Enhanced Concentration-Dependent Cytotoxic Effect of the Dinuclear Copper(II) Complex of L-Carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, Compared to L-Carnitine or Copper Chloride Dihydrate, in Human Leukemic Cell Lines

Hellinida Thomadaki,[†] Alexandra Karaliota,[‡] Charalambos Litos,[‡] and Andreas Scorilas*[,†]

Department of Biochemistry and Molecular Biology, University of Athens, Panepistimiopolis, 15701 Athens, Greece, and Department of Inorganic Chemistry, Faculty of Chemistry, University of Athens, Panepistimiopolis, 15771 Athens, Greece

Received November 26, 2007

We studied the antitumor properties of the dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, as well as those of L-carnitine and copper chloride dihydrate, in human leukemic cells. The complex was synthesized and characterized using EPR, ^1H NMR, ^{13}C NMR, IR, and UV−vis analyses. Its cytotoxic effect on the human leukemia cell lines HL-60 and K562 was studied by assessing the metabolic activity of cells (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT method), the structural integrity of cell membrane using Trypan blue assay, and the proliferation capacity of cells studying growth curves. Both leukemia cell lines showed a concentration-specific increased cytotoxicity of the complex, compared to L-carnitine or copper chloride dihydrate, with distinct underlying mechanisms, which were decreased proliferation efficiency for HL-60 cells and increased necrotic phenomena for K562 cells. Our results are indicative of a concentration-specific enhanced antileukemic effect of the complex, implying its value as a tool in the implementation of leukemia.

Introduction

L-Carnitine, a trimethylated amino acid (β-hydroxy-γ-butyrobetaine) with a structure quite similar to that of choline, plays an essential biological role in the transport of long-chain fatty acids (after conversion to acylcarnitines) into mitochondria where β-oxidation takes place.[1–4] Its relevance to the long-chain fatty acids degradation and the consequent energy production makes it a widespread dietary supplement for athletic performance as well as for weight loss.[5–7]

L-Carnitine and its derivatives also exhibit a noticeable antidiabetic activity because the long-chain free fatty acids transport by carnitine is connected to the glucose metabolism.[8–11] L-Carnitine is also an antioxidant resulting in satisfactory protection against oxidative stress related diseases such as lipid peroxidation,[12] Down syndrome,[13,14] heart failure,[15,16] aging,[17] and HIV.[18] Carnitine deficiency is connected to cardiac symptoms[19,8] and anemia,[20,21] and its supplementation has a positive effect on these dysfunctions. The role of L-carnitine in carcinogensis is evident by data that support its inhibitory role on cancer development[22–24] as well as its supportive role in prevention or minimization of side effects from cisplatin induced injury of kidney and intestine.[25]

Copper has an important biological role in all living organisms as an essential trace element. Because of the redox behavior of the Cu(II)−Cu(I) system and the interaction of copper complexes with O_2, biomimetic complexes of copper ions with biologically interesting ligands have been investigated in detail. Copper is involved in the activation and hydroxylation of various substrates via free radical reactions.[26,27] Copper(II) complexes with diverse drugs and their synergetic activity have been the subject of a large number of research studies.[28–34]

Additionally, in HL-60 and Jurkat leukemia cell lines, L-carnitine treatment prevented GP7 (4-[4″-(2″,2″,6″,6″-tetramethyl-l″-piperidinyloxy)amino]-4′-demethyl epipodophyllotoxin)-induced caspase-3 activation, with subsequent inhibition of GP7-induced apoptotic internucleosomal DNA fragmentation only in HL-60 cells.[35] According to another study, L-carnitine treatment of the same two cell lines prevented IDA (idarubicin)-induced caspases 3 and 7 activation, without affecting IDA-induced internucleosomal DNA fragmentation.[36] Furthermore, it has been found that L-carnitine promotes differentiation of a certain fraction of HL-60 leukemic population, yielding a small number of atypical cells for the myeloid lineage cells.[37] Such an observation may account for the inhibitory effect of L-carnitine over GP-7 and IDA-induced distinct apoptotic hallmarks. What is more, the anticancer drug methylglyoxal bis(guanylhydrazone) (mitoguazone) depresses carnitine-dependent oxidation of long-chain fatty acids in cultured mouse leukemia cells.[38] Similarly, exposure of the leukemia cells to the drug in the presence of carnitine abolished the inhibition of fatty acid oxidation and prevented the drug-induced mitochondrial damage.[39]

Two binuclear copper(II) complexes of L-carnitine were synthesized and characterized, with the first reported X-ray structural characterization of a Cu(II) paddle-wheel complex.[40] In the intestine and kidney the L-carnitine transport is Na^+-dependent.[41] The Ca^{2+}-dependent mitochondrial damage is mediated in the presence of L-carnitine.[42] Only a Zn^{2+} complex of L-carnitine has been isolated and characterized with infrared spectroscopy and elemental analysis. This complex demonstrates antidiabetic activity.[43] The possible chelation of iron with propionyl-L-carnitine explains the reduction of ischemic injury.[44,45]

Recently, Chang et al. reported that oxidative stress elicited from copper accumulation increases the amount of free fatty acids, thereby inducing mitochondrial dysfunction. L-Carnitine treatment can inhibit this mitochondrial injury.[46] Therefore, the investigation of copper and L-carnitine interaction is biologically important.

* To whom correspondence should be addressed. Phone: +30-210-727-4360. Fax: +30-210-727-4158. E-mail: ascorilas@biol.uoa.gr or scorilas@netscape.net.

[†] Department of Biochemistry and Molecular Biology.

[‡] Department of Inorganic Chemistry.

10.1021/jm701473x CCC: $40.75
Published on Web 06/05/2008

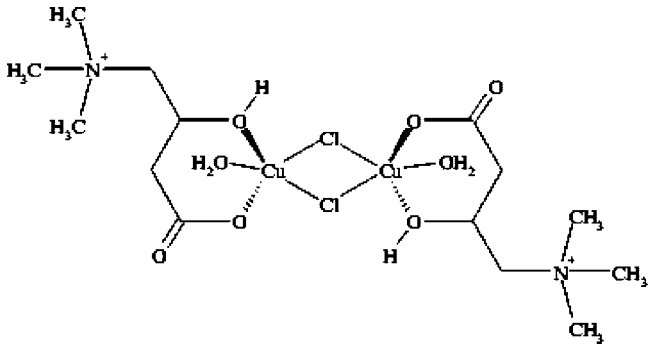

Figure 1. Proposed structure of the complex.

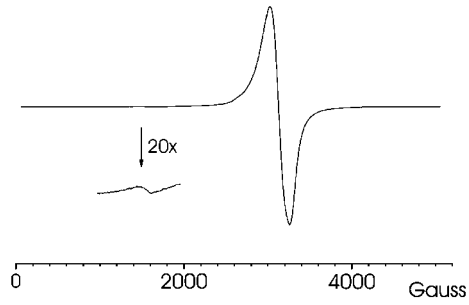

Figure 2. Low-temperature (11 K) EPR spectrum of the complex in aqueous solution.

In the present work, we examined the cytotoxic effect of the dinuclear copper(II) complex of L-carnitine, $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$, on two leukemia cell lines, HL-60 and K562, against L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$). We found that this compound could sensitize and potentiate the leukemia cells tested to cell death, in comparison to L-carnitine or copper chloride dihydrate, alone.

Results

Preparation and Characterization of the Compound $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$. The L-carnitinatocopper(II) complex $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$ was prepared in ethanolic solution via the reaction of equimolar quantities of copper(II) chloride dehydrate and L-carnitine and resulted as a green solid soluble in water and DMSO. The structure of the dinuclear copper(II) complex of L-carnitine is presented in Figure 1. In the IR spectrum the most characteristic bands at 1619 and 1421 cm^{-1} correspond to the asymmetric and symmetric stretching vibrations of the carboxylate group, respectively. The two bands at 561 and 431 cm^{-1} are indicative of the Cu−O vibrations.[47,48]

The complex dissolved in water is EPR silent at room temperature. At lower temperatures (11 K) the spectrum reveals two signals at $g \approx 2$, around 3100 G, and at $g \approx 4$, around 1500 G. The first one is an unsaturated signal without hyperfine interactions, indicating a significant interdimer interaction between the two copper(II) ions (Figure 2). The appearance of an additional very weak signal (Figure 2, inset) at lower field (near half-field) is characteristic of binuclear copper complexes due to a singlet−triplet transition of $\Delta M_s = 2$.[49]

The electronic absorption spectrum of the complex in aqueous solution demonstrates $\pi \rightarrow \pi^*$ transitions within the ligands at 240 nm. A ligand-to-metal charge transfer band, through the chloride ions, is observed at 300 nm. The complex exhibits also a d−d transition as a weak and broad band at 790 nm, which is typical of a distortion of a square pyramidal geometry toward a equilibrium trigonal bipyramidal geometry.[50,51]

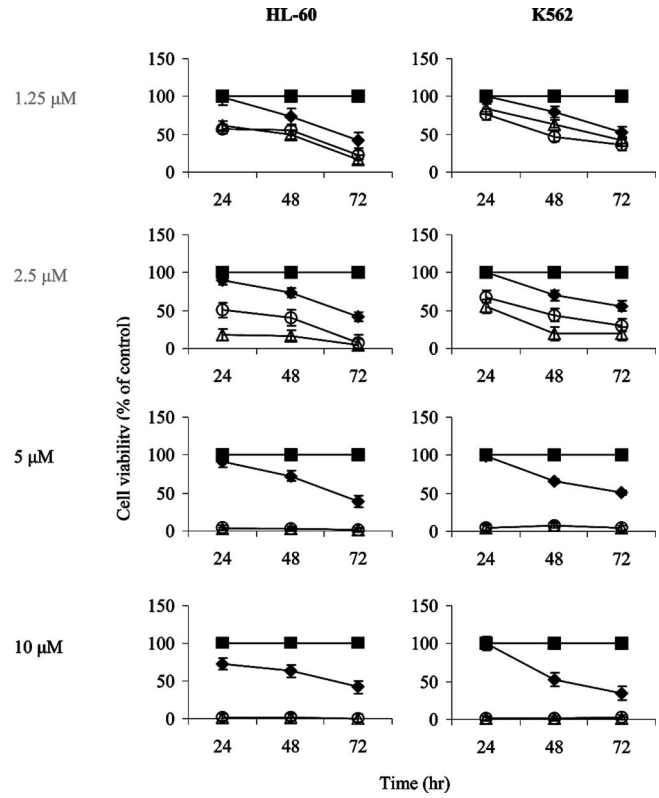

Figure 3. Treatment of HL-60 and K562 cell lines with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$, displayed enhanced cytotoxic effect compared to L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$). The cells were treated in the absence (■) or presence of various doses of the complex (△) or of L-carnitine (◆) or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) (○), as individual entities, for 24, 48, and 72 h, and the cell viability was measured by the MTT assay. Each data point represents the mean of three separate experiments (mean ± SD).

In the ^1H NMR spectrum, it is shown that the ligand is coordinated to the metal by a single-bonded carboxylate oxygen atom and a hydroxylate one. The peaks at 1.29, 3.24, and 3.87 ppm correspond to the $-C\underline{H}_2-COO^-$, $(C\underline{H}_3)_3N^+$, and $-C\underline{H}-OH$ protons, respectively. In the ^{13}C NMR spectrum, the peaks at 19.6, 56.6, 59.8, and 81.7 ppm correspond to the $-\underline{C}H_2-COO^-$, $(\underline{C}H_3)_3N^+$, $(CH_3)_3N^+-\underline{C}H_2-$, and $-\underline{C}H-OH$ carbon atoms, respectively.

Effect of $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$ on Growth and Viability of Leukemia Cells. To compare the effect of the compound $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$, in relation to that of L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), on HL-60 and K562 leukemia cell lines, the cells were exposed to increasing doses of $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$ or L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities (1.25, 2.5, 5, and 10 μM), for increasing periods of time (24, 48, and 72 h), and cell viability was measured by the MTT[a] assay. As shown in Figure 3, treatment of both cell lines with either the complex $[Cu_2(\text{L-carnitine})_2Cl_2(H_2O)_2]Cl_2$ or copper chloride dihydrate at ≥5 μM led to almost 0% cell viability since 24 h of treatment, whereas L-carnitine alone caused a more restricted time-dependent down-regulation of cell viability. Exposure of the cell to concentrations of ≤2.5 μM led to a time-

[a] Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; *BCL2*, B cell lymphoma gene 2; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

dependent negative regulation of cell viability by all three compounds under study, with a more pronounced effect compared with that of the compound $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, or of copper chloride dihydrate compared to L-carnitine as individual entity (Figure 3). At the median concentration of 2.5 μM only, there was a more predominant effect of the complex compound $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$ compared to the effect of both copper chloride dihydrate and L-carnitine as individual entities, in both cell lines, still being more evident in the HL-60 cell line (Figure 3). Such observation could indicate concentration dependence. Furthermore, the sensitivity of both cell lines to L-carnitine alone, in terms of cell viability as assayed by the MTT assay, was mainly time-dependent (Figure 3).

Apart from the MTT method, which assesses quantitatively cell viability but cannot distinguish between growth arrest or necrosis, the effect of the compounds under study on cell growth and the percentage (%) of Trypan blue +ve cells (necrosis) in the cell lines mentioned above were evaluated. The cell growth curves exhibited mainly a concentration-dependent and to a lesser extent a time-dependent inhibition of cell proliferation, with important differences among the complex and L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) at 2.5 μM in the HL-60 cell line. Yet more evident and enhanced decrease in cell number appeared in the presence of the complex and copper chloride dihydrate at concentrations of ≥ 5 μM, in relation to L-carnitine alone, in both cell lines examined (Figure 4), whereas at the smallest concentration of 1.25 μM all three compounds had similar effects (Figure 4).

The Trypan blue staining revealed that in HL-60 cells the complex displayed similar necrotic effects as copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), still higher than L-carnitine at 5 μM, in some cases causing even 100% necrosis of the cells (Figure 5, Tables 1 and 2). In the K562 cell line, the complex displayed enhanced necrotic effects compared to copper chloride dihydrate and L-carnitine, only at the 2.5 μM concentration. Concentrations of ≥ 5 μM presented extensive toxicity for both the complex compound under study and the copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) in both cell lines under study, whereas the concentration of 1.25 μM caused almost no necrotic phenomena (Figures 3–5, Tables 1 and 2).

Discussion

L-Carnitine has been well-known for its protective action over ischemia/reperfusion injury of the heart,[52] its beneficial effect against Alzheimer's disease[53] and AIDS,[54] and its antiapoptotic action.[55] Mutomba[55] showed that L-carnitine protected Jurkat leukemia cells against Fas-mediated apoptosis, yet without inhibiting IDA-induced apoptotic DNA fragmentation.

In the past few years a lot of action has taken place concerning more successful confrontation of distinct types of leukemia, mainly with new chemotherapeutic regimens[56,57] for better therapeutic interventions and more accurate prognostic biomarkers.[58–62] An important parameter in the evaluation of chemotherapeutic agents is cancer cell growth inhibition and cancer cell death, with fewer possible side effects for the patient. The unsuccessful action of many chemotherapeutic drugs reflects, at cellular level, the inability of specific drugs to induce cell growth arrest and cell death.[63–71]

In the present research, the responses of two leukemia cell lines (HL-60 and K562) to the compound $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$ and to L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, were studied and evaluated. The selection of these two leukemia cell lines was not accidental, since they cover a wide range of cell responses to chemotherapy, from the endogenous predisposition to cell death (HL-60) to the resistance to it (K562).

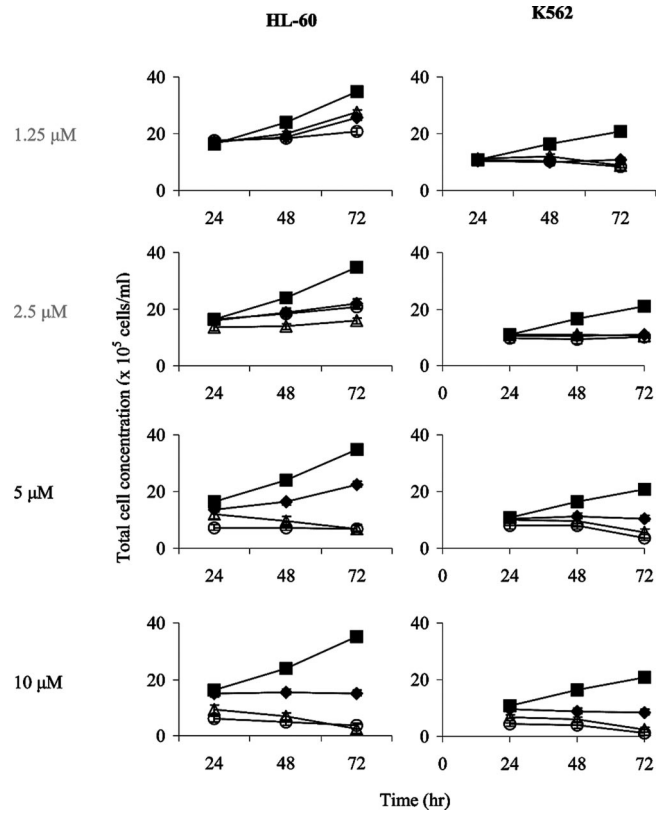

Figure 4. Treatment of HL-60 and K562 cell lines with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) displayed antiproliferative effect in both cell lines. The cells were treated in the absence (■) or presence of various doses of the complex (△) or of L-carnitine (◆) or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) (○), as individual entities, for 24, 48, and 72 h, and total cell concentration was measured using a hemeocytometer. The results were expressed as the total cell concentration of cells. Each data point represents the mean of three separate experiments (mean ± SD).

Compound $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$ was synthesized and consisted of two subunits, L-carnitine and copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$). Our results revealed that a specific concentration of the compound, which was estimated as 2.5 μM, could sensitize both HL-60 and K562 human leukemia cells to a more pronounced decrease of cell viability, as assayed by MTT, in comparison to L-carnitine and copper chloride dehydrate alone, accompanied by increased necrotic phenomena in the case of K562 cells and yet with more extensive decreased cell proliferation in the case of HL-60 cells. The effect of this concentration will be discussed, since the distinctive effect of the complex in comparison to the metal ion and the ligand was more evident.

The enhanced decrease in cell viability in HL-60 cells, after treatment with 2.5 μM $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, in comparison to treatment with either L-carnitine or copper chloride dihydrate as individual entities, was mainly due to decreased cell proliferation efficiency (Figure 4), whereas in the case of K562 cells only, increased necrotic phenomena appeared along with decrease in cell proliferation (Figure 5, Tables 1 and 2). Therefore, this overall intense cytotoxic activity of the complex (2.5 μM) in HL-60 cells seems to be due to its enhanced cell proliferation inhibitory activity as a possible synergistic effect of the compounds that exists in the complex

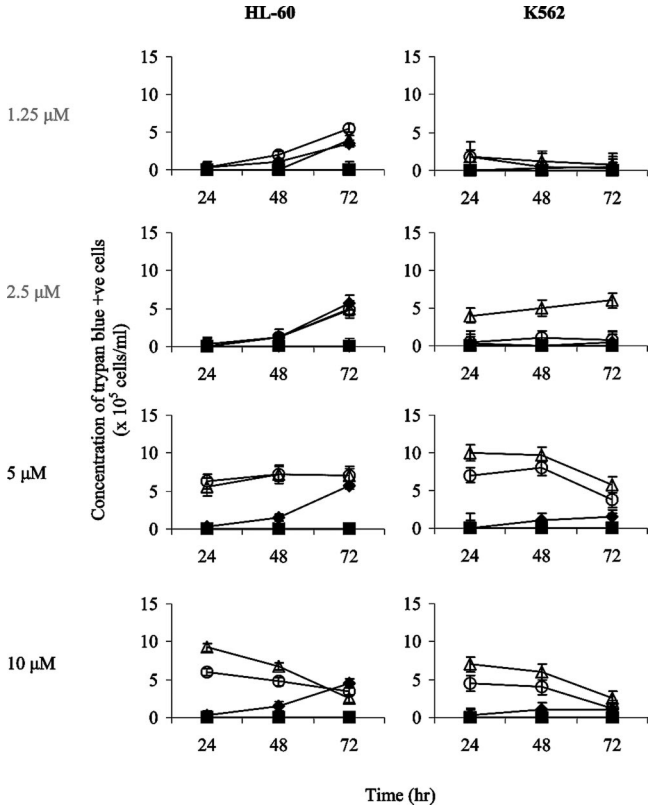

Figure 5. Treatment of HL-60 and K562 cell lines with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, displayed enhanced necrotic phenomena compared to L-carnitine or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) in K562 cells but not in HL-60 cells. The cells were treated in the absence (■) or presence of various doses of the complex (△) or of L-carnitine (◆) or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$) (○), as individual entities, for 24, 48, and 72 h, and necrotic phenomena were detected by Trypan blue staining. The results were expressed as the concentration of trypan blue +ve cells. Each data point represents the mean of three separate experiments (mean ± SD).

and to a much lesser extent due to necrotic phenomena, which are quite limited. Such a cell proliferation inhibitory effect of the compound and to a lesser extent of L-carnitine may be considered to be more due to the previously reported differentiation action of L-carnitine on HL-60 cells[37] than on DNA or a consequence of cell death, since L-carnitine possesses an inhibitory effect over apoptosis hallmarks induced by distinct drugs (GP-7 and IDA), such as DNA fragmentation, mitochondrial damage, and caspase activation.[35,36,39]

In addition, the enhanced decrease in cell number caused by the complex (2.5 μM), due to necrotic phenomena in the K562 cell line, could be interpreted by the more resistant nature of K562 cells to drug-induced programmed cell death mechanisms, which resulted in the cell selection of a distinct and more adverse type of cell death, necrosis. Cells under stress have to choose among distinct fates according to complicated biochemical pathways. For K562 cells and the compound concentration of 2.5 μM, necrosis was the fate pathway possibly because L-carnitine possesses a known apoptosis inhibitory activity. However, at 1.25 μM, although there were no important differences in the degree of decrease in cell viability among the compounds L-carnitine and copper chloride dehydrate, such a decrease was due to decreased cell proliferation efficiency and not due to necrotic phenomena. Such a result revealed that the concentration of the complex above a certain limit is deleterious for K562 cells, ultimately leading to necrosis, whereas at lower concentrations where its effect is due to decreased cell proliferation efficiency (like in HL-60 cells) almost to the same extent as L-carnitine and copper chloride dehydrate, differentiation may be the underlying mechanism.

Distinct results were observed in the case of cell treatment with L-carnitine (2.5 μM) as an individual entity, where HL-60 and not K562 cells responded with the appearance of limited necrotic phenomena, with both cell lines showing decreased proliferation efficiency, probably due to induction of differentiation. Additionally, copper chloride dihydrate (2.5 μM) alone caused the same effect on both cell lines as L-carnitine but to a different extent.

Taken together, these data indicate that in HL-60 cells the complex augmented the decrease in cell proliferation efficiency caused by both L-carnitine and copper chloride dihydrate, as individual entities. However, in K562 cells, the complex at 2.5 μM but not at 1.25 μM induced necrotic phenomena, which did not appear after cell treatment with L-carnitine or copper chloride dihydrate alone.

Overall, the compound $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$ showed mainly a concentration-specific mode of antileukemic action that was enhanced compared to L-carnitine or copper chloride dihydrate, as individual entities, providing valuable information about its potential as a tool in cancer chemotherapy, although further work is required.

Experimental Section

Chemistry. The reagents L-carnitine, $CuCl_2 \cdot 2H_2O$, and the solvent ethanol were purchased from Aldrich Chemical Co. and used without purification. All the reactions took place under aerobic conditions and room temperature.

Synthesis of the L-Carnitinatocopper(II) Complex $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$. A green solid of $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$ was prepared after the addition of $CuCl_2 \cdot 2H_2O$ (90.0 mg, 0.5 mmol) to an ethanolic solution of L-carnitine (80.0 mg, 0.5 mmol) and was filtered and dried overnight in vacuum over $CaCl_2$.[40] Anal. ($C_{14}H_{34}O_8N_2Cl_4Cu_2$) C, H, N.

IR Analysis. The infrared spectral data were recorded on a Perkin-Elmer 880 IR spectrophotometer. The solid was in a KBr pellet, and the frequency range was 4000−250 cm^{-1}. IR data (ν, cm^{-1}): 3418, 3021, 2955, 2918, 1619, 1421, 561, 431.

EPR Analysis. EPR spectra in aqueous solution (298 and 11 K) were recorded on a Bruker ER-200D-SRC X-band spectrometer interfaced to a personal computer and equipped with an Oxford EPR 900 cryostat, an Anritsu MF76A frequency counter, and a Bruker 035N NMR gauss meter.

UV−Visible Analysis. The UV−vis spectrum was recorded on a Hitachi U-2000 spectrophotometer. The complex was dissolved in water and was transferred to a 1 cm cuvette. UV−vis data (λ_{max}, nm): 240, 400, 790.

NMR Analysis. ^1H and ^{13}C NMR spectra were recorded on a Varian UNITYplus spectrometer operating at 300 MHz in D_2O with TSP as an internal reference (0.0 ppm). ^1H NMR (δ values in ppm): 1.29, 3.24, 3.87. ^{13}C NMR (δ values in ppm): 19.6, 56.6, 59.8, 81.7.

Cell Culture Conditions. HL-60 (peripheral blood human promyelocytic leukemia) and K562 (human chronic myelogenous leukemia) cell lines were maintained in an atmosphere of 95% air/5% CO_2 with 100% humidity at 37 °C and cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 200 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 2 mM $NaHCO_3$. Cells were seeded at 4×10^5 cells/mL, a density that allowed logarithmic growth of cells throughout the experiments. They were incubated at 37° for 24 h and treated with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, while in exponential growth phase, for the indicated

Table 1. Percentage (%) of Trypan Blue + Cells versus Total Number of Cells, in the Cell Line HL-60

	24 h		48 h		72 h	
sample	%	SD[a]	%	SD[a]	%	SD[a]
		1.25 μM				
control	0	0.003	0	0.002	0	0.001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	0	0.001	0	0.05	14.5	0.04
L-carnitine	1.4	0.005	5.3	0.01	13.6	0.01
Copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	1.4	0.001	10.8	0.05	26.5	0.01
		2.5 μM				
control	0	0.003	0	0.002	0	0.001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	0	0.001	8.8	0.005	26.3	0.008
L-carnitine	0	0.002	7.8	0.08	28.4	0.05
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	1.5	0.002	6.8	0.06	22.6	0.025
		5 μM				
control	0	0.003	0	0.002	0	0.001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	100	0.002	75	0.1	100	0.05
L-carnitine	1.8	0.04	9.2	0.0001	25.6	0.03
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	86.2	0.08	100	0.0001	100	0.025
		10 μM				
control	0	0.003	0	0.002	0	0.001
the dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	100	0.01	100	0.001	100	0.005
L-carnitine	1.7	0.005	9.7	0.05	30	0.05
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	100	0.002	100	0.001	100	0.005

[a] SD, standard deviation.

Table 2. Percentage (%) of Trypan Blue + Cells versus Total Number of Cells, in the Cell Line K562

	24 h		48 h		72 h	
sample	%	SD[a]	%	SD[a]	%	SD[a]
		1.25 μM				
control	0	0.0005	0	0.001	0	0.0001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	15.6	0.05	10.4	0.01	8.3	0.05
L-carnitine	0	0.001	2.5	0.025	4.7	0.025
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	16.3	0.04	4.9	0.05	3	0.05
		2.5 μM				
control	0	0.0005	0	0.001	0	0.0001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	36.4	0.01	46.5	0.05	57.1	0.025
L-carnitine	2.4	0.01	0	0.001	4.7	0.05
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	5.1	0.05	10.8	0.01	7.5	0.05
		5 μM				
control	0	0.0005	0	0.001	0	0.0001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	100	0.02	100	0.03	100	0.01
L-carnitine	0	0.0008	8.9	0.01	14.3	0.02
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	87.5	0.03	100	0.01	100	0.003
		10 μM				
control	0	0.0005	0	0.001	0	0.0001
dinuclear copper(II) complex of L-carnitine $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$	100	0.05	100	0.01	100	0.01
L-carnitine	2.4	0.03	11.1	0.02	11.8	0.001
copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$)	100	0.05	100	0.01	100	0.01

[a] SD, standard deviation.

time periods. The compounds were added to the cell medium, whose pH was 7−7.2, where they remained constantly throughout the experiments.

Assessment of Cell Viability by MTT. The sensitivity of the two cell lines under study to the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, was assessed using the MTT colorimetric assay, as described previously.[72] Briefly, exponentially growing human leukemia cells were seeded at a density of 4×10^5 cells/mL in triplicate, and 24 h later they were treated with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. This was followed by addition of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma Chemical Co, St. Louis, MO) at a final concentration of 0.5 mg/mL in the presence of which they were further incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere to allow MTT to form formazan crystals in metabolically active cells. Following the solubilization of formazan

crystals overnight at 37 °C, in a solution containing 12.5% SDS (Sigma Chemical Co, St. Louis, MO) and 45% (v/v) formamide (ACROS Organics, Geel, Belgium), the absorbance of each cell lysate solution was measured at 545 nm, with a reference wavelength of 690 nm. The results were expressed as the percentage (%) of treated cells versus untreated cells.

Determination of Necrotic Phenomena by Trypan Blue Staining. Exponentially growing human leukemia cells were seeded at a density of 4×10^5 cells/mL in triplicate, and 24 h later they were treated with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. An amount of 50 μL from each sample was then diluted 10× in PBS (1×), and an amount of 2 μL of 0.4% (w/v) Trypan blue stain (Sigma Chemical Co., St. Louis, MO) was added to 18 μL of 10× diluted cell suspension. They were mixed thoroughly and allowed to stand for 5 min at room temperature. The total number of cells and the number of blue-stained cells were counted on a hemocytometer, using a microscope. The determination of necrotic or secondary necrotic (late apoptotic) cell populations is based on the interaction of the negatively charged Trypan blue dye with only damaged cellular membranes, resulting in their penetration by the blue-colored dye and the staining of the cells.[73] The results were expressed as the concentration of Trypan blue stained cells (cells/mL) and as the percentage of the total cell number of cells. Each data point represents the mean of three separate experiments (mean ± SD).

Cell Growth Assay. The effect of the compounds under study on the proliferative capacity of leukemia cells was studied, which concerned the determination of the number of cells that were divided in the culture in the presence or absence of the compounds under study, as well as the creation of growth curves. For that purpose, exponentially growing human leukemia cells were seeded at a density of 4×10^5 cells/mL in triplicate, and 24 h later they were treated with the dinuclear copper(II) complex of L-carnitine, $[Cu_2(L\text{-carnitine})_2Cl_2(H_2O)_2]Cl_2$, L-carnitine, or copper chloride dihydrate ($CuCl_2 \cdot 2H_2O$), as individual entities, at 37 °C in a humidified 5% CO_2 atmosphere for the indicated time periods. Then a 50 μL aliquot from each sample was mixed thoroughly with 450 μL of complete medium without serum (10× dilution) and total cell number was determined manually under a light microscope, using a hemocytometer. The results were expressed as the total cell concentration of cells, with each data point representing the mean of three separate experiments (mean ± SD).

Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Ramsay, R. R.; Gandour, R. D.; van der Leij, F. R. Molecular enzymology of carnitine transfer and transport. *Biochim. Biophys. Acta* **2001**, *1546*, 21–43.

(2) Harpaz, S. L-Carnitine and its attributed functions in fish culture and nutrition. *Aquaculture* **2005**, *249*, 3–21.

(3) Arenas, J.; Rubio, J. C.; Martín, M. A.; Campos, Y. Biological roles of L-carnitine in perinatal metabolism. *Early Hum. Dev.* **1998**, *53 Suppl*, S43–50.

(4) Walter, P.; Schaffhauser, A. O. L-Carnitine, a "vitamin-like substance" for functional food. Proceedings of the symposium on L-carnitine, April 28 to May 1, 2000, Zermatt, Switzerland. *Ann. Nutr. Metab.* **2000**, *44*, 75–96.

(5) Karlic, H.; Lohninger, A. Supplementation of L-carnitine in athletes: does it make sense? *Nutrition* **2004**, *20*, 709–715.

(6) Speich, M.; Pineau, A.; Ballereau, F. Minerals, trace elements and related biological variables in athletes and during physical activity. *Clin. Chim. Acta* **2001**, *312*, 1–11.

(7) Arenas, J.; Ricoy, J. R.; Encinas, A. R.; Pola, P.; D'Iddio, S.; Zeviani, M.; Didonato, S.; Corsi, M. Carnitine in muscle, serum, and urine of nonprofessional athletes: effects of physical exercise, training, and L-carnitine administration. *Muscle Nerve* **1991**, *14*, 598–604.

(8) Carvajal, K.; Moreno-Sánchez, R. Heart metabolic disturbances in cardiovascular diseases. *Arch. Med. Res.* **2003**, *34*, 89–99.

(9) Giancaterini, A.; De Gaetano, A.; Mingrone, G.; Gniuli, D.; Liverani, E.; Capristo, E.; Greco, A. V. Acetyl-L-carnitine infusion increases glucose disposal in type 2 diabetic patients. *Metabolism* **2000**, *49*, 704–708.

(10) Giannessi, F.; Pessotto, P.; Tassoni, E.; Chiodi, P.; Conti, R.; De Angelis, F.; Dell'Uomo, N.; Catini, R.; Deias, R.; Tinti, M. O.; Carminati, P.; Arduini, A. Discovery of a long-chain carbamoyl aminocarnitine derivative, a reversible carnitine palmitoyltransferase inhibitor with antiketotic and antidiabetic activity. *J. Med. Chem.* **2003**, *46*, 303–309.

(11) Mingrone, G. Carnitine in type 2 diabetes. *Ann. N.Y. Acad. Sci.* **2004**, *1033*, 99–107.

(12) Dayanandan, A.; Kumar, P.; Panneerselvam, C. Protective role of L-carnitine on liver and heart lipid peroxidation in atherosclerotic rats. *J. Nutr. Biochem.* **2001**, *12*, 254–257.

(13) Thiel, R. J.; Fowkes, S. W. Down syndrome and epilepsy: a nutritional connection. *Med. Hypotheses* **2004**, *62*, 35–44.

(14) Seven, M.; Cengiz, M.; Tüzgen, S.; Iscan, M. Y. Plasma carnitine levels in children with Down syndrome. *Am. J. Hum. Biol.* **2001**, *13*, 721–725.

(15) Calò, L. A.; Pagnin, E.; Davis, P. A.; Semplicini, A.; Nicolai, R.; Calvani, M.; Pessina, A. C. Antioxidant effect of L-carnitine and its short chain esters: relevance for the protection from oxidative stress related cardiovascular damage. *Int. J. Cardiol.* **2006**, *107*, 54–60.

(16) Arsenian, M. A. Carnitine and its derivatives in cardiovascular disease. *Prog. Cardiovasc. Dis.* **1997**, *40*, 265–286.

(17) Savitha, S.; Tamilselvan, J.; Anusuyadevi, M.; Panneerselvam, C. Oxidative stress on mitochondrial antioxidant defense system in the aging process: role of DL-alpha-lipoic acid and L-carnitine. *Clin. Chim. Acta* **2005**, *355*, 173–180.

(18) Moretti, S.; Alesse, E.; Di Marzio, L.; Zazzeroni, F.; Ruggeri, B.; Marcellini, S.; Famularo, G.; Steinberg, S. M.; Boschini, A.; Cifone, M. G.; De Simone, C. Effect of L-carnitine on human immunodeficiency virus-1 infection-associated apoptosis: a pilot study. *Blood* **1998**, *91*, 3817–3824.

(19) Bueno, R.; Alvarez de Sotomayor, M.; Perez-Guerrero, C.; Gomez-Amores, L.; Vazquez, C. M.; Herrera, M. D. L-Carnitine and propionyl-L-carnitine improve endothelial dysfunction in spontaneously hypertensive rats: different participation of NO and COX-products. *Life Sci.* **2005**, *77*, 2082–9207.

(20) Hurot, J. M.; Cucherat, M.; Haugh, M.; Fouque, D. Effects of L-carnitine supplementation in maintenance hemodialysis patients: a systematic review. *J. Am. Soc. Nephrol.* **2002**, *13*, 708–714.

(21) Matsumoto, Y.; Amano, I.; Hirose, S.; Tsuruta, Y.; Hara, S.; Murata, M.; Imai, T. Effects of L-carnitine supplementation on renal anemia in poor responders to erythropoietin. *Blood Purif.* **2001**, *19*, 24–32.

(22) Wenzel, U.; Nickel, A.; Daniel, H. Increased carnitine-dependent fatty acid uptake into mitochondria of human colon cancer cells induces apoptosis. *J. Nutr.* **2005**, *135*, 1510–1514.

(23) Yeh, C. S.; Wang, J. Y.; Cheng, T. L.; Juan, C. H.; Wu, C. H.; Lin, S. R. Fatty acid metabolism pathway play an important role in carcinogenesis of human colorectal cancers by microarray-bioinformatics analysis. *Cancer Lett.* **2006**, *233*, 297–308.

(24) Peluso, G.; Nicolai, R.; Reda, E.; Benatti, P.; Barbarisi, A.; Calvani, M. Cancer and anticancer therapy-induced modifications on metabolism mediated by carnitine system. *J. Cell. Physiol.* **2000**, *182*, 339–350.

(25) Chang, B.; Nishikawa, M.; Sato, E.; Utsumi, K.; Inoue, M. L-Carnitine inhibits cisplatin-induced injury of the kidney and small intestine. *Arch. Biochem. Biophys.* **2002**, *405*, 55–64.

(26) Feng, C. J.; Luo, Q. H.; Wang, Z. L.; Shen, M. C.; Wang, H. W.; Zhao, M. H. A study on a novel superoxide dismutase mimic bis(dioxotetraamine) copper(II) complex—kinetics of catalytic dismutation of superoxide anion by pulse radiolysis and solution equilibrium. *J. Inorg. Biochem.* **1999**, *75*, 1–6.

(27) Zhou, L.; Sorenson, J. R. J. Formation of Cu or Fe thiobarbiturate complexes interfere with the determination of malondialdehyde. *J. Inorg. Biochem.* **1998**, *72*, 217–225.

(28) Efthimiadou, E. K.; Sanakis, Y.; Raptopoulou, C. P.; Karaliota, A.; Katsaros, N.; Psomas, G. Crystal structure, spectroscopic, and biological study of the copper(II) complex with third-generation quinolone antibiotic sparfloxacin. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 3864–3867.

(29) Efthimiadou, E. K.; Sanakis, Y.; Katsarou, M.; Raptopoulou, C. P.; Karaliota, A.; Katsaros, N.; Psomas, G. Neutral and cationic mononuclear copper(II) complexes with enrofloxacin: structure and biological activity. *J. Inorg. Biochem.* **2006**, *100*, 1378–1388.

(30) Karaliota, A.; Kretsi, O.; Tzougraki, C. Synthesis and characterization of a binuclear coumarin-3-carboxylate copper(II) complex. *J. Inorg. Biochem.* **2001**, *84*, 33–37.

(31) Melnik, M. Study of the relation between the structural data and magnetic interaction in oxo-bridged binuclear copper(II) compounds. *Coord. Chem. Rev.* **1982**, *42*, 259–293.

(32) Kato, M.; Muto, Y. Factors affecting the magnetic properties of dimeric copper(II) complexes. *Coord. Chem. Rev.* **1988**, *92*, 45–83.
(33) Weder, J. E.; Dillon, C. T.; Hambley, T. W.; Kennedy, B. J.; Lay, P. A.; Biffin, J. R.; Regtop, H. L.; Davies, N. M. Copper complexes of non-steroidal anti-inflammatory drugs: an opportunity yet to be realized. *Coord. Chem. Rev.* **2002**, *232*, 95–126.
(34) Rajendiran, V.; Karthik, R.; Palaniandavar, M.; Stoeckli-Evans, H.; Periasamy, V. S.; Akbarsha, M. A.; Srinag, B. S.; Krishnamurthy, H. Mixed-ligand copper(II)-phenolate complexes: effect of coligand on enhanced DNA and protein binding, DNA cleavage, and anticancer activity. *Inorg. Chem.* **2007**, *46*, 8208–8221.
(35) Qi, S. N.; Yoshida, A.; Wang, Z. R.; Ueda, T. GP7 can induce apoptotic DNA fragmentation of human leukemia cells through caspase-3-dependent and -independent pathways. *Int. J. Mol. Med.* **2004**, *13*, 163–167.
(36) Qi, S. N.; Yoshida, A.; Ueda, T. Activation of caspases-3/7 is dispensable for idarubicin-induced apoptotic DNA fragmentation in human leukemia cells. *Int. J. Oncol.* **2003**, *22*, 1123–1128.
(37) Vassiliadis, S.; Evangeliou, A.; Basta, E.; Dionyssopoulou, E.; Dimitriou, H.; Stiakaki, E.; Kalmanti, M.; Athanassakis, I. The role of L-carnitine on a restricted number of myeloid leukemia progenitor cells: generation of atypical cell types. *Haematologia* **2002**, *32*, 341–353.
(38) Nikula, P.; Alhonen-Hongisto, L.; Seppänen, P.; Jänne, J. Inhibition of long-chain fatty acid oxidation by methylglyoxal bis(guanylhydrazone). *Biochem. Biophys. Res. Commun.* **1984**, *120*, 9–14.
(39) Nikula, P.; Ruohola, H.; Alhonen-Hongisto, L.; Jänne, J. Carnitine prevents the early mitochondrial damage induced by methylglyoxal bis(guanylhydrazone) in L1210 leukaemia cells. *Biochem. J.* **1985**, *228*, 513–516.
(40) Litos, C.; Parsons, S.; Karaliota, A. Synthesis of two dicopper(II) complexes of L-carnitine: the first structural determination of a metal complex containing L-carnitine. *Polyhedron* **2007**, *26*, 1397–1403.
(41) Durán, J. M.; Peral, M. J.; Calonge, M. L.; Ilundáin, A. A. Functional characterization of intestinal L-carnitine transport. *J. Membr. Biol.* **2002**, *185*, 65–74.
(42) Mokhova, E.; Arrigoni-Martelli, E.; Bellei, M.; Dedukhova, V.; Muscatello, U.; Starkov, A.; Bobyleva, V. The protecting effect of L-carnitine on Ca(2+)-loaded rat liver mitochondria. *FEBS Lett.* **1991**, *289*, 187–189.
(43) Yoshikawa, Y.; Ueda, E.; Sakurai, H.; Kojima, Y. Anti-diabetes effect of Zn(II)/carnitine complex by oral administration. *Chem. Pharm. Bull. (Tokyo)* **2003**, *51*, 230–231.
(44) Reznick, A. K.; Kagen, V. E.; Ransey, R.; Tsuchiya, M.; Serbinova, E. A.; Packer, L. Protection of the heart against ischemic reperfusion injury. The possible role of ion chelation. *Arch. Biochem. Biophys.* **1992**, *296*, 349–401.
(45) Packer, L.; Valenza, M.; Serbinova, E.; Starke-Reed, P.; Frost, K.; Kagan, V. Free radical scavenging is involved in the protective effect of L-propionyl-carnitine against ischemia-reperfusion injury of the heart. *Arch. Biochem. Biophys.* **1991**, *288*, 533–537.
(46) Chang, B.; Nishikawa, M.; Nishiguchi, S.; Inoue, M. L-Carnitine inhibits hepatocarcinogenesis via protection of mitochondria. *Int. J. Cancer.* **2005**, *113*, 719–729.
(47) Mathey, Y.; Greig, D. R.; Shriver, D. F. Variable-temperature Raman and infrared spectra of the copper acetate dimer $Cu_2(O_2CCH_3)_4(H_2O)_2$ and its derivatives. *Inorg. Chem.* **1982**, *21*, 3409–3413.
(48) Karaliota, A.; Kretsi, O.; Tzougraki, C. Synthesis and characterization of a binuclear coumarin-3-carboxylate copper(II) complex. *J. Inorg. Biochem.* **2001**, *84*, 33–37.
(49) Alves, W. A.; De Almeida Santos, R. H.; Paduan-Filho, A.; Becerra, C. C.; Borin, A. C.; Da Costa Ferreira, A. M. *Inorg. Chim. Acta* **2004**, *357*, 2269–2278.
(50) Tirado-Guerra, S.; Cuevas-Garibay, N. A.; Sosa-Torres, M. E.; Zamorano-Ulloa, R. Ferromagnetic exchange interaction of the Cu−Cu dimer $[Cu_2(picditn)(CH_3CO_2)(H_2O)_2Cl][ZnCl_4]\cdot 0.5H_2O$ [picditn = 1,11-bis(2-pyridyl)-2,6,10-triazaundecane]. *J. Chem. Soc., Dalton Trans.* **1998**, 2431–2436.
(51) Rodriguez, M.; Llobet, A.; Corbella, M. A theoretical analysis of how geometrical distortions on $Cu(\mu\text{-}Cl)_2Cu$ dimers influence their electronic and magnetic properties. *Polyhedron* **2000**, *19*, 2483–2491.
(52) Gottlieb, R. A.; Burleson, K. O.; Kloner, R. A.; Babior, B. M.; Engler, R. L. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J. Clin. Invest.* **1994**, *94*, 1621–1628.
(53) Cotman, C. W.; Anderson, A. J. A potential role for apoptosis in neurodegeneration and Alzheimer's disease. *Mol. Neurobiol.* **1995**, *10*, 19–45.
(54) Gelbard, H. A.; James, H. J.; Sharer, L. R.; Perry, S. W.; Saito, Y.; Kazee, A. M.; Blumberg, B. M.; Epstein, L. G. Apoptotic neurons in brains from paediatric patients with HIV-1 encephalitis and progressive encephalopathy. *Neuropathol. Appl. Neurobiol.* **1995**, *21*, 208–217.
(55) Mutomba, M. C.; Yuan, H.; Konyavko, M.; Adachi, S.; Yokoyama, C.; Esser, V.; McGarry, J. D.; Babior, B. M.; Gottlieb, R. A. Regulation of the activity of caspases by L-carnitine and palmitoylcarnitine. *FEBS Lett.* **2000**, *478*, 19–25.
(56) Thomadaki, H.; Karaliota, A.; Litos, C.; Scorilas, A. Enhanced antileukemic activity of the novel complex 2,5-dihydroxybenzoate molybdenum(VI) against 2,5-dihydroxybenzoate, polyoxometalate of Mo(VI), and tetraphenylphosphonium in the human HL-60 and K562 leukemic cell lines. *J. Med. Chem.* **2007**, *50*, 1316–1321.
(57) Efthimiadou, E. K.; Thomadaki, H.; Sanakis, Y.; Raptopoulou, C. P.; Katsaros, N.; Scorilas, A.; Karaliota, A.; Psomas, G. Structure and biological properties of the copper(II) complex with the quinolone antibacterial drug *N*-propyl-norfloxacin and 2,2′-bipyridine. *J. Inorg. Biochem.* **2007**, *101*, 64–73.
(58) Floros, K. V.; Thomadaki, H.; Lallas, G.; Katsaros, N.; Talieri, M.; Scorilas, A. Cisplatin-induced apoptosis in HL-60 human promyelocytic leukemia cells: differential expression of BCL2 and novel apoptosis-related gene BCL2L12. *Ann. N.Y. Acad. Sci.* **2003**, *1010*, 153–158.
(59) Floros, K. V.; Thomadaki, H.; Katsaros, N.; Talieri, M.; Scorilas, A. mRNA expression analysis of a variety of apoptosis-related genes, including the novel gene of the BCL2-family, BCL2L12, in HL-60 leukemia cells after treatment with carboplatin and doxorubicin. *Biol. Chem.* **2004**, *385*, 1099–1103.
(60) Thomadaki, H.; Tsiapalis, C. M.; Scorilas, A. Polyadenylate polymerase modulations in human epithelioid cervix and breast cancer cell lines, treated with etoposide or cordycepin, follow cell cycle rather than apoptosis induction. *Biol Chem.* **2005**, *386*, 471–480.
(61) Floros, K. V.; Thomadaki, H.; Florou, D.; Talieri, M.; Scorilas, A. Alterations in the mRNA expression of the apoptosis-related genes BCL2, BAX, FAS, CASPASE-3 and the novel member, BCL2L12, after treatment of human leukemic cell line HL60 with the antineoplasitic agent etoposide. *Ann. N.Y. Acad. Sci.*, in press.
(62) Thomadaki, H.; Scorilas, A Breast cancer cells response to the antineoplastic agents cisplatin, carboplatin and doxorubicin, at the mRNA expression levels of distinct apoptosis-related genes, including the new member, BCL2L12. *Ann. N.Y. Acad. Sci.*, in press.
(63) Reed, J. C. Dysregulation of apoptosis in cancer. *J. Clin. Oncol.* **1999**, *17*, 2941–2953.
(64) Seller, W. R.; Fisher, D. E. Apoptosis and cancer drug targeting. *J. Clin. Invest.* **1999**, *104*, 1655–1661.
(65) Fisher, D. E. Apoptosis in cancer therapy: crossing the threshold. *Cell* **1994**, *78*, 539–542.
(66) Kerr, J. F.; Winterford, C. M.; Harmon, B. V. Apoptosis. Its significance in cancer and cancer chemotherapy. *Cancer* **1994**, *73*, 2013–2026.
(67) Belamy, C. O.; Malcomson, R. D.; Harrison, D. J.; Wyllie, A. H. Cell death in health and disease: the biology and regulation of apoptosis. *Semin. Cancer Biol.* **1995**, *6*, 3–16.
(68) McDonnell, T. J.; Meyn, R. E.; Robertson, L. E. Implications of apoptosis cell death regulation in cancer therapy. *Semin. Cancer Biol.* **1995**, *6*, 3–16.
(69) Eastman, A. Survival factors, intracellular signal transduction and the activation of endonucleases in apoptosis. *Cancer Biol.* **1995**, *6*, 53–60.
(70) Shah, A. M.; Schwartz, G. K. Cell cycle-mediated drug resistance:an emerging concept in cancer therapy. *Clin. Cancer Res.* **2001**, *7*, 2168–2181.
(71) Kanduc, D.; Mittelman, A.; Serpico, R.; Sinigaglia, E.; Sinha, A. A.; Natale, C.; Santacroce, R.; Di Corcia, M. G.; Lucchese, A.; Dini, L.; Pani, P.; Santacroce, S.; Simone, S.; Bucci, R.; Farber, E. Cell death: apoptosis versus necrosis. *Int. J. Oncol.* **2002**, *21*, 165–170.
(72) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
(73) Freshney, R. *Culture of Animal Cells: A Manual of Basic Techniqu*; Alan R. Liss, Inc.: New York, 1987; p 117.

JM701473X