAP70

hsp70

β-Actin

G. Primary CLL, 8 hrs

0 1.0 0 1.0 µM, AF

0 0 20 20 nM, CZ

ZAP70

c-RAF

HDAC6

β-Actin
Auranofin induces lethal oxidative and endoplasmic reticulum stress and exerts potent preclinical activity against chronic lymphocytic leukemia

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