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Design, development, and validation of a high-throughput drug-screening assay for targeting of human leukemia

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Abstract

Background—We introduce an *ex vivo* methodology to perform drug library screening against human leukemia.

Method—Our strategy relies on human blood or bone marrow cultures under hypoxia; under these conditions, leukemia cells deplete oxygen faster than normal cells, causing a hemoglobin oxygenation shift. We demonstrate several advantages: (I) partial recapitulation of the leukemia microenvironment, (ii) use of native hemoglobin oxygenation as real-time sensor/reporter, (iii) cost-effectiveness, (iv) species-specificity, and (v) format that enables high-throughput screening.

Results—As a proof-of-concept, we screened a chemical library (size ~20,000) against human leukemia cells. We identified 70 compounds ("hit" rate=0.35%; Z-factor=0.71) with activity; we examined 20 to find 18 true-positives (90%). Finally, we show that carbonohydraxonic diamide group-containing compounds are potent anti-leukemia agents that induce cell death in leukemia cells and patient-derived samples.

Conclusions—This unique functional assay can identify novel drug candidates as well as find future applications in personalized drug selection for leukemia patients.

Keywords

blood; bone marrow; chemical library; drug screening; hypoxia; leukemia targeting; tumor microenvironment

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Introduction

Functional screening platforms that can assess drug candidates within the appropriate tumor microenvironment are needed. First, mouse bone marrow is generally less sensitive to many cytotoxics than is human bone marrow, thus often rendering murine leukemia models inadequate in emulating myelosuppression in patients.[1] Second, the co-existence of non-malignant host cells (immune, stromal, mesenchymal) and leukemia stem cells provoke complex tumor-host interactions that may affect drug resistance, self-tolerance, angiogenesis, tumor growth, and response to therapy.[2] Species-specific differences between human leukemia cells and mouse stromal cells can alter the tumor growth and drug responses in murine models,[3] and the deficiency of functional immune system in these models may also interfere with the outcome.[2]

Ex vivo models utilizing human tissue as a screening platform are valuable preclinical tools. In human solid tumors, multicellular tumor spheroid models have shown to recapitulate *in vivo*-like growth and have proven to be excellent *in vitro* 3D-models for high-throughput drug discovery.[4] Here, we describe a new functional high-throughput *ex vivo* screening assay against leukemia, which is based on culturing leukemia cells in human blood or bone marrow under hypoxic conditions. We reasoned that these co-cultures mimic the disease microenvironment and therefore partially recapitulate at least some attributes of leukemia in patients. Moreover, the oxygenation state of native hemoglobin reliably and reproducibly serves as a "built-in" indicator of leukemia cell growth and/or viability, therefore overcoming the need for elaborate detection methods in a multicellular setting.

As a proof-of-concept, we have used this assay for a chemical library screening on established leukemia cell lines to select "microenvironment-stable" drugs with potential for translation into clinical applications. Via using this assay, we identified a subset of carbonohydraxonic diamide group-containing compounds that markedly and specifically inhibited several leukemia cell lines and a panel of clinical samples obtained from leukemia patients. Together, these data suggest that testing of libraries of compounds or candidate drugs in this new *ex vivo* model may yield compounds against human leukemia, which are potentially active in the circulation and/or bone marrow microenvironment.

Methods

Cell culture

OCI-AML3, Kasumi-1, THP-1, HL-60, MOLT-4, CCRF-CEM, HL-60, RPMI-8226, SR-786, U937, KBM7, K562 and K562-luc2 Bioware® Ultra (Caliper LifeSciences) were maintained in humidified hypoxia chambers (HeraCell 150, Thermo Electron Corporation) with 5% CO_2 and 5% oxygen at 37° C in RPMI1640 containing 10% fetal bovine serum (FBS), penicillin, and streptomycin.

Blood and bone marrow samples from leukemia patients and normal volunteers

The Institutional Review Board of M. D. Anderson Cancer Center approved the use of whole blood and bone marrow obtained from patients or healthy donors. Peripheral blood and bone marrow samples were obtained from patients with AML who had signed an

informed consent in accordance with the Declaration of Helsinki. Blood samples from healthy volunteers were obtained through the hospital's Blood Bank and Transfusion Services. We used anonymized blood samples, which had been previously tested and proven negative against blood-transmittable diseases. These specimens were stored at 4°C for 24 h before use. Heparin was used as an anti-coagulant. Pre-tested whole blood and bone marrow were also obtained from commercial sources (Innovative Research or AllCells).

Assays containing human peripheral whole blood and bone marrow

Leukemia cells were plated at 20,000 per well in 100 μl of RPMI containing 10% human whole blood, heparin (100 μg/ml), L-glutamine (0.292 mg/ml), penicillin (100 units/ml), and streptomycin (100 units/ml) in 96-well plates with flat-bottomed wells (Becton Dickinson). 10% blood specimens from patients with AML and 5-10% bone marrow aspirates were diluted in either RPMI or complete StemPro TM-34SFM (GibcoBRL) culture medium containing heparin, L-glutamine, penicillin, and streptomycin. The microplates were incubated under hypoxia (without shaking) and the optical density at 600 nm (OD_{600}) was measured at the starting point, 20 h and/or 40 h incubation. The Micros60 analyzer (ABX Diagnostics) was used to count white blood cells (WBC), granulocytes, monocytes, lymphocytes, red blood cells (RBC), platelets, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and red blood cell distribution width.

Chemical library and drug screening against leukemia cell lines

We used the DiverSet Chemical Library (Chembridge) formatted in 96-well plates and containing small-molecule compounds with drug-like properties. We screened 20,000 individual compounds from the chemical library, each at 20 μM, against OCI-AML3 cell lines. Primary compounds that decreased the OD_{600} by at least 0.2 units were selected for secondary screening and analysis; moreover, structural analogues with at least 50% similarity to the primary compounds were commercially obtained (Chembridge) and subsequently evaluated.

Leukemia proliferation, viability, and cell death assays

Proliferation of luciferase-transfected K562 leukemia cells was determined with substrate D-luciferin (Xenogen) incubated at 150 μg/ml per well for 1 h followed by measurement of the luminescence (SpectraMax 5; SoftMax Pro 5). Cell proliferation and viability were measured with a lactate dehydrogenase (LDH) activity assay (DHL, AnaSpec). To measure incorporation of 5-bromo-2′-deoxyuridine (BrdU) (Calbiochem) cells were incubated for 2 h with BrdU, red blood cells were lysed with lysis buffer (Roche), and the remaining cells were pelleted and fixed for immuno-determination of incorporated BrdU. For the assessment of cell apoptosis and necrosis, a total of 10^6 OCI-AML3 cells were incubated for 4 h at 37°C in hypoxia status in a volume of 5 ml in the presence of each test compound at 20 μM. After washing, the cells were stained with an FITC-conjugated Annexin V antibody plus propidium iodide (Sigma) and subsequently analyzed by flow cytometry (BD Canto II).

Statistical analysis

All data are reported as the mean ± standard deviation (SD). Student's *t*-tests (unpaired) were used to determine statistical significance ($n = 3$, unless otherwise specified). P-values considered statistically significant are indicated with asterisks: less than 0.05*, less than 0.01**, or less than 0.001***. Correlations were calculated with the Pearson correlation coefficient. The Z-factor was calculated according to the described formula[5].

Results

Real-time leukemia cell monitoring in an *ex vivo* blood-containing assay

A panel of leukemia cells (K562, OCI-AML3, Molt-4, THP-1, Kasumi-1) were found to proliferate in a medium containing 1:10 (vol/vol) whole blood from healthy donors under 5% oxygen (**data not shown**). Under these conditions, live leukemia cells induced a hemoglobin color switch to dark purple. The hemoglobin purple color switch was reversible to bright red when the samples were returned to 21% oxygen, and shaking accelerated this change, a result indicative that the color change depends on the oxygenation state of hemoglobin (Fig. 1A). We hypothesized that this assay might be a suitable platform for screening of anti-leukemia compounds stable in the presence of blood. A schematic presentation of the screening is shown (Fig. 1B) along with an example of a 96-microwell plate containing a newly discovered leukemia cell inhibitor (Fig. 1C).

Optimization of the blood-containing *ex vivo* assay for leukemia cells

Time-dependent proliferation of OCI-AML3 cells in the *ex vivo* cultures was demonstrated with a blood count analyzer, which showed an increase in total white blood cells (Fig. 2A). In addition to the hemoglobin-mediated color switch, leukemia cell status was also evaluated by three other independent assays: cellular LDH activity, BrdU incorporation, and activity of transfected luciferase (Fig. 2B-D). Leukemia cell growth above background became detectable after culturing for 20 h or longer. We used whole blood samples from more than 200 individual healthy donors, all of them supporting leukemia cell growth.

OD_{600} measurements were substantially higher in leukemia *ex vivo* assays cultured in hypoxia rather than in normoxia after 40 h (Fig. 2E). Both cultures were next oxygenated by mixing and the differences in OD_{600} measurements virtually disappeared, indicating that the higher OD_{600} can be observed after the leukemia cells deplete oxygen from the microenvironment. Background levels of OD_{600} varied little (range 1.7-1.9) at 0 h. The OD_{600} increased less than 0.2 units in leukemia-free control assays after incubation for 40 h; in contrast, the presence of leukemia cells generally increased OD_{600} by at least 0.4 to 0.6 units (Fig. 2E). To exclude the possibility that different cell growth rates in hypoxia versus normoxia affected the OD_{600} measurements, we showed that leukemia cells grew similarly in both conditions (Fig. 2F). In addition to normal peripheral blood, we also established that leukemia cells could be cultured in medium containing human bone marrow samples obtained from healthy volunteer donors (Fig. 2G). In these samples, the oxygen consumption of live cells could also be determined by measuring the OD_{600} (Fig. 2H). Correspondingly, the OD_{600} decreased upon oxidation of these cultures (Fig. 2I). To confirm that the blood-containing assay could be used to detect anti-leukemia compounds,

we used etoposide, a topoisomerase II inhibitor with known cytotoxic activity,[6] as a positive control (Fig. 2J).

To verify the dependence of OD_{600} values on leukemia cell viability and growth, we determined both OD_{600} and WBC counts in two different donor blood samples in the presence or absence of several anti-leukemia compounds. As measured by the blood count analyzer, the increase in OD_{600} values correlated with the increase in total WBC counts (r =0.81 and r =0.79), granulocytes (r=0.82 and r=0.84), and to a lesser extent monocytes (r=0.66 and r=0.65) (Fig. 3). Therefore, we concluded that the absorbance of hemoglobin[7,8] at OD_{600} may be reliably used as an initial indicator of leukemia viability and/or cell growth in these assays.

Screening of anti-leukemia compounds in the presence of human whole blood

As a proof-of-concept, we next screened a 20,000 small molecular weight compound library (ChemBridge) against OCI-AML3 leukemia cells. We observed a ∼0.35% "hit rate" to identify 70 potential anti-leukemia compounds (**data not shown**) that decreased the OD_{600} by at least 0.2 units. The Z-factor,[5] calculated for positive hits against background in a particular blood sample, was 0.71 (n=30). We examined a subset of 20 randomly selected compounds out of the initial "hit pool" and demonstrated that 18 out of 20 (90%) retained activity against OCI-AML3 cells at a 10 μM concentration in standard culture conditions in the absence of blood (**data not shown**). Next, we selected four structurally different test compounds out of the 18 active candidates for subsequent studies (termed #1, #2, #3, and #4), and compared their efficacy to inhibit leukemia cells in the presence of blood by using different cell proliferation/viability detection methods (Fig. 4A). Of these, the compound #1 (N″-{4-[(4-bromo-2,3,5,6-tetramethylbenzyl)oxy] -3-methoxy-benzylidene} carbonohydrazonic diamide hydrochloride) had the most robust activity. To identify the chemical group(s) required for the anti-leukemia activity, we evaluated a panel of structural analogues of compounds #1, #2, #3, and #4 with the criterion that similarity to the parent compound must be at least 50%. These compound analogues were tested on both the OCI-AML3 and K562 leukemia cells in the blood-containing assay (Fig. 4B), and on other leukemia and lymphoma cell lines in standard culture (Fig.5). These experiments showed that five analogues of compound #1, all of which contained a carbonohydrazonic diamide group, were the most effective inhibitors of leukemia cells both in the presence and absence of blood. Several analogues, which lacked carbonohydrazonic diamide or had other modifications, were inactive in the presence of blood. The statistically significant result (Fisher's exact test, p=0.0217) indicates that carbonohydrazonic diamide is required for the growth inhibitory activity of this class of compounds.

Effects of carbonohydrazonic diamide-containing compounds on leukemia cells

We further analyzed a panel (n=7) of carbonohydrazonic diamide-containing compounds along with the negative control compound #1N (Fig. 6A). We found that the carbonohydrazonic diamide-containing compounds inhibited the increase in OD_{600} and the leukemia cell counts, and reduced the WBC count to background levels at the 20 h time point with <0.05-0.001% probability (Fig. 6B). We next examined apoptosis induction in OCI-AML3 cells by these compounds. The analog compounds #1A, #1C, #1D, and #1E

induced apoptosis more efficiently (>70-80%) than the original compound #1 (>50%) as determined by Annexin V and propidium iodide staining after 4 h incubation (Fig. 6C). The compound #1B showed only a slight increase (∼5%) in apoptosis compared to control cells. The control compound #1N, which does not contain carbonohydrazonic diamide, did not induce apoptosis.

Next, we analyzed the effects of carbonohydrazonic diamide-containing compounds on different blood cell populations at 0, 20, and 40 h via blood cell counter. In general, OCI-AML3 leukemia cell growth increased the total WBC ∼2-fold after incubation for 40 h, and the increase was detected mainly in the granulocyte population and to a lesser extent in the monocytes. The assay analysis of compound #1A is shown as an example: the compound #1A at 10 μM prevented the increase in WBC counts, and had no clear inhibitory effect on lymphocytes or RBC (Fig. 7A).

Carbonohydrazonic diamide-containing compounds also inhibited leukemia cells in the assay with aspirated human bone marrow. As in the blood-containing assay, the example compound #1A (Fig. 7B) inhibited the increase in OD_{600} and in leukemia cell counts in the bone marrow-containing assay. Notably, the compound #1A (at 10 μM) sufficed to inhibit leukemia cell growth in the blood-containing medium but 20 μM was required for efficient inhibition in the bone marrow-containing medium, even when adjusted for the lower vol/vol used (i.e., 5% for bone marrow rather than 10% for blood).

Carbonohydrazonic diamide-containing compounds inhibit primary AML cells

To validate the translational potential of the blood assay, we used peripheral blood obtained from AML patients, and observed that carbonohydrazonic diamide-containing compounds inhibited primary leukemia cell growth and/or viability in the assays with patient blood. Specifically, of the six compounds with activity against leukemia cell lines, compounds #1A and #1B, which differ only by one methyl group, proved to be the most effective, reducing the OD_{600} in all samples evaluated (Fig. 8A). Notably, the activities of compounds #1, #1C, and #1E varied in different AML samples, suggesting that the efficacy of these compounds may be different in every patient. In contrast, the compound #1D was without detectable effects against primary AML. The negative control #1N did not reduce the OD_{600} (Fig. 8A) while the positive controls (etoposide, actinomycin D, and staurosporin) consistently reduced the OD_{600} significantly (Fig. 8B). To determine whether the effects of the carbonohydrazonic diamide-containing compounds were leukemia-specific, we measured their effects in *ex vivo* assays of normal blood of healthy donors. In two of three normal blood samples the compounds had no statistically significant effects but in the third sample the compound #1A reduced the OD_{600} slightly, suggesting individual-specific differences in susceptibility (Fig. 8C).

To confirm that the increase in OD_{600} correlated with the WBC counts also in the patient sample setting, we tested the effect of the leukemia cell counts on OD_{600}. WBCs from AML patient samples were isolated and then re-inserted into the *ex vivo* cultures at concentrations which enriched the WBC count ∼2-fold. This resulted into a corresponding increase in OD_{600}, indicating that the WBC counts are a fundamental factor determining the OD_{600} (Fig. 8D).

Finally, the compounds were evaluated on primary AML cells cultured in standard culture media. In these conditions, all the carbonohydrazonic diamide-containing compounds, except #1D, were efficient inhibitors of primary cells isolated from AML patients (n=3) (Fig. 8E). In effect, isolated primary AML cells grown in the absence of blood gave results similar to those obtained with established leukemia and lymphoma cell lines in standard culture conditions (Fig. 5).

Discussion

In this study, we introduce a novel *ex vivo* assay for leukemia, which provides a high-throughput screening platform for identification of compounds that are active in the presence of human blood and bone marrow under hypoxia. This assay allows detection of live leukemia cells by using the hemoglobin oxygenation state as an internal readout system that can be measured via OD_{600} based on hemoglobin absorption spectra[7,8], and therefore does not require introduction of either chemically active exogenous markers to measure leukemia cell growth or oxygen probes to estimate cancer cell metabolic rates. Specifically, we observed that viable human leukemia cells would rapidly deplete oxygen from the medium and predominantly contribute to the high OD_{600} values detected; in contrast, control normal blood incubated with no leukemia cells yielded only slightly elevated levels. This increase in OD_{600} correlates well with the increase in leukemia cell counts, which indicates that the levels of deoxyhemoglobin in the medium increase in accordance with the oxygen consumption by leukemia cells. Moreover, the OD_{600} values could be manipulated with the oxygen levels – independent of the other variables – confirming that the oxygenation state of the hemoglobin directly contributed to the OD_{600} value measured.

Empirically, we detected clear differences in the drug activities of several individual compounds when leukemia cells were grown in standard cultured media versus the blood-containing medium. Thus, we focused this original study on the discovery of drugs with robust activity in the presence of human whole blood. The “Z-factor” – a coefficient that reflects both the assay signal dynamic range and the data variation associated with the signal measurements[5] – was calculated to be around 0.7 for our assay, which indicates that the results are reproducible and accurate. Notably, differences among donor blood samples can contribute to observed variations in efficacy of drug candidate evaluations.

As an initial proof-of-concept for the assay, we screened a library of ∼20,000 individual chemical compounds against OCI-AML3 cell line. We identified 70 lead compounds with activity against the human leukemia cell lines (library/assay “hit” rate=0.35%); most of the compounds had not been previously described in the literature but a few of them had structurally similarity to compounds with anti-cancer, anti-inflammatory, or anti-microbial activity. A few specific examples of promising drug leads merit mention. Compound #2 is active against the blood-borne protozoan parasite *Trypanosoma cruzi*[9] and another compound selectively induces apoptosis in tumor cells independently of P-glycoprotein status.[10] Notably, certain structural moieties were common within the discovered compounds, such as piperazine, hydrazide, and hydrazone groups. Some of these derivatives had also been reported with anti-tumor activity.[11] Moreover, from the 70 “hits” in our initial

screen, we re-examined an arbitrarily chosen subset of 20 compounds and found that 18 of them (90%) were reproducibly true-positives for anti-leukemia activity.

Several limitations of this proof-of-concept for assay development and candidate drug discovery study merit further comment. First, although specificity and toxicity of the selected drugs are beyond the scope of this initial work, we observed the requirement of relatively high molar concentrations; this might be at least in part due to loss of drug activities in the chemical library setting secondary to presence of impurities and salts or degradation (i.e., non-GMP grade materials). Indeed, the simplest carbonohydraxonic diamide, aminoguanidine, has already been examined in clinical trials as a potential anti-diabetic agent,[12] a fact suggesting that such compounds may be tolerated in mammals *in vivo*. To begin to address this possibility, we have *de novo* re-synthesized compound #1A and found it to be active and stable in low micromolar concentrations in the presence of whole blood, suggesting that translational applications with this compound may actually be feasible. Moreover, our whole blood assay is certainly not sufficient to address the issue of cell specificity (e.g., normal versus malignant cells or leukemia versus non-leukemia tumor cells); comprehensive toxicology evaluation in bone marrow, lymphatic vasculature as well as other non-hematopoetic organs will be needed to assess the safety and potential therapy applications of any of these compounds. Finally, whether or not the growth inhibitory effect of carbonohydraxonic diamide group-containing compounds is specific remains an open question. Further mechanistic studies will be needed to determine the specificity of carbonohydraxonic diamide-containing compounds towards AML cells as several potential molecular targets for these type of compounds have been reported such as glucose-mediated protein dimerization,[12] nitrix oxide synthase,[13] furin,[14] and E2 ubiquitin conjugating enzyme.[15,16] Ultimately, formal toxicology GLP studies in animals will be required in future studies to determine whether or not a first-in-human and/or phase zero clinical trial will go forward.

Our results indicate that the unique functional drug-screening assay introduced here has the capability to identify novel microenvironment-stable drug candidates. This assay may also find future applications in personalized drug selection for leukemia patients as therapeutic compound responses are correlated to specific cancer genotypes,[17] which can greatly vary from individual to individual. In effect, in the pilot experiments, we were able to optimize the blood-containing assay for blood samples directly from AML patients. Furthermore, similar studies with human bone marrow co-cultures showed promise that drug efficacy could also be individually validated in a bone marrow-like microenvironment, in which resistance is often encountered, possibly due to increased protection of tumor cells by bone marrow stroma.[2] Together, our results support the idea that this new methodology could potentially be used as a tool in predicting drug efficacy and/or response in each leukemia patient.

Acknowledgments

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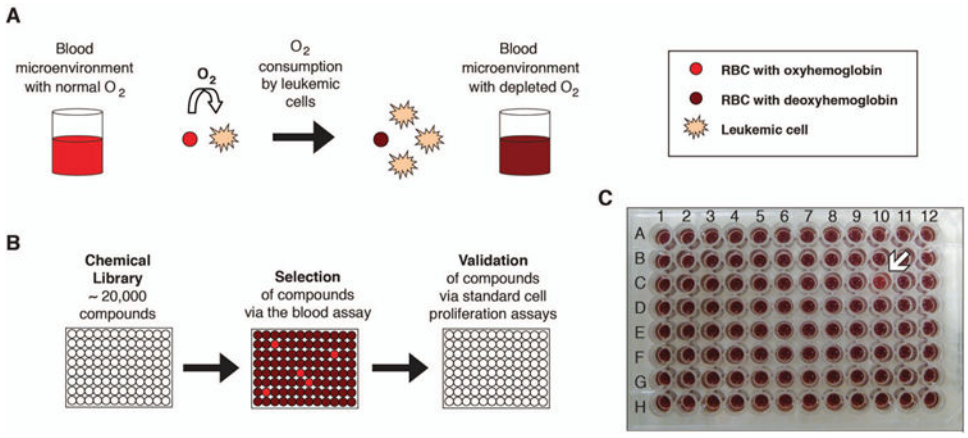

Figure 1.

Monitoring leukemia cell viability in a human blood-containing assay. (a) A schematic presentation of oxygen consumption of leukemic cells in the presence of blood. The oxygen consumption of metabolically active leukemia cells is seen as a color change in the presence of blood under hypoxia, as leukemia cells deplete the surrounding oxygen, causing hemoglobin to shift to its deoxyhemoglobin conformation. (b) A schematic presentation of a large chemical library screening, with bright red wells displaying leukemia cell cultures that were not metabolically active. (c) A 96-well plate with different compounds tested in each well. The arrow indicates a compound of interest, which inhibited the color shift and was therefore selected for further studies.

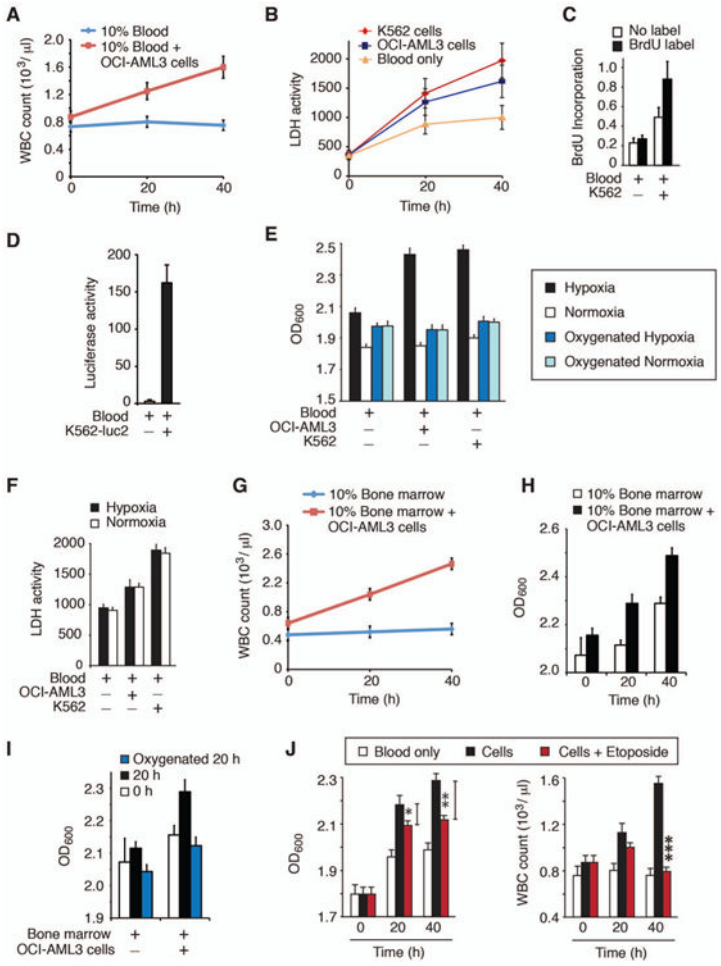

Figure 2.

Leukemia cell cultures in the presence of human whole blood or bone marrow aspirate. K562 or OCI-AML3 cell lines were cultured at 20,000 cells per well in a culture medium containing 10% normal human blood or bone marrow aspirate as described. (a) Increase in leukemia cell counts in the presence of blood was determined by measuring total white blood cell (WBC) counts. (b) Cell viability and growth were determined by measuring LDH activity, and (c) dividing cells were assessed via BrdU incorporation. (d) Increase in luciferase activity was measured in stably transfected K562-luc2 cells. (e) The color shift was detected via the optical density at 600 nm (OD_{600}). OD_{600} was measured both in hypoxia and normoxia cultures grown for 40 h. To assess the dependence of the color shift on the oxygen tension, both cultures were oxygenated (by gently pipetting up and down) and OD_{600} was measured again. (f) LDH activity was compared between cells grown in hypoxia and normoxia for 40 h. (g) Increase in leukemia cell counts in the presence of bone marrow aspirate was determined by measuring total WBC counts. (h) OD_{600} of cultures with bone marrow aspirates was determined. (i) The absorbances were read again after oxygenating the cultures as previously. (j) Etoposide (10 µM), which inhibits the color shift due to its anti-leukemia activity, served as a positive control. The results represent means ± standard deviations (SD) from triplicate wells. In this and other figures: The following symbols were used: * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

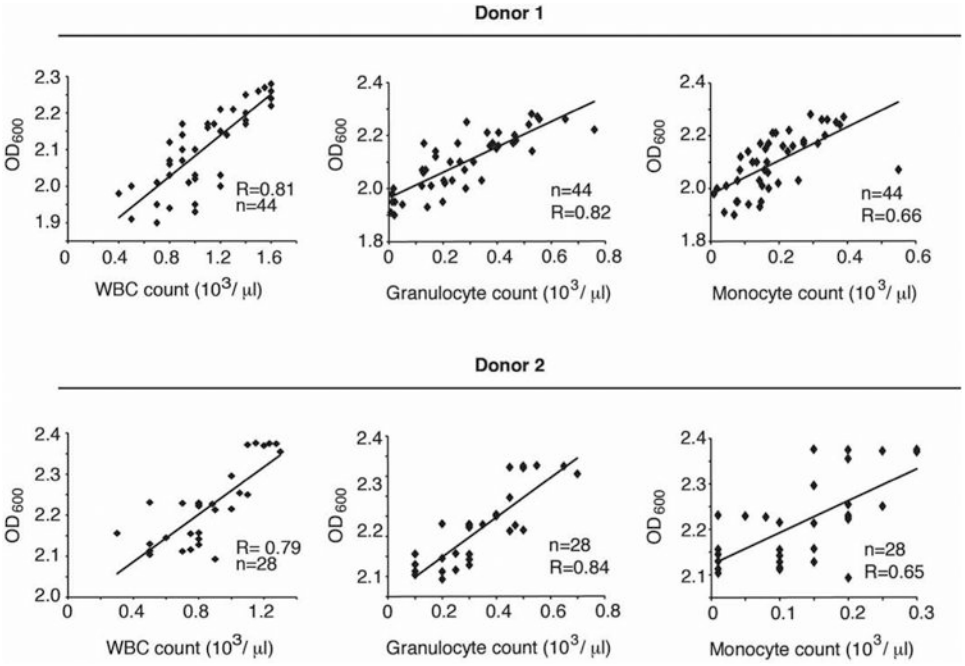

Figure 3.

Relationship between the OD_{600} value and counts determined by automatic blood analyzer of total WBC, and the subsets of granulocytes and monocytes in two different donor blood specimens. Correlation was determined by culturing OCI-AML3 in 10% blood for 20 h and 40 h in the absence and presence of inhibitors (n=10 for donor 1 and n=7 for donor 2, each at 10 and 20 μM) that had previously been discovered in the screening process.

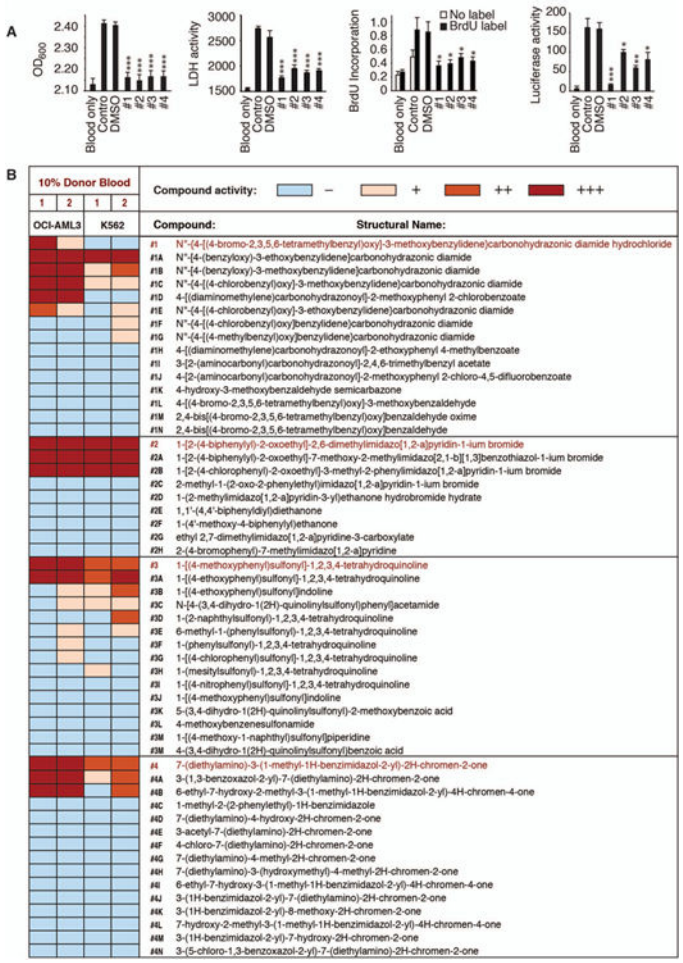

Figure 4.

Evaluation of the four selected primary compounds and their analogs. (A) Activities of the compounds were compared with the OD_{600}, LDH, BrdU incorporation, and luciferase assays. OCI-AML3 or K562 cells were cultured in RPMI containing 10% human blood. (B) Heat-map presentation of the activities of the four selected primary compounds and their corresponding structural analogs in OCI-AML3 or K562 cells cultured in 10% blood. Two different donor bloods were used. The following symbols were used: +++ $\Delta OD_{600} \leq 0.3$, ++ $\Delta OD_{600} \leq 0.2$, + $\Delta OD_{600} \leq 0.1$

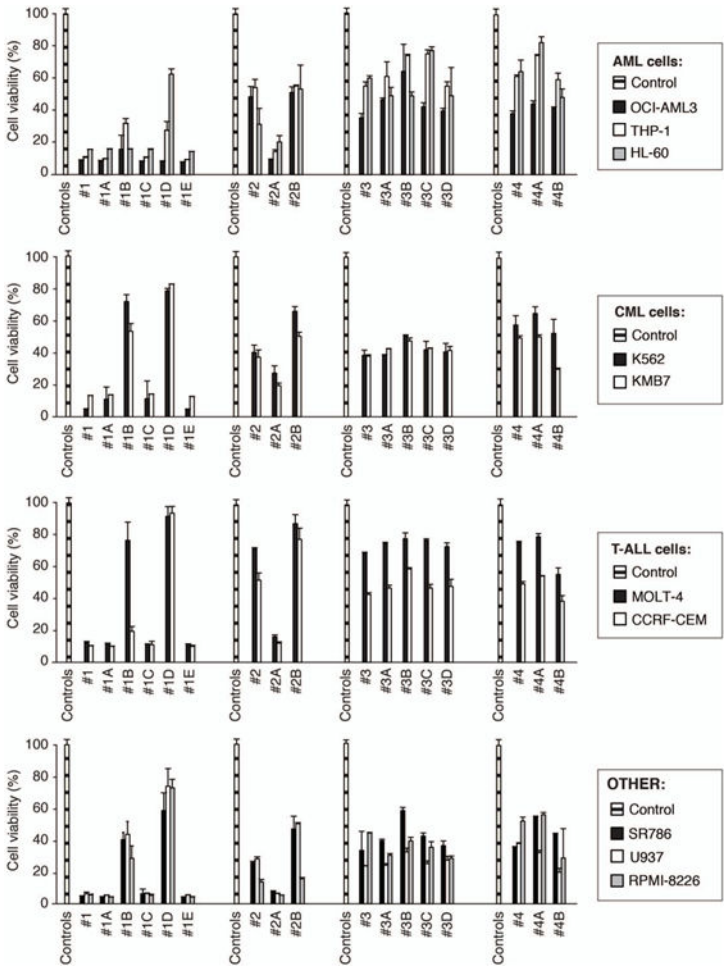

Figure 5.

Activities of the selected structural analogs on a representative panel of human leukemia and lymphoma cell lines (n=10) in standard culture in the absence of blood. Compounds are numbered according to Figure 3b. Cell viability and proliferation were determined by the LDH assay. The results represent means ± SD from triplicate wells.

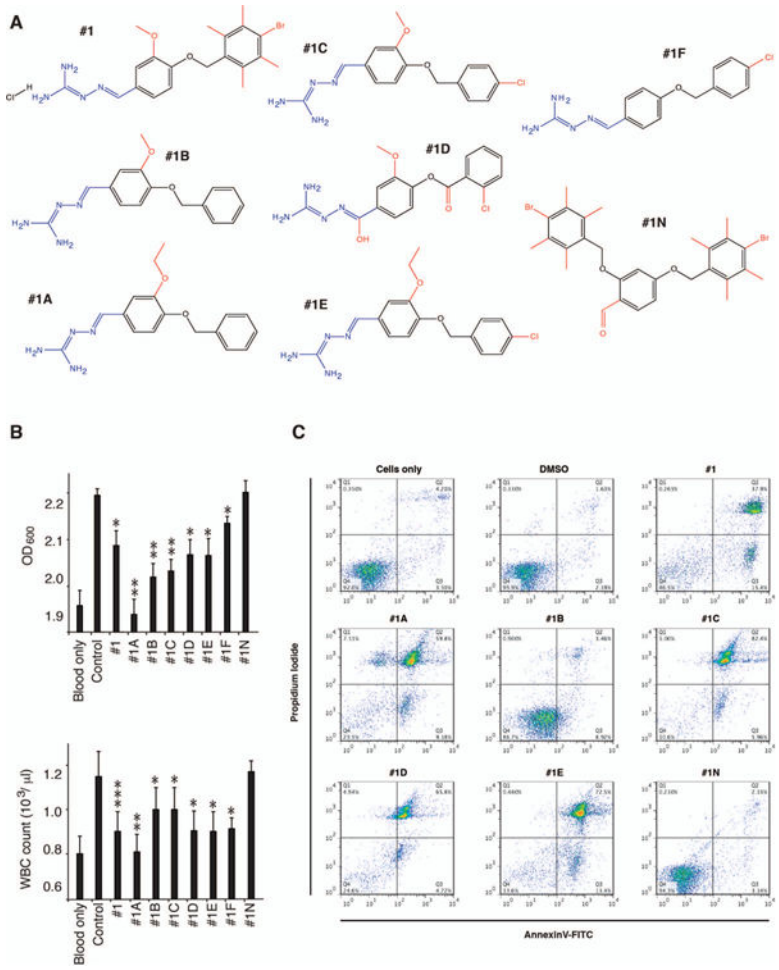

Figure 6.

Carbonohydraxonic diamide-containing compounds. (a) Chemical structures of six active carbonohydraxonic diamide-containing compounds. The carbonohydrazonic diamide group is depicted in blue and the differences in structures between different compounds are depicted in red. (b) The effects of carbonohydrazonic diamide-containing compounds (at 10 μM each) are plotted for OCI-AML3 cells cultured in media containing human blood. OD_{600} and WBC counts were determined at 20 h time point. The results represent means ± SD from triplicate wells. (c) Induction of cell apoptosis and necrosis by carbonohydrazonic diamide-containing compounds as indicated. Cells were analyzed by flow cytometry after staining with Annexin-V-FITC and propidium iodide.

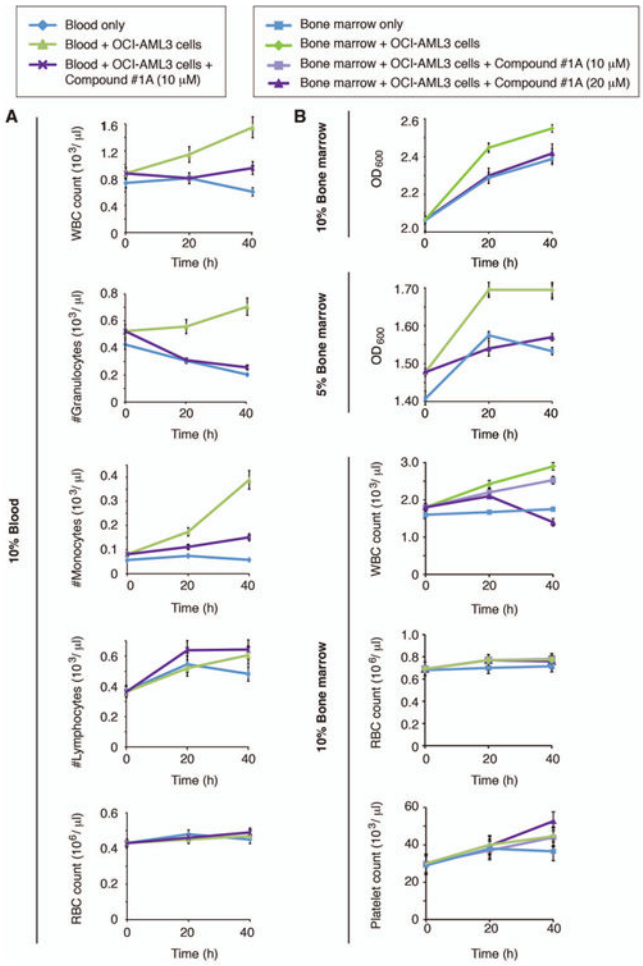

Figure 7.

Effects of compound #1A on individual blood cell populations. (a) OCI-AML3 cells were cultured at 20,000 cells per well in culture medium containing 10% blood in the absence or presence of compound #1A (10 μM). WBC, lymphocytes, monocytes, granulocytes and RBC were determined via blood cell counter at the time points 0, 20 and 40 h. (b) OCI-AML3 cells were cultured at 20,000 cells per well in culture medium containing normal human bone marrow aspirate (5 or 10%) in the absence or presence of compound #1A (10 or 20 μM). Bone marrow without treatment served as a negative control. The OD_{600} was determined at the time points 0, 20 and 40 h. WBC, RBC, and platelets were determined. The results represent means ± SD from at least triplicate wells.

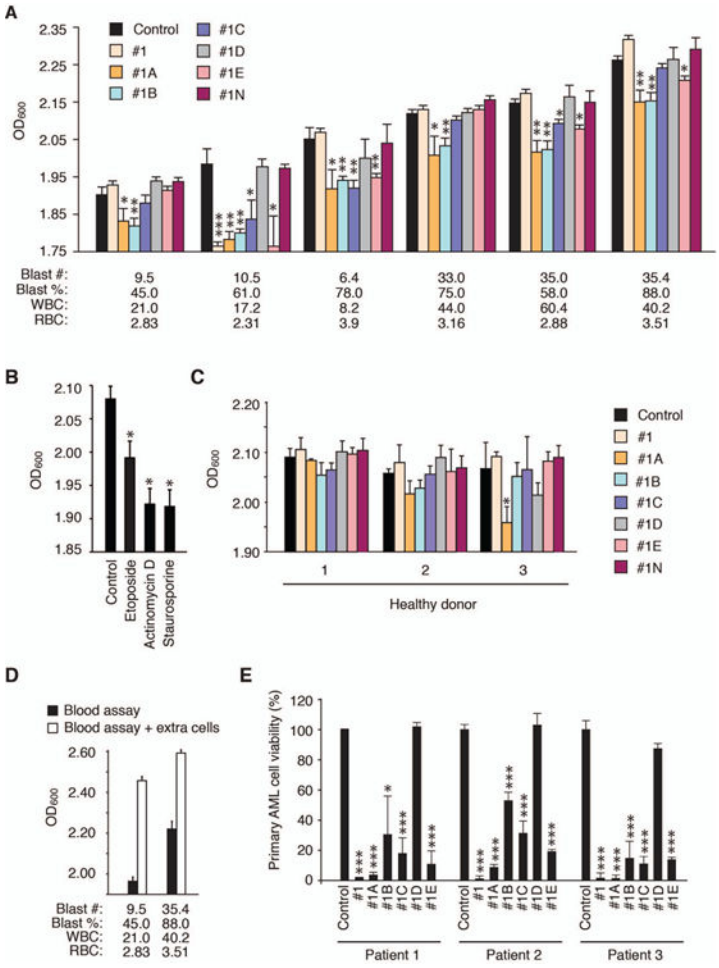

Figure 8.

Inhibition of primary AML cells by carbonohydraxonic diamide-containing compounds. (a) Blood samples from AML patients were diluted to 10% and incubated in the absence or presence of compounds as indicated (at 10 μM) for 20 h, and OD_{600} was measured. The initial blast number (blast #) (10^3/μl), blast %, WBC (10^3/μl), and RBC (10^6/μl) are shown for each patient sample. (b) Etoposide, actinomycin D, and staurosporin (at 10 μM), which have cytotoxic activities, served as positive controls for the patient blood assay. (c) Effect of carbonohydrazonic diamide-containing compounds on OD_{600} values of blood from healthy donors was measured after 20 h cultivation. (d) Effects of blast number and sample volume on OD_{600} values. Mononuclear cells were isolated and re-implanted in the patient blood samples. The initial blast number (blast #) (10^3/μl), blast %, WBC (10^3/μl), and RBC (10^6/μl) are shown for both patient samples. (e) Efficacy of carbonohydrazonic diamide-containing compounds on primary AML cells under standard tissue culture conditions. Cells were cultured for 24 h, after which cell viability was determined by the LDH assay. The results represent means ± SD from at least triplicate wells.