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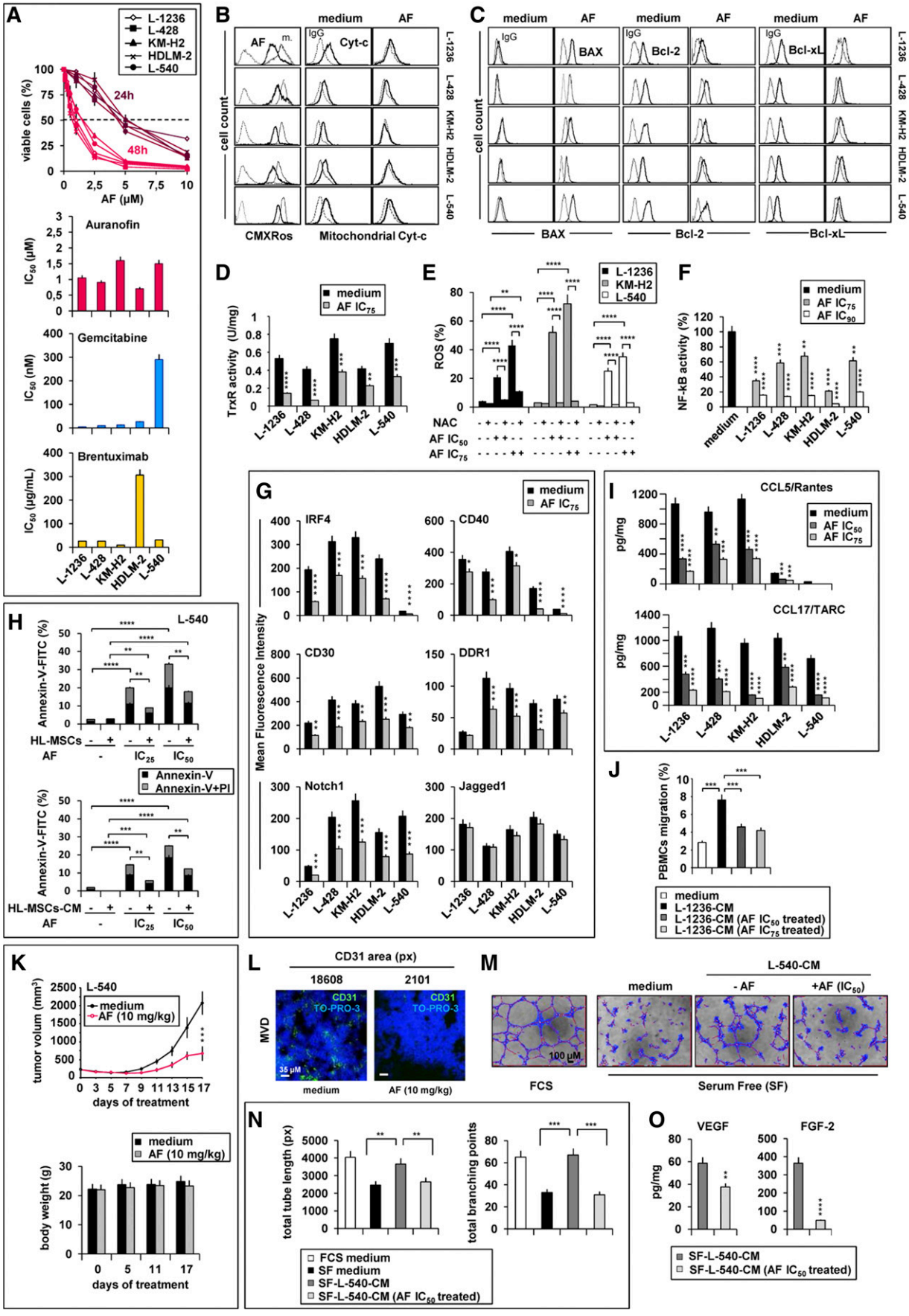
To the editor:

Preclinical activity of the repurposed drug auranofin in classical Hodgkin lymphoma

The treatment of classical Hodgkin lymphoma (cHL) patients with refractory/relapsed disease remains a clinical challenge.[1] To find a new therapeutic option for cHL, we investigated the preclinical activity of the repurposed drug auranofin (AF).[2] AF is an anti-inflammatory drug used for rheumatoid arthritis and is now considered a potential anti-cancer drug. AF, approved by the US Food and Drug Administration for clinical trials in chronic lymphocytic leukemia and in ovarian and lung cancer, seems to have application also in bacterial and parasitic infections as well as in HIV/AIDS.[3]

With the goal of repurposing AF for refractory cHL, we demonstrated its antitumoral activity in in vitro and in vivo tumor models. AF inhibited proliferation (Figure 1A) and clonogenic growth (supplemental Figure 1A, available on the *Blood* Web site) of L-1236, L-428, KM-H2, HDLM-2, and L-540 cHL-derived cell lines with IC50 ranging from 0.7 to 1.5 μM (supplemental Table 1). AF was also active in L-540 gemcitabine-resistant and HDLM-2 brentuximab-resistant cells (Figure 1A; supplemental Table 1). AF induced cytotoxic effects by promoting apoptotic stimuli: it decreased the mitochondrial membrane potential (Figure 1B) and induced cyt-c release (Figure 1B), upregulated BAX (Figure 1C) and downregulated the antiapoptotic Bcl-2 (Figure 1C) and Bcl-xL (Figure 1C) molecules, and induced caspase 3 activation (supplemental Figure 1B) and DNA fragmentation (supplemental Figure 1C). AF reduced TrxR activity (Figure 1D) and induced the accumulation of ROS (Figure 1E), which was inhibited by the ROS scavenger N-acetyl-cysteine (NAC) (Figure 1E). NAC reverted apoptotic effects by AF (supplemental Figure 1D).

AF not only could exert a direct cytotoxic activity but also could counteract the survival signals from the microenvironment dependent on: (1) inflammatory cells[4] expressing CD40L or CD30L; (2) collagen secreted by stromal cells and capable of activating DDR1; and (3) Jagged1 expressed by endothelial, smooth muscle, and epithelioid cells.[5] In fact, AF inhibited nuclear factor κB (Figure 1F),



constitutively active in cHL cells, and downmodulated its target genes IRF4 and CD40 (Figure 1G).[6] It decreased DDR1 (Figure 1G) and CD30 (Figure 1G); it downmodulated the Notch1 receptor but not its ligand Jagged1 (Figure 1G).

To mimic the effects of the microenvironment, we evaluated AF activity in the presence of HL-MSCs and sCD40L. Cocultivation of cHL cells for 48 hours with HL-MSCs or conditioned medium from HL-MSCs (HL-MSCs-CM) reduced the proapoptotic effects of AF of about 50% (Figure 1H) with a minor effect of sCD40L (supplemental Figure 1E).

The cHL microenvironment is dominated by an extensive inflammatory-cell infiltrate that plays a crucial role in the pathobiology of the disease and is thought to be responsible for the minimal residual disease leading to drug resistance and relapse.[4] AF decreased the secretion by cHL cells of cytokines involved in proliferation/survival, angiogenesis, and recruitment of inflammatory cells (IL-13, TNF-α, TGF-β1, VEGF, FGF-2, CCL5, CCL17, and IL-6)[4] (Figure 1I; supplemental Figure 1F), leading to a reduced capability to recruit PBMCs (Figure 1J).

AF exerted a clear synergistic activity with 3 chemotherapeutic drugs widely used in cHL treatment—doxorubicin, cisplatin, and gemcitabine (supplemental Table 2, supplemental Figure 1G)—thus suggesting that it could be used in combination with drugs with different mechanisms of actions to reduce dose and toxicity and to overcome drug resistance.[7]

AF led to a 70% tumor growth reduction of L-540 gemcitabine-resistant–derived tumor xenografts, with minimal weight loss (Figure 1K)[8] and decreased microvessel density (CD31 staining) in tumor xenograft of about 90% (Figure 1L).

Little is known about the angiogenic potential of cHL cells.[9] We found for the first time that L-540-CM, obtained in SF conditions to avoid FCS activity, increased human umbilical vein endothelial cell tubulogenesis (Figure 1M) as evaluated by the total tube length and branching points (Figure 1N), whereas L-540-CM from AF-treated cells (Figure 1N), consistent with the decreased secretion of both VEGF and FGF-2 (Figure 1O), was ineffective. Thus AF could inhibit angiogenesis by cytotoxic effect on endothelial cells[10] and by decreasing proangiogenic factors secreted by Hodgkin and Reed-Sternberg cells.

In conclusion, in light of the activity observed also in the gemcitabine- and brentuximab-resistant cells, the very significant reduction of tumor mass obtained with AF used alone, the low toxicity, and the inhibition of angiogenesis and microenvironmental interactions, our results provide the rationale for the clinical assessment in cHL of AF as single or as combination therapy.

Figure 1. Antitumoral activity of AF in cHL. A panel of human cHL-derived cell lines (L-1236, L-428, KM-H2, HDLM-2, and L-540) were obtained from an authenticated source (DSMZ, German collection of microorganisms and cell cultures, Germany) and cultured in RPMI medium supplemented with 10% fetal calf serum (FCS). (A) cHL cells (2.0×10^5 cells/mL) were treated for 24 or 48 hours with increasing concentrations of AF (Sigma) (0.1 to 10 μM), for 48 hours with gemcitabine (Actavis) (1 to 500 nM), and for 72 hours with brentuximab (Takeda) (1 to 500 μg/mL), and then viable cells were evaluated by trypan blue dye exclusion assay. Histograms show the 50% inhibitory concentration (IC_{50}) values calculated using the CalcuSyn software (Biosoft). AF concentration (IC_{25}, IC_{50}, IC_{75} and IC_{90}, the drug concentration required for 25, 50, 75 and 90% growth inhibition in vitro) used in all experiments referred to the dose response obtained after 48 hours incubation with the drug (see Figure 1A). (B) (2.0×10^5/mL) cHL cells were treated for 24 hours with AF IC_{75} and then changes in the mitochondrial membrane potential were evaluated using the Mito-Tracker Red CMXRos (Invitrogen) by flow cytometry. Mitochondrial cytochrome-c (cyt-c) release was assessed on permeabilized and fixed cells. After washing twice with phosphate-buffered saline (to eliminate cyt-c in the cytoplasm), the residual cyt-c was detected using the mouse anti–cyt-c antibody (Becton-Dickinson [BD]) followed by phycoerythrin (PE)-conjugated goat anti-mouse immunoglobulins (IgG) (BD). (C) Bcl-2 associated X protein (BAX), B-cell lymphoma/leukemia-2 protein (Bcl-2), and B-cell lymphoma-extra large protein (Bcl-xL) were analyzed using anti-Bax generated from Bax-α (BD), followed by PE-conjugated goat anti-mouse human IgG (BD), anti–Bcl-2-fluorescein isothiocyanate (FITC) (clone 124) (DAKO), and anti-human Bcl-xL (Cell Signaling) followed by goat anti-rabbit IgG-FITC (Jackson ImmunoResearch). Fluorescence-activated cell sorter (FACS) histograms are representative of 1 of 3 different experiments. Dotted lines indicate background fluorescence of cells, as determined by isotype-matched immunoglobulins (Ig). X- and Y-axes indicate the logarithms of the relative intensity of fluorescence and relative cell number, respectively. (D) Thioredoxin reductase (TrxR) activity was evaluated and expressed as enzyme unit per milligram of total protein (U/mg) in cHL cells (2.0×10^5/mL) treated for 24 hours with AF. TrxR (EC 1.8.1.9) activity was assessed using the TrxR Assay Kit (Sigma) in cell lysates. The relative activity was standardized by protein concentration using the protein assay dye reagent (Bio-Rad Laboratories). (E) Mitochondrial reactive oxygen species (ROS) production in the presence or absence of the antioxidant *N*-acetyl-cysteine (NAC; 5 mM) (Sigma) was evaluated by flow cytometry using MitoSox reagent (Molecular Probes). Results represent the mean ± standard error of the mean (SEM) of 3 replicate wells from 3 independent experiments. (F). Nuclear factor κB p65 transcription factor activity in nuclear extracts of cHL cells (2.0×10^5/mL) treated for 12 hours with AF was analyzed using the Transcription Factor Kit (p65) (Pierce Biotechnology). Values in the bar graph represent the mean ± SEM of 3 different experiments and standardized by protein concentration using the protein assay dye reagent (Bio-Rad Laboratories). (G) IRF4, CD40, CD30, discoidin domain receptor 1 (DDR1), Notch1, and Jagged1 expression were evaluated in cHL cells incubated with AF for 12 hours by flow cytometry using anti-IRF4 (M-17) (Santa Cruz Biotechnology), anti–CD40-PE (BD), anti–CD30-FITC (DAKO), anti-DDR1-PE (48B3) (Santa Cruz Biotechnology), anti–Notch1-PE (R&D System), and anti-Jagged1 (J1G53-3) (AdipoGen) antibodies. Histograms represent the mean fluorescence intensity. (H) (2.0×10^5/mL) L-540 cells were treated with AF in the presence or in the absence of a confluent layer of primary Hodgkin lymphoma-derived mesenchymal stromal cells (HL-MSCs), obtained from 3 separate freshly excised lymph node preparations from a patient affected by nodular sclerosis cHL. Alternatively, L-540 cells were incubated with HL-MSCs-conditioned medium (CM) for 2 hours, and then were treated with AF for 2 additional days. Apoptosis was evaluated by Annexin-V-FITC/PI staining after 48 hours. Data are expressed as mean ± SEM of 3 separate experiments. (I) The release of CCL5/Rantes and CCL17/TARC in CM of cHL cells (2.0×10^5/mL) cultured with AF for 48 hours was measured using commercially available kits according to manufacturer's instructions (Pierce Biotechnology and R&D System, respectively). Results are expressed as pg/mg of proteins and represent the mean ± SEM of 3 different experiments. (J) Migration of peripheral blood mononuclear cells (PBMCs) from healthy donors was assessed using the fluorescence-assisted transmigration invasion and motility assay (FATIMA). 2.0×10^5 PBMCs/insert were seeded in fibronectin-coated Boyden chamber inserts. L-1236-CM treated or untreated with AF for 48 hours was used as a chemoattractant. The percentages of transmigrated cells were measured using a computer-interfaced GeniusPlus microplate reader (Tecan). Histograms represent the percentage of migrated cells after 2 hours. Values represent the mean ± SEM of 3 different experiments. (K) L-540 (20×10^6 cells) derived tumor volume was measured with a caliper in female athymic nu/nu (nude) mice purchased from Charles River after intraperitoneal injection (3 times a week) of either drug-free medium or with 10 mg/kg AF. Histograms represent the weight of mice measured at different time points during treatment. Data represent the mean ± SEM of 8 animals per group. (L) Angiogenesis in vivo. Excised tumors were included in the optimal cutting temperature compound (Kaltek) and were frozen. Tumors were sectioned (7 μm) and subjected to immunofluorescence staining with anti-mouse CD31 (BD) and TO-PRO-3 (Invitrogen). Representative pictures were acquired with a LEICA TCS SP2 confocal system (Leica Microsystems GmbH), and vessel density was calculated using the image processing in Java software ImageJ. (M) Tube formation. Human umbilical vein endothelial cells (1×10^4/well) on Matrigel (growth factor reduced; BD) were cultured with RPMI (10% FCS) (positive control), serum-free (SF) medium, CM from AF treated and untreated L-540 cells for 12 hours. Representative photographs (Eclipse TS/100, Nikon, with photomicrographic systems DS Camera Control Unit DS-L2) showing tube formation with a different medium or supernatants. Purple lines identify tubes, white dots show branching points, blue areas are cells (magnification ×4). (N) Bar graphs represent total tube length (px, pixel) and branching points connecting the tubes quantified using the tool WimTube (Wimasis Image Analysis, GmbH). Data are expressed as mean ± SEM of 3 independent experiments. (O) Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) released by AF (48 hours) treated and untreated L-540 cells, cultured in SF medium. Cytokine concentrations were measured using commercially available kits, according to manufacturer's instructions (VEGF, Immunological Sciences, and FGF-2, Peprotech). Results are expressed as pg/mg of proteins and represent the mean ± SEM of 1 of 3 different experiments. Statistical analysis was performed using GraphPad Prism 6 Software (GraphPad). The significance of differences was determined by Student *t* test for comparison between 2 groups. Analysis of variance was used to evaluate the correlation of data among 3 or more groups; consecutive multiple comparison analysis was performed using Dunnett or Tukey tests. $P < .05$ was considered statistically significant. $^*P < .05$; $^{**}P < .01$; $^{***}P < .001$; $^{****}P < .0001$.

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Contribution: M.C., C.B., N.C., M.M., A.P., and X.U.K. generated and interpreted data; M.C., N.C., M.S., A.C., and D.A. wrote and/or revised the manuscript; and D.A. supervised the study.

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