published techniques [30,31]. The purity of sorted cells amounted to >98%. The following AML cell lines were used: HL60, U937, and KG1. Cell lines were obtained from the DSMZ Institute (Braunschweig, Germany) and maintained in RPMI 1640 medium with 10% FCS at 37°C.

^{3}H-thymidine incorporation assay

Unfractionated AML cells (MNC), sorted CD34+/CD38- and CD34+/CD38+ AML cells, and cell lines were cultured in 96-well microtiter plates (1-5 x 10^4 cells/well) in the absence or presence of PEG-ZnPP or SMA-ZnPP (100 nM to 50 μM) at 37°C for up to 48 hours. Unfractionated MNC were kept with or without GM-CSF (100 ng/ml). Proliferation of sorted AML progenitor cells was induced by a cytokine-cocktail containing SCF (100 ng/ml), IL-3 (100 ng/ml), GM-CSF (100 ng/ml), and G-CSF (100 ng/ml). After exposure to drugs, 0.5 μCi ^{3}H-thymidine was added (12 hours). Cells were then harvested on filter membranes in a Filtermate 196 harvester (Packard Bioscience, Meriden, CT). Filters were air-dried, and the bound radioactivity was measured in a β-counter (Top-Count NXT, Packard Bioscience). In a separate set of experiments, AML cell lines were incubated with various concentrations of ZnPP (0.1-10 μM) or with combinations of Hsp-32-targeting drugs and ARA-C at a fixed dose-ratio (48 hours). All experiments were performed in triplicates.

Immunocytochemistry

Immunocytochemistry was performed on cytospin-slides prepared from primary leukemic cells (BM or PB MNC from 43 patients) as reported [25] using a polyclonal rabbit anti-HO-1 antibody. Newfuchsin (Nichirei, Tokyo, Japan) was used as chromogen. In control experiments, the primary antibody was omitted, was pre-incubated with control buffer, or was preincubated with a HO-1-specific blocking peptide (Stressgen Biotechnologies, Victoria, BC, Canada) prior to staining.

Quantitative PCR (qPCR)

PCR experiments were performed on RNA from unfractionated MNC as well as sorted CD34+/CD38+ and CD34+/CD38- cells (AML, n=10, cord blood cells pooled from 3 donors). mRNA levels were quantified on a 7900HT Fast Real-Time PCR System from Applied Biosystems (Foster City, CA), using iTaq SYBR Green Supermix with ROX from Bio-Rad (Hercules, CA) and Abl as reference gene as reported [26,32]. In addition, conventional PCR was performed. Primers used are shown in Table 2. In a separate set of experiments, unfractionated AML cells (n=6 donors) were exposed to control medium or hemin (10 μM) at 37°C for 4 hours prior to RNA isolation and qPCR analysis. Stable HO-1 knock-down in U937 cells was generated by transduction with a microRNA (miRNA) adapted retroviral vector as described [33]. Briefly, the LMP-miHO1 vector was used to produce recombinant VSV-G pseudotyped retroviruses. Cells were transduced in the presence of polybrene (7 μg/mL) and GFP positve cells were sorted on a FACSAria (Becton Dickinson Biosciences). Knock-down of HO-1 was confirmed by Western blotting.

Preparation of cell lysates and Western blotting

Western blot experiments were performed on primary AML cells and AML cell lines as described [25]. Cell lines were incubated in control medium with or without hemin

(10 μM) at 37°C for 4 hours. After washing cells, protein-fractions were separated by SDS-polyacrylamide gel-electrophoresis and transferred to nitrocellulose membranes. For detection of Hsp32, a rabbit polyclonal anti-HO-1 antibody (Stressgen) and goat anti-rabbit IgG (Amersham) were applied. To confirm equal loading, membranes were re-probed with a rabbit anti-ß-actin antibody (Sigma-Aldrich).

Evaluation of apoptosis by microscopy and Tunel assay
Primary AML cells and cell lines were incubated with PEG-ZnPP or SMA-ZnPP (each 1–20 μM) at 37°C for up to 48 hours. In a separate set of experiments, leukemic cells were incubated with various concentrations of ZnPP (1-50 μM) for up to 48 hours. Apoptotic cells were identified as reported [34] and quantified on Wright-Giemsa-stained cytospin preparations. In select experiments, the pan-caspase-inhibitor Z-VAD-FMK (50 μM) (Enzo Life Sciences, Farmingdale, NY, USA) was added for 24 hours to AML cell lines exposed to SMA-ZnPP. To confirm apoptosis, electron microscopy was performed as reported [35] using U937 cells incubated with SMA-ZnPP (20 μM) or control medium for 48 hours. Sections were viewed under a JEOL 1200 EX II electron microscope (JEOL, Tokyo, Japan). Typical signs of apoptosis including nuclear fragmentation and chromatin condensation were recorded. To confirm apoptosis in drug-exposed cells, a Tunel assay was performed as reported [35]. In brief, AML cell lines were cultured in control medium, PEG-ZnPP (20 μM) or SMA-ZnPP (20 μM) at 37°C for 3 days. Thereafter, cells were recovered, fixed in 1% formaldehyde, treated with 70% ice-cold ethanol for 1 hour, washed, and incubated in terminal-transferase reaction-solution containing $CoCl_2$, DNA deoxy-nucleotidyl-exotransferase, and biotin-16-2′-deoxy-uridin-5′-triphosphate (Boehringer Mannheim, Germany) at 37°C for 10 minutes [35]. Then, cells were washed, incubated with Streptavidin Fluorescein, and analyzed with a Nikon Eclipse E 800 fluorescence microscope (Tokyo, Japan).

Detection of active caspase 3 by flow cytometry
AML cell lines were incubated in control medium or various concentrations of SMA-ZnPP or PEG-ZnPP at 37°C for 48 hours. Then, cells were fixed in 2% formaldehyde for 10 minutes, permeabilized in 100% methanol at -20°C (15 minutes), washed in PBS plus BSA (0.1%), then stained with FITC-conjugated mAb C92-605 (Becton Dickinson Biosciences) directed against active caspase 3 (1 hour), and then analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Biosciences). In select experiments, cell cycle distribution in drug-exposed AML cells was examined by flow cytometry after permeabilization of cells with 0.1% Triton X-100 and DNA-staining with propidium iodide (PI).

Repopulation of AML cells in NOD-SCID IL-2Rgamma^{null} (NSG) mice
Primary AML cells (n=3 experiments) were incubated in control medium or in SMA-ZnPP (20 μM) with 10% FCS at 37°C for 2 hours. After incubation, AML cells were fully viable cells without signs of apoptosis as assessed by trypan blue exclusion and Wright-Giemsa staining (not shown). Drug exposed cells were washed, resuspended in 0.15 ml PBS with 2% FCS, and injected into the tail vein of adult female NSG mice (2-5 x 10^6 per mouse, 4 mice per group) (The Jackson Laboratory, Bar Harbor, ME). Twenty four hours prior to injection, mice were irradiated (2.4 Gy). After injection,

mice were inspected daily and sacrificed as soon as they developed disease-symptoms or after a maximum observation period of 8-10 weeks. Event-free survival was defined as survival without symptoms of disease. Bone marrow cells were obtained from flushed femurs, tibias, and humeri. Leukemic AML cells were detected in BM samples by multicolor flow cytometry using mAb against CD19, CD33, CD38, CD34, and CD45. AML-repopulation was defined as detection of a population of CD45+/CD34+/CD19- cells (at least 0.1% CD45+ cells) in mouse BM by flow cytometry. AML engraftment was confirmed by immunohistochemistry using mAb QBEND10 against CD34 (Immunotech, Marseilles, France). Flushed BM cells were spun on cytospin slides and examined for Hsp32 expression by immunocytochemistry. Animal studies were approved by the ethics committee of the Medical University of Vienna, and carried out in accordance with guidelines for animal care and protection and protocols approved by the Austrian law (GZ 66.009/0040-II/10b/2009).

Statistical analysis
To determine the significance in differences in mRNA expression levels and differences in growth and apoptosis, the student's t test was applied. Results were considered statistically significant when p was <0.05.

RESULTS

AML cells express Hsp32 mRNA and the Hsp32 protein
As assessed by PCR, Hsp32 mRNA was expressed in leukemic cells in all AML patients and all cell lines tested (Fig. **1A-1D**). Hsp32 mRNA was detectable in unfractionated AML cells as well as in highly enriched CD34+/CD38+ and CD34+/CD38- cells in all patients (n=10; Fig. **1C**). In untreated cells, the levels of Hsp32 mRNA detected by qPCR were relatively low. Hemin, a potent inducer of Hsp32 was found to upregulate expression of Hsp32 mRNA in primary AML cells in all primary cell samples (Fig. **1A** and **1B**) as well as in the 3 AML cell lines tested (Fig. **1D**). In qPCR experiments, hemin induced a 5-28 fold increase in Hsp32 mRNA expression in primary AML cells (Fig. **1B**). Corresponding results were obtained by Western blotting, where Hsp32 was found to be expressed at relatively low levels in unstimulated AML cells. Preincubation with hemin resulted in an increased expression of Hsp32 (Fig. **2A**). Immunocytochemistry confirmed expression of Hsp32 in AML cell lines (Fig. **2B**), primary AML MNC (Fig. **2C**), and sorted CD34+/CD38- cells (Fig. **2D**). In 33 of all 43 MNC samples tested (77%), and in 8 of 10 patients with relapsed/refractory AML (80%), leukemic cells stained positive for Hsp32 (Table 1). Preincubation of the anti-Hsp32 antibody with Hsp32-blocking peptide resulted in a negative stain (Fig. **2D**), and the same result was obtained when the primary antibody was ommitted (Fig. **2B** and **2C**). Expression of Hsp32 in AML cells did not correlate with anemia or WBC (Supplemental Table S1). Together, these data show that AML cells and AML progenitor cells express Hsp32.

Effects of Hsp32-targeting drugs on growth of AML cells
As assessed by ^{3}H-thymidine-incorporation, PEG-ZnPP and SMA-ZnPP were found to inhibit the spontaneous as well as GM-CSF-induced proliferation of primary AML

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cells (Fig. **3A**). The effects of PEG-ZnPP and SMA-ZnPP on spontaneous and cytokine-dependent growth of primary AML cells were dose-dependent, with IC_{50} values ranging between <5 µM and 20 µM (Fig. **3A**). No major differences were found when comparing IC_{50} values in GM-CSF-exposed cells with AML cells kept in control medium. Fig. (**3B**) shows typical examples of dose-responses obtained with primary AML cells and Hsp32-targeting drugs. We next attempted to correlate drug-responses with AML subtypes. In most FAB categories except M4, AML cells were found to respond well to the Hsp32-targeting drugs. However, in about 50% of the AML M4 samples tested, blast cells showed only a minor or no response to PEG-ZnPP and SMA-ZnPP, although Hsp32 was detectable in leukemic cells. We also asked whether blast cells in secondary or relapsed AML would respond differentially to Hsp32-inhibitors. However, PEG-ZnPP and SMA-ZnPP produced growth inhibition in AML blasts in all patients with secondary or relapsed AML. We were also able to show that PEG-ZnPP and SMA-ZnPP inhibit ^{3}H-thymidine uptake in all 3 AML cell lines tested, with similar IC_{50} values (Fig. **3D**). Supplemental Table S2 shows a summary of IC_{50} values obtained with SMA-ZnPP, PEG-ZnPP, primary AML cells, and the three cell lines examined. An shRNA against HO-1 also induced growth-inhibition in AML cells (Supplemental Fig. **S1**). Finally, we were able to show that the non-conjugated HO-1 blocker ZnPP induced apoptosis and growth inhibition in all 3 AML cell lines tested (Supplemental Fig. **S2A and S2B**). The HO-1-targeting drugs did not alter cell cycle distribution in AML cells (Supplemental Fig. **S2C**), suggesting that apoptosis-induction is a primary mechanism of drug-induced growth inhibition.

Hsp32-targeting drugs inhibit proliferation of CD34+/CD38+ and CD34+/CD38- AML cells

To demonstrate growth-inhibitory effects of SMA-ZnPP on AML progenitor cells, CD34+/CD38+ cells (more mature phenotype) and CD34+/CD38- (immature) cells were highly enriched in 3 patients (AML M1, M5, secondary AML). In all three patients, Hsp32-targeting drugs were found to inhibit cytokine-induced uptake of ^{3}H-thymidine and thus proliferation in CD34+/CD38+ and CD34+/CD38- cells (Fig. **3C**). PEG-ZnPP was tested in one patient and found to act on CD34+/CD38+ and CD34+/CD38- cells in the same way as SMA-ZnPP (not shown).

Targeting of Hsp32 in AML cells is associated with induction of apoptosis

PEG-ZnPP and SMA-ZnPP were found to induce apoptosis in primary AML cells as well as in all 3 AML cell lines tested (Fig. **4A**, Supplemental Table S3). The effects of PEG-ZnPP and SMA-ZnPP were dose dependent (Fig. **4A**) and were seen within 24 hours of incubation (Supplemental Fig. **S3**). ZnPP was also found to induce dose- and time-dependent apoptosis in AML cells. Apoptosis-inducing effects of PEG-ZnPP and SMA-ZnPP were confirmed by electron microscopy (Fig. **4B**) and by Tunel assay (Fig. **4C**). Moreover, SMA-ZnPP induced caspase-3-activation in AML cell lines (Fig. **4D**). Finally, we were able to show that the pan-caspase inhibitor Z-VAD-FMK blocks SMA-ZnPP-induced apoptosis in the AML cell lines tested (Supplemental Fig. **S4**). We next asked whether SMA-ZnPP cooperates with other anti-leukemic agents in AML cells. In these experiments, we were able to show that SMA-ZnPP and ARA-C synergize in producing growth inhibition in AML cells (Fig. **5**). Confirming previous

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data [36], ARA-C was found to upregulate HO-1 expression in AML cells, suggesting that synergistic effects were mediated by target-induction (Fig. **5D**).

Targeting of Hsp32 in AML cells interferes with leukemia-initiation in NSG mice
To explore whether Hsp32 inhibitors interfere with repopulation of AML cells *in vivo*, a xenotransplant model was established using NSG mice. We found that AML MNC reproducibly initiate leukemias in these mice after several weeks. The percentage of leukemic cells in the BM of engrafted mice ranged from 0.1% to >95%. Preincubation of AML MNC with SMA-ZnPP was found to interfere with AML-formation (Fig. **6A**). Moreover, mice injected with SMA-ZnPP-pretreated AML cells were found to have a better event-free survival compared to mice injected with control cells, i.e. AML MNC preincubated in control medium (Fig. **6B**). However, incubation of AML cells with SMA-ZnPP did not completely suppress AML-formation in mice. In one control experiment, AML cells were incubated with SMA-ZnPP (20 μM) as well as ARA-C (20 μM) and fludarabine (20 μM) before injected into NSG mice. In this experiment, no AML-formation was observed in mice injected with AML MNC exposed to all three drugs, whereas mice injected with SMA-ZnPP alone were found to develop AML after a few weeks (Fig. **6A**). In all mice, engrafted AML cells were found to be blast cells by morphology and to express CD34 as well as Hsp32 by immunostaining (Fig. **6C**). In a control experiment, these engrafted AML cells were injected into 5 secondary recipient NSG mice. In all mice examined, AML cells were found to repopulate secondary recipient NSG mice with full blown AML (not shown).

DISCUSSION

Heat shock proteins may be interesting new therapeutic targets in hematology [14-16,25,26]. In the present study, we show that AML cells display Hsp32/HO-1 in a constitutive manner and that the Hsp32-targeting drugs PEG-ZnPP and SMA-ZnPP exert major growth-inhibitory effects and apoptosis in primary AML cells and 3 AML cell lines. Furthermore, our data show that Hsp32-targeting drugs act on CD34+/CD38+ as well as CD34+/CD38- AML cells and inhibit leukemia-initiation in NSG mice. These data suggest that Hsp32 may serve as a target in AML.
A number of recent data suggest that Hsp32 is expressed in neoplastic cells in various hematopoietic and non-hematopoietic malignancies [19-26]. In most cases, the levels of Hsp32/HO-1 expressed are low but detectable, and can be upregulated by addition of hemin [25]. Similarly, in the current study, AML cells displayed low but detectable levels of Hsp32 at baseline, and hemin was found to promote Hsp32 expression in leukemic cells. No differences in Hsp32 baseline expression or hemin-induced upregulation were found when comparing various FAB/WHO subtypes of AML, various cytogenetic subgroups, or *de novo* with secondary or relapsed AML. However, in a few patients, resting AML blasts displayed only trace amounts of Hsp32. These observations suggest that Hsp32 may not be the only critical factor relevant to blast cell survival in AML. An interesting concept is that Hsp32 may serve as a survival factor in AML cells under stress situations or when exposed to chemotherapy.
Recently, two water-soluble Hsp32-inhibitors have been developed, namely PEG-ZnPP and SMA-ZnPP [23,24,27]. In the current study, both drugs were found to block growth of primary AML cells in a majority of AML patients as well as growth in all 3

AML cell lines. The effects of SMA-ZnPP and PEG-ZnPP on growth of AML cells were dose-dependent with IC_{50} values corresponding to pharmacologic concentrations at which these drugs reportedly inhibit tumor formation in experimental animals without producing major toxicity [22-24,26]. The mechanism of action of these drugs is not completely understood. Although both drugs reportedly suppress HO-1 activity, growth inhibitory effects exerted by such drugs may also involve other drug targets. Likewise, La et al. reported that ZnPP inhibits the expression of various proteins involved in proliferation of cancer cells, including cyclin D1 [37]. On the other hand, not all cancer cells may be killed by Hsp32 inhibitors. In this regard it is noteworthy that both Hsp32 inhibitors were found to exert growth-inhibitory effects on primary AML blasts even when Hsp32 levels were rather low, and vice versa, some of the non-responding AML blasts were found to express rather high levels of Hsp32. All in all these observations suggest that SMA-ZnPP and PEG-ZnPP exert anti-leukemic effects through multiple mechanisms and targets.

In AML, the malignant clone is considered to be organized hierarchically with more mature cells programmed to undergo apoptosis after a variable number of cell divisions and immature primitive cells that have self-renewal and leukemia-initiating capacity [38,39]. Although the exact phenotype of AML stem cells remains uncertain, several studies have suggested that NOD/SCID-repopulating AML stem cells reside within the CD34+/CD38- fraction of the leukemic clone [38,39]. Other studies have shown that NSG-repopulating AML stem cells reside in both the CD34+/CD38+ and CD34+/CD38- cell-fractions [40]. The results of our study show that SMA-ZnPP and PEG-ZnPP produce dose-dependent growth inhibition in CD34+/CD38+ as well as in CD34+/CD38- AML progenitor cells, supporting the assumption that Hsp32 is expressed in AML progenitor cells. In line with this observation, we found that incubation of AML MNC with SMA-ZnPP before injection into NSG mice inhibits *in vivo* leukemia-initiation. These data are in favour of the notion that Hsp32 might be a new potential target in AML. However, preincubation of AML cells with SMA-ZnPP did not completely block AML formation in NSG mice, suggesting that this drug depleted some but did not all AML stem cells. One possible explanation for this observation would be that not all leukemia-initiating AML (stem) cells expressed Hsp32. Alternatively, some of the AML stem cells (subclones) display Hsp32 but exhibit intrinsic resistance against SMA-ZnPP. Another explanation might be that some of the AML stem cells expressed other survival factors sufficient for their survival so that the knock-down of Hsp32 had little if any effect on survival and growth and thus engraftment in NSG mice. In a control experiment, pre-incubation of AML cells with a combination of SMA-ZnPP, ARA-C, and fludarabine resulted in a complete depletion of AML-repopulating cells. Based on this observation we also performed *in vitro* experiments combining ARA-C and SMA-ZnPP. Indeed, this combination was found to produce synergistic anti-proliferative effects in all 3 AML cell lines tested. These data suggest that therapy with drug combinations are required to produce optimal anti-leukemic effects in AML.

Another interesting observation in this regard was that Hsp32-targeting drugs did not produce *in vitro* growth inhibition in AML blast cells in all patients. Especially in AML M4, responses of blast cells to SMA-ZnPP or PEG-ZnPP were only seen in approximately 50% of all cases. The biochemical basis of resistance to Hsp32-targeting drugs in these patients remains unknown. In fact, in all these patients, blast

cells were found to display Hsp32, and these cells were also responsive to other anti-leukemic drugs. One possibility for resistance against Hsp32-targeting drugs may be that these drugs did not accumulate in leukemic cells because of low (insufficient) uptake or enhanced drug efflux. An alternative explanation might be that apart from Hsp32, other more important survival factors were employed by leukemic cells. Finally, it cannot be excluded that these cells exhibited a modified (mutated) form of Hsp32 that was not recognized by the Hsp32-targeting drugs applied. Studies are under way to clarify the biochemical basis of resistance to Hsp32-targeting drugs in AML.

Hsp32 supposedly serves as an important survival factor in leukemic cells and supposedly mediates resistance against apoptosis [26,36,41]. Therefore, we were interested to learn whether targeting of Hsp32 in leukemic cells would be associated with signs of apoptosis. Indeed, the results of our study show that PEG-ZnPP and ZnPP and SMA-ZnPP were demonstrable by light microscopy and electron microscopy as well as in a Tunel assay. Moreover, we were able to show that SMA-ZnPP induces caspase 3 activation in the 3 AML cell lines examined. Finally, the pan-caspase inhibitor Z-VAD-FMK was found to block SMA-ZnPP-induced apoptosis. Together, these data suggest that Hsp32 is an essential survival factor in AML cells, which is in line with our recent data obtained in CML and other hematopoietic and non-hematopoietic malignancies [26,36]. These data also show that the primary mechanism of drug-induced cell death is apoptosis-induction, whereas we were unable to show major effects of Hsp32/HO-1 inhibitors on cell cycle-distribution on AML cells. These data are somehow in contrast to the data published by Rushworth et al. [41,42]. In their manuscript, major growth-inhibitory effects on AML cells were only seen when ZnPP was combined with the NFκB inhibitor BAY 11-7082. This discrepancy may be explained by the different cell lines used, varying culture conditions, or by the different read outs. A selective effect of the water-soluble drugs could be excluded. Notably, ZnPP was found to induce apoptosis and growth arrest in AML cells in the same way as the water soluble derivatives SMA-ZnPP and PEG-ZnPP.

In summary, our data show that Hsp32 is a new potential target in AML and that Hsp32-targeting drugs induce growth arrest and apoptosis in leukemic cells. Moreover, our data suggest that Hsp32-targeting drugs can inhibit growth and leukemia-initiation in AML progentitor cells. Whether Hsp32-targeting drugs also counteract leukemic cell growth *in vivo* in patients with AML remains to be determined in clinical trials.

LIST OF ABBREVIATIONS:

AML	acute myeloid leukemia
APC	allophycocyanin
HO-1	heme oxygenase-1
NSG mice	NOD/SCID IL2R gamma chain deficient mice
Hsp32	heat shock protein 32
FAB	French-American-British
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
pb	peripheral blood
bm	bone marrow
MNC	mononuclear cells
BSA	bovine serum albumin
PBS	phosphate buffered saline
PE	phycoerythrin
PEG-ZnPP	pegylated zinc protoporphyrin
PerCP	Peridinin chlorophyll protein
SMA-ZnPP	styrene maleic acid-copolymer-micelle-encapsulated zinc protoporphyrin

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TABLES

Table 1

Patients' characteristics

n	diagnosis FAB	f:m	age year	WBC x10^9/L	Hb g/dL	plt x10^9/L	% blasts BM	% blasts PB	Hsp32 expression by ICC
					Median values				% positive patients (n/n)
04	M0	1:3	50	2.1	10.1	123	58	33	50% (2/4)
14	M1	5:9	66	27.4	10.8	27	82	77	60% (6/10)
12	M2	6:6	54	13.2	9.6	46	58	46	80% (8/10)
11	M4	6:5	70	62.2	9.4	50	52	31	100% (7/7)
03	M4eo	0:3	53	3.9	13.0	48	63	47	67% (2/3)
07	M5	2:5	62	40.3	9.6	39	74	65	80% (4/5)
01	M6a	0:1	68	6.1	9.4	20	93	93	n.t.
06	sec. AML	1:5	60	24.4	10.2	37	29	30	100% (4/4)
17	ref. AML	4:13	60	23.3	10.0	24	71	26	80% (8/10)
58	all pts	21:37	62	32.2	9.9	41	67	49	77% (33/43)

Abbreviations: n, number of patients examined; FAB, French-American-British cooperative study group; WBC, white blood count; Hb, hemoglobin; plt, platelets; BM, bone marrow; PB, peripheral blood. sec.AML, secondary AML; ref.AML, refractory or relapsed AML; pts, patients; ICC, immunocytochemistry; n.t., not tested.

Table 2

PCR primer sequences

gene	forward sequence	reverse sequence
hu-β-actin	5'-TCGACAACGGCTCCGGCATG-3'	5'-CCTCTCTTGCTCTGGGCCTCGTC-3'
hu-Hsp32	5'-CAGGATTTGTCAGAGGCCCTGAAGG-3'	5'-TGTGGTACAGGGAGGCCATCACC-3'
hu-Abl	5'-TGTATGATTTGTGGCCAGTGGAG-3'	5'-GCCTAAGACCCGGAGCTTTTCA-3'

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LEGENDS TO FIGURES

Figure 1
Expression of Hsp32 mRNA in AML cells
A, RT-PCR analysis of expression of Hsp32 mRNA in leukemic cells in 2 patients with AML M1. AML cells were incubated with control medium (Co) or hemin (10 μM) at 37°C for 4 or 8 hours. RT-PCR was performed using primers specific for Hsp32 (upper panel) or β-Actin (lower panel). *B*, qPCR performed with AML cells (6 patients) incubated in control medium (Co) or hemin (10 μM) at 37°C for 4 hours. Results show relative Hsp32 mRNA levels (fold of Co) and represent the mean±S.D. from 6 donors. Asterisk: p<0.05. *C*, qPCR performed with sorted CD34+/CD38+ (upper panel) and CD34+/CD38- cells (lower panel) obtained from 10 AML patients and cord blood MNC (sorted cells pooled from 3 donors). Hsp32 transcript levels are expressed as percent of Abl mRNA levels. *D*, qPCR analysis of AML cell lines. Cells were kept in control medium (Co) or hemin (10 μM) at 37°C for 4 hours before examined. Results show relative Hsp32 mRNA levels (fold of control) and represent the mean±S.D. from 3 independent experiments.

Figure 2
Detection of the Hsp32 protein in AML cells
A, Primary AML cells (FAB M4, M4, M5a, and two secondary AML) and cell lines were incubated in control medium (-) or hemin, 10 μM (+) for 4 hours. Thereafter, cells were harvested and subjected to Western blotting using anti-Hsp32 antibody (upper lane) and an antibody against β-actin (lower lane). *B,C*, Immunocytochemistry of AML cells. Cell lines (*B*) and primary AML cells (*C*) (one AML M1, two M2, and one M4) (without hemin-preincubation) were spun on cytospin slides and examined for expression of Hsp32 by immunocytochemistry. Antibody-omission control is shown in the lower panel. (*D*) CD34+/CD38- AML cells were spun on cytospin slides and stained with an anti-Hsp32 antibody preincubated with control buffer (upper panel) or Hsp32-blocking peptide (lower panel).

Figure 3
SMA-ZnPP and PEG-ZnPP inhibit the proliferation of AML cells
A, Primary AML cells kept in GM-CSF, 100 ng/ml (left panels) or without GM-CSF (right panels) were incubated in control medium (Co) or various concentrations of SMA-ZnPP or PEG-ZnPP at 37°C for 48 hours. Thereafter, ^{3}H-thymidine-uptake was measured. Results show the percent ^{3}H-thymidine uptake in drug-exposed cells relative to control (100%) and represent the mean±S.D. from at least 12 donors. Asterisk: p<0.05. *B*, Dose-dependent effects of SMA-ZnPP (left panels) and PEG-ZnPP (right panel) on ^{3}H-thymidine uptake (percent of control) of AML cells in individual donors. *C*, Effects of SMA-ZnPP on cytokine (SCF/IL-3/G-CSF/GM-CSF)-induced proliferation of highly purified CD34+/CD38- and CD34+/CD38+ AML cells. Cells were incubated with various concentrations of SMA-ZnPP for 48 hours. Results show the percent ^{3}H-thymidine uptake in drug-exposed cells relative to control (100%) and represent the mean±S.D. of 3 patients. *D*, Dose-dependent effects of SMA-ZnPP (left panels) and PEG-ZnPP (right panels) on proliferation of AML cell lines. Cells

were kept in control medium (Co) or various concentrations of SMA-ZnPP or PEG-ZnPP at 37°C for 48 hours. Thereafter, ^{3}H-thymidine uptake was measured. Results show the percent ^{3}H-thymidine uptake in drug-exposed cells relative to control (100%) and represent the mean±S.D. from at least 3 independent experiments. Asterisk: $p<0.05$.

Figure 4
Induction of apoptosis in AML cells by PEG-ZnPP and SMA-ZnPP

A, Primary AML cells (upper panels) were cultured in RPMI 1640 medium plus 10% FCS in the absence (Co) or presence of various concentrations of SMA-ZnPP (upper left; AML M2) or PEG-ZnPP (upper right; AML M4eo) at 37°C for 48 hours. After incubation, the percentages of apoptotic cells were determined by light microscopy. Results represent the mean±S.D. from triplicates. Asterisk: $p<0.05$. AML cell lines (lower panel) were also examined for the percentage of apoptotic cells after incubation with SMA-ZnPP (1-10 μM, 37°C, 48 hours). Results represent the mean±S.D. from three independent experiments. Asterisk: $p<0.05$. *B,* Electron microscopy analysis of U937 cells kept in control medium (Co, left panel), PEG-ZnPP (20 μM) or SMA-ZnPP (20 μM). As visible, drug-exposed cells exhibited clear signs of apoptosis, including cell shrinkage, nuclear fragmentation, and chromatin condensation. *C,* U937 cells (left panel) and HL60 cells (right panel) were incubated in control medium or medium containing PEG-ZnPP (20 μM) or SMA-ZnPP (20 μM) at 37°C for 48 hours (upper panel) or 72 hours (lower panel). Then, cells were examined by Tunel assay. *D,* U937 cells (left panel), HL60 cells (middle panel), and KG1 cells (right panel) were incubated with increasing concentrations of SMA-ZnPP (37°C, 48 hours) as indicated. Then, the percent of active caspase 3-positive cells was determined by flow cytometry. Results represent the mean±S.D. from at least 3 independent experiments. Asterisk: $p<0.05$.

Figure 5
Effects of drug combinations on growth of AML cell lines

AML cell lines (*A*: HL60, *B*: KG1, *C*: U937) were incubated in control medium (0) or with cytosine arabinoside (ARA-C), SMA-ZnPP, or a combination of both drugs at various concentrations (fixed ratio) as indicated for 48 hours (37°C). Then, ^{3}H-thymidine uptake was measured. Results are expressed as percent of control and represent the mean±S.D. of triplicates. As visible, both drugs produced clear cooperative effects in all three cell lines examined. *D,* U937 cells were kept in control medium (Co), hemin (10 μM), or ARA-C (1 μM) at 37°C for 24 hours. Thereafter, Western blotting was performed using antibodies specific for Hsp32 or β-actin (loading control).

Figure 6
### Effect of SMA-ZnPP on engraftment of primary AML cells in NSG mice

A, Before being injected into NSG mice, AML cells (MNC) were incubated in Control medium (●), SMA-ZnPP, 20 μM (▲), or SMA-ZnPP (20 μM) plus ARA-C (20 μM) plus Fludarabine (20 μM) (◆) at 37°C for 2 hours. Then, 2 x 10^6 cells were injected into the tail vein of irradiated NSG mice. Engraftment of AML was determined after 6-7 weeks and was quantified by measuring the percentage of human AML cells in
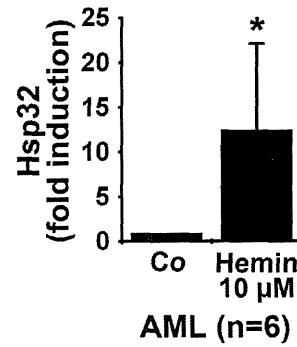
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mouse bone marrow by flow cytometry. *B*, Event-free survival of NSG mice injected with 5 x 10^6 AML cells preincubated in control medium (□) or in SMA-ZnPP, 20 μM (--) for 2 hours. *C*, Demonstration of engraftment of human AML in NSG mouse bone marrow by CD34 immunohistochemistry (left panel) and analysis of cytospin preparations by Giemsa staining (right upper panel) and immunocytochemistry using an antibody against CD34 (middle panel) and Hsp32/HO-1 (lower panel).
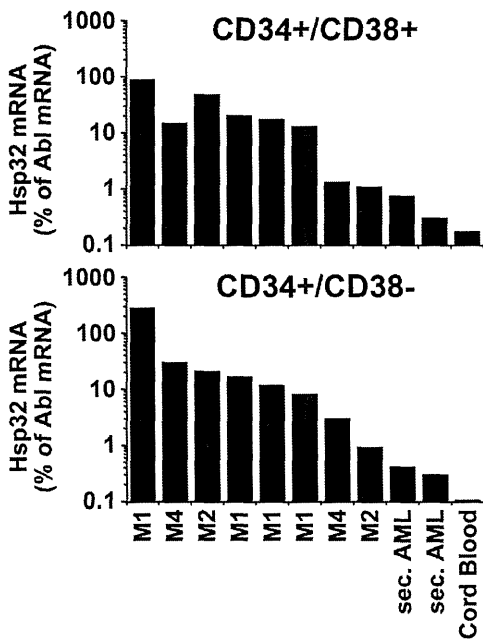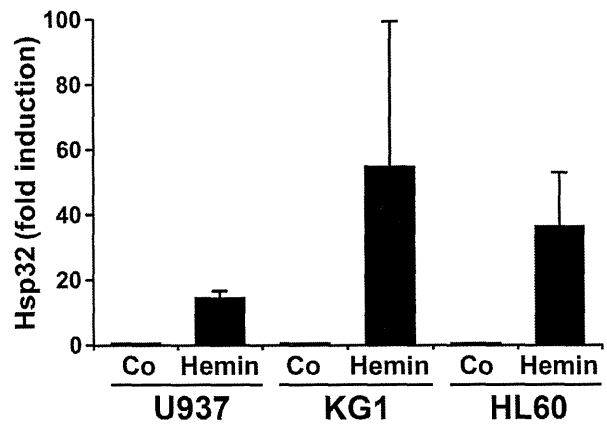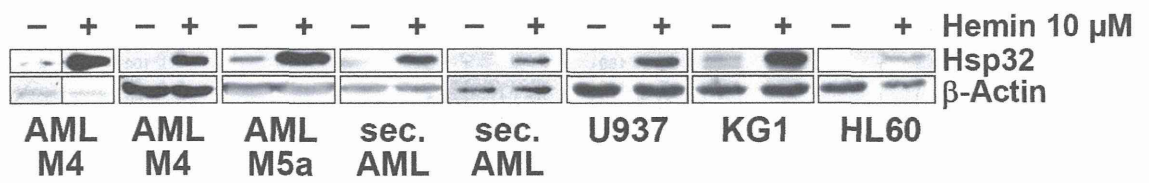
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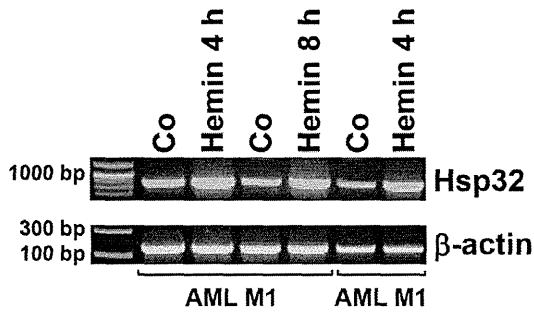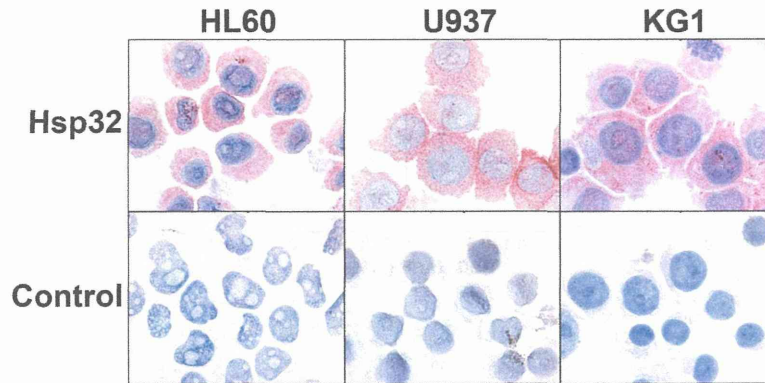

Herrmann et al, Figure 1A

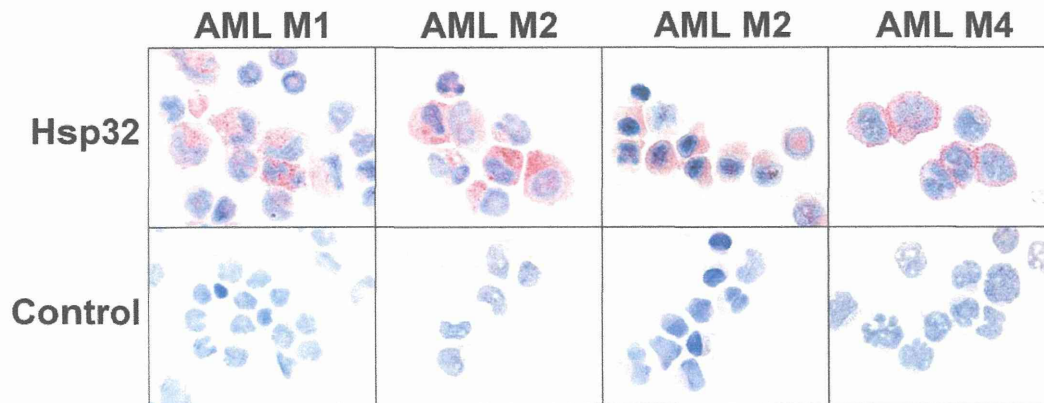Herrmann et al, Figure 1B

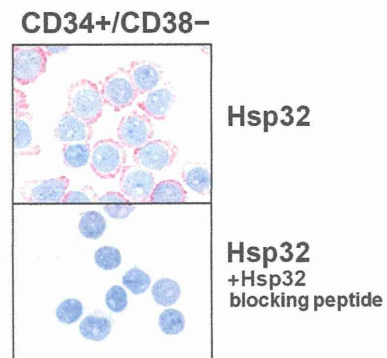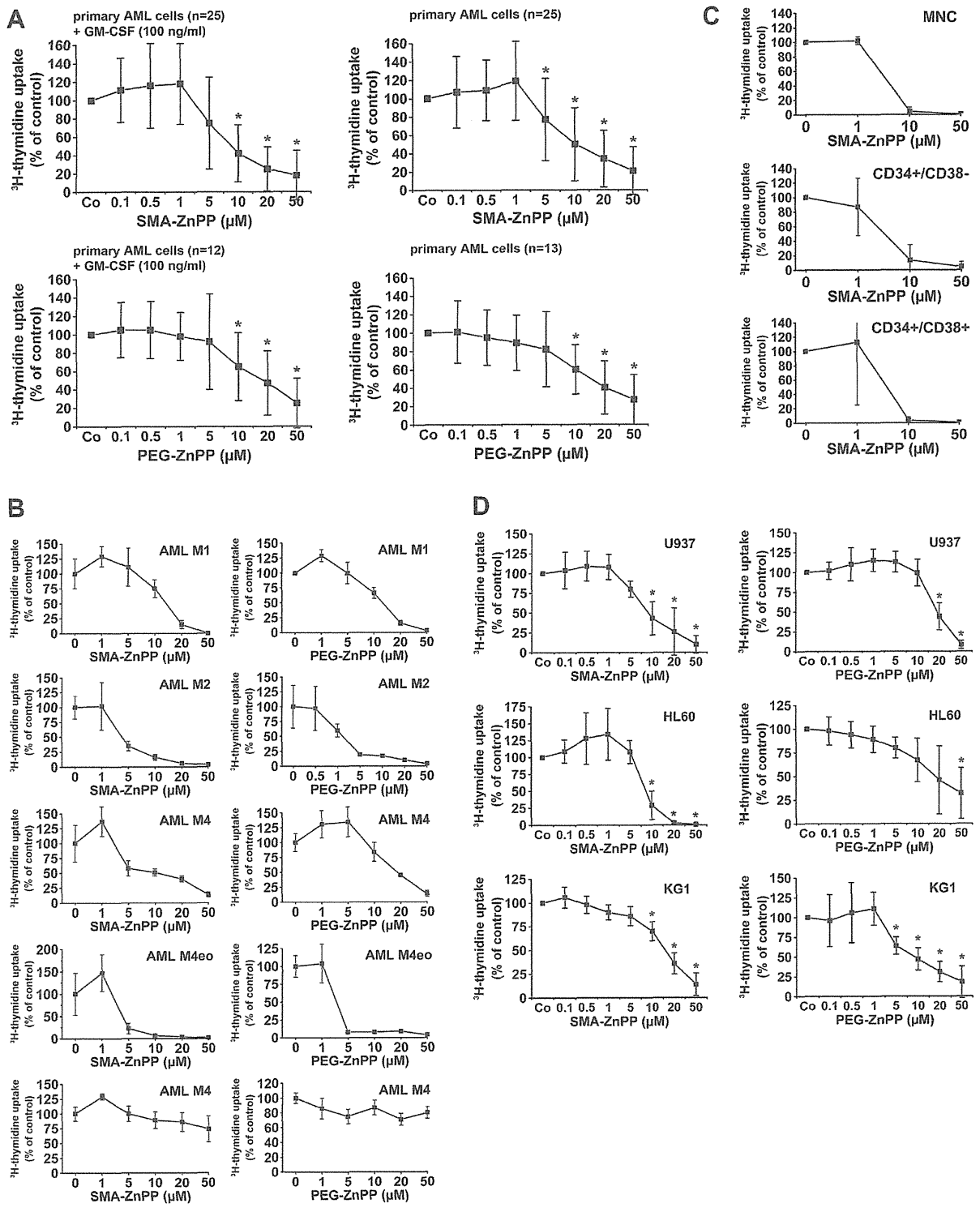Herrmann et al, Figure 1C

Herrmann et al, Figure 1D

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− + − + − + − + − + − + − + − + Hemin 10 μM
Hsp32
β-Actin

AML M4 AML M4 AML M5a sec. AML sec. AML U937 KG1 HL60

Herrmann et al, Figure 2A

HL60   U937   KG1

Hsp32

Control

Herrmann et al, Figure 2B

AML M1   AML M2   AML M2   AML M4

Hsp32

Control

Herrmann et al, Figure 2C

CD34+/CD38−

Hsp32

Hsp32
+Hsp32
blocking peptide

Herrmann et al, Figure 2D

Herrmann et al, Figure 3

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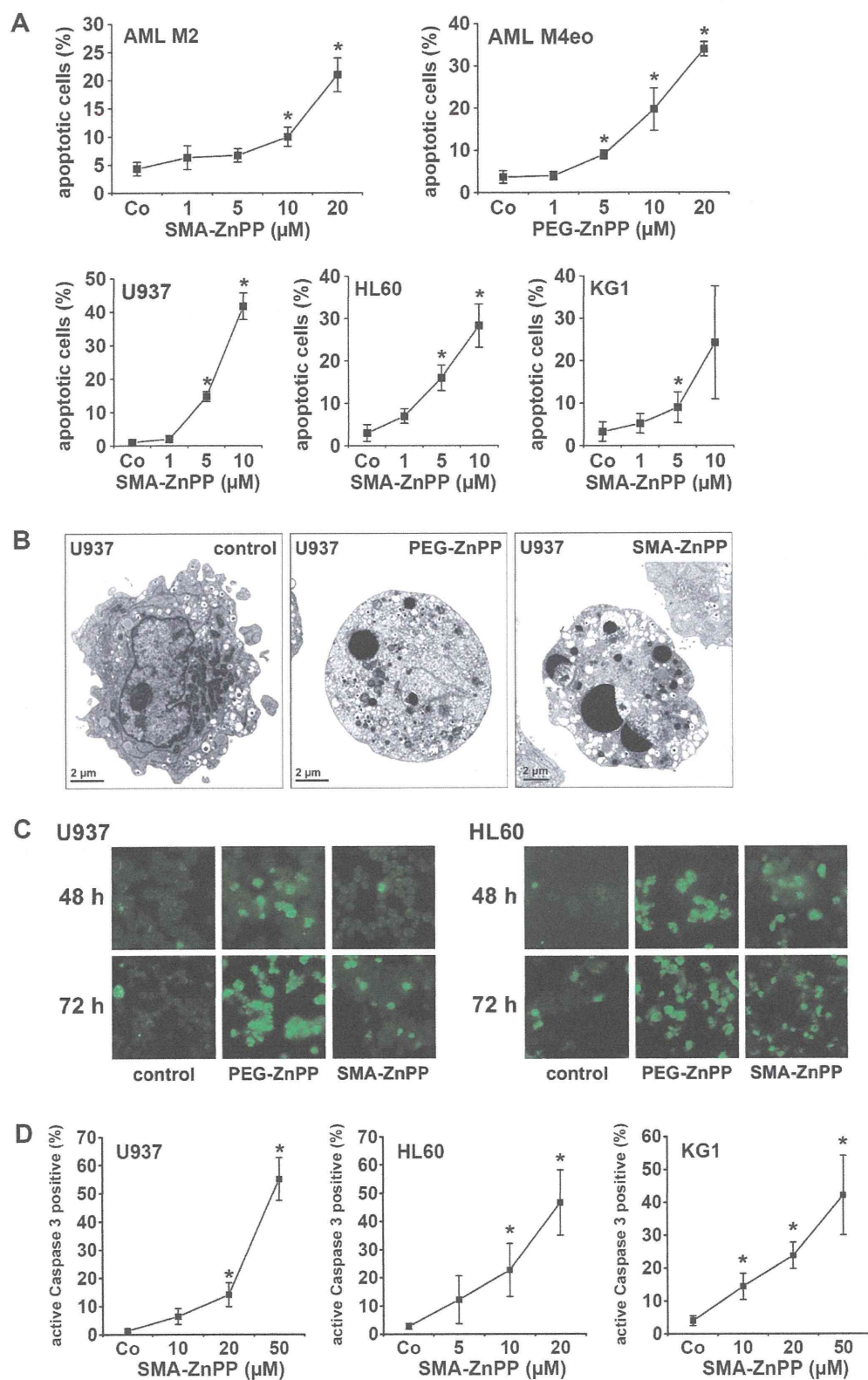Herrmann et al, Figure 4