N-hydroxy-N'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine, a novel resveratrol analog, inhibits ribonucleotide reductase in HL-60 human promyelocytic leukemia cells: Synergistic antitumor activity with arabinofuranosylcytosine

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Abstract. Resveratrol (3,4',5,-trihydroxystilbene, RV), an ingredient of wine, exhibits a broad spectrum of antiproliferative effects against human cancer cells. In order to develop a derivative with comparable effects, we modified the molecule by introducing additional methoxyl groups. The resulting novel RV analog, *N*-hydroxy-*N*'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine (KITC), was investigated in HL-60 human promyelocytic leukemia cells. The induction of apoptosis was determined employing a specific Hoechst/propidium iodide double staining method and cell cycle distribution was evaluated by FACS. KITC's influence on the concentration of deoxyribonucleoside triphosphates, the products of ribonucleotide reductase (RR), was determined using the HPLC method. In addition, we analyzed the effects of KITC treatment on the incorporation of ^{14}C-cytidine into the DNA of tumor cells in order to quantify the loss of RR *in situ* activity. To reveal a potential value of KITC for supporting conventional chemotherapy, we also examined whether a combination of KITC with arabinofuranosylcytosine (Ara-C) could yield synergistic growth inhibitory effects. KITC caused a dose-dependent induction of apoptosis, whereas no remarkable changes of the cell cycle distribution were observed. Incubation with KITC resulted in a significant depletion of intracellular dTTP and dATP pools and was also found to remarkably reduce the *in situ* activity of RR, the key enzyme of *de novo* DNA synthesis. In addition, KITC exhibited synergistic combination effects when applied sequentially with Ara-C. Due to these promising results, KITC deserves further preclinical and *in vivo* testing.

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Introduction

Naturally occurring compounds with putative cancer chemopreventive properties, such as the phytoalexin resveratrol (3,4',5-trihydroxy-*trans*-stilbene; RV), guide the design of novel agents with improved pharmacologic potential. RV displays remarkable cytostatic and cytotoxic activity against a multitude of human cancer cell lines (1-4). RV induces apoptosis via the activation of caspases as well as through the release of mitochondrial cytochrome c (5,6) and was also identified as an effective inhibitor of ribonucleotide reductase (RR). RR catalyzes the rate limiting step of *de novo* DNA synthesis and is highly upregulated in rapidly proliferating tumor cells in comparison with non-malignant cell populations, which makes the enzyme an excellent target for cancer chemotherapy (7,8). Other clinically established inhibitors of RR, such as hydroxyurea or fludarabine are applied successfully in combination chemotherapy regimens for the treatment of leukemia. These compounds have been proven to exert synergistic effects with the first-line antileukemic agent arabinofuranosylcytosine (Ara-C) (9-14). In addition, RV has been shown to inhibit DNA polymerase and to arrest

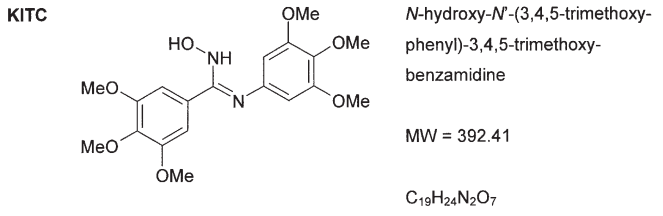

Figure 1. Structural formula of KITC including nomenclature, molecular weight and empirical formula.

cells in the S and G2 phases of the cell cycle (15-19), and to reduce the growth of various myeloma cell lines by a mechanism involving apoptosis (20). RV has been reported to be effective in inhibiting platelet aggregation and lipid peroxidation, altering eicosanoid synthesis, modulating lipoprotein metabolism (21-22), and exhibiting vasorelaxing and anti-inflammatory activities (23). A number of methoxylated RV derivatives have also displayed remarkable *in vitro* growth inhibition and apoptotic activity in HL-60 cells (24). MR-4, a tetramethoxystilbene, specifically activated the mitochondria-mediated apoptotic pathway in WI38VA cells (25) and was proven to possess even stronger antiproliferative properties than RV in human HCA-7 colon cancer cells (26). Another methoxylated RV molecule, 3,4',5-trimethoxystilbene, exerted cytotoxic effects by depleting the intracellular polyamine pools and by inhibiting tubulin polymerization in Caco-2 cells (27).

In this context, we prepared a series of polymethoxylated RV analogs with the aim of discovering new agents with potential clinical relevance. Here, the antitumor effects of *N*-hydroxy-*N*'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine (KITC) are presented. The activity of KITC was tested in HL-60 human promyelocytic leukemia cells. Its cytotoxicity was evaluated by growth inhibition assays and the induction of apoptosis was determined employing a specific Hoechst/propidium iodide double staining method. Cell cycle distribution after exposure to KITC was investigated by FACS analysis. Since we have shown previously that RV depletes intracellular dNTP concentrations (28), the products of RR, we now investigated whether KITC had comparable effects on dNTP pools. In addition, we analyzed the effects of KITC treatment on the incorporation of ^{14}C-cytidine into the DNA of HL-60 cells in order to quantify the loss of RR *in situ* activity. To determine a potential value of KITC in supporting conventional chemotherapy of human malignancies, we finally examined whether a combination of KITC with Ara-C could yield synergistic growth inhibitory effects in HL-60 cells.

Materials and methods

Chemicals and supplies. The investigated *N*-hydroxy-*N*'-(3,4,5-trimethoxyphenyl)-3,4,5-trimethoxybenzamidine (KITC) was synthesized and provided by Dr T. Erker, University of Vienna, Austria (structural formula is shown in Fig. 1). All other chemicals and reagents were commercially available (Sigma-Aldrich, Vienna, Austria) and of the highest purity.

Cell culture. The HL-60 human promyelocytic leukemia cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 1% L-Glutamine and 1% Penicillin-streptomycin in a humidified atmosphere consisting of 5% CO_2. All media and supplements were obtained from Life Technologies (Paisley, UK). Cell counts were determined using a microcellcounter CC-108 (SYSMEX, Kobe, Japan). Cells in the logarithmic phase of growth were used for all experiments described below.

Growth inhibition assay. HL-60 cells ($0.1x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of KITC at 37˚C under cell culture conditions. Cell counts and IC_{50} values were determined after 72 h using the microcellcounter CC-108. The viability of cells was determined by staining with trypan-blue. Results were calculated as number of viable cells.

Hoechst dye 33258 and propidium iodide double staining. The Hoechst staining was performed according to the method described by Grusch and coworkers (29). HL-60 cells ($0.4x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and exposed to increasing concentrations of KITC for 48 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, cells were examined on a Leica DMR XA fluorescence microscope (Leica, Wetzlar, Germany) equipped with appropriate filters for Hoechst 33258 and PI. This method allows to distinguish between early apoptosis, late apoptosis, and necrosis. Cells were judged according to their morphology and the integrity of their cell membranes, which can easily be seen after propidium iodide staining. Cells were counted under the microscope (150 cells per flask) and the number of apoptotic cells was given as percentage value.

Cell-cycle distribution analysis. HL-60 cells ($0.4x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of KITC at 37˚C under cell culture conditions. After 24 h, cells were harvested and suspended in 5 ml cold PBS, centrifuged, resuspended and fixed in 3 ml cold ethanol (70%) for 30 min at 4˚C. After two washing steps in cold PBS RNAse A and propidium iodide were added to a final concentration of 50 μg/ml each and incubated at 4˚C for 60 min before measurement. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell-cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA).

Determination of deoxyribonucleoside triphosphates (dNTPs). Logarithmically growing HL-60 ($0.4x10^6$ cells per ml) cells were incubated with 25, 50, and 100 μM KITC for 24 h. Afterwards, $5x10^7$ cells were separated for the extraction of dNTPs according to the method described by Garrett and Santi (30). Cells were centrifuged at 1800 rpm for 5 min and then resuspended in 100 μl phosphate-buffered saline. In this suspension, cells were lysed by addition of 10 μl of trichloroacetic acid and the mixture was vortexed for 1 min. The lysate

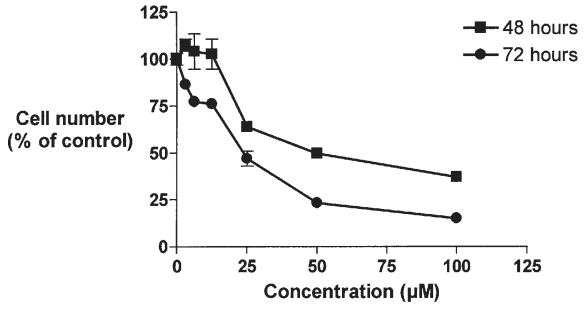

Figure 2. Growth inhibition of human HL-60 promyelocytic leukemia cells after incubation with KITC for 48 and 72 h. HL-60 cells ($0.1x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of KITC at 37˚C under cell culture conditions. Cell counts and IC_{50} values were determined using the microcellcounter CC-108. Viability of cells was determined by trypan-blue staining. Results were calculated as number of viable cells. Data are means ± standard errors of three determinations of one representative experiment.

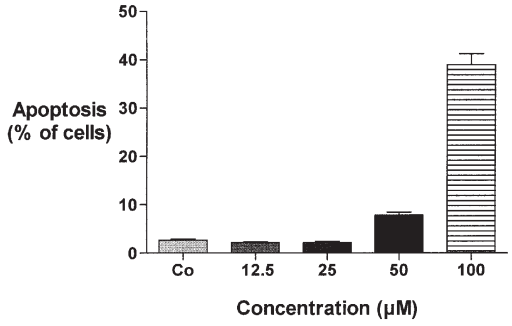

Figure 3. Induction of apoptosis in human HL-60 promyelocytic leukemia cells after incubation with KITC for 48 h. HL-60 cells ($0.4x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and exposed to increasing concentrations of KITC for 48 h. Hoechst 33258 (HO, Sigma, St. Louis, MO, USA) and propidium iodide (PI, Sigma) were added directly to the cells to final concentrations of 5 and 2 μg/ml, respectively. After 60 min of incubation at 37˚C, cells were counted under a fluorescence microscope and the number of apoptotic cells was given as percentage value. Data are means ± standard errors of three determinations of one representative experiment.

was rested on ice for 30 min and then the protein was separated by centrifugation at 15000 rpm for 10 min in an Eppendorf microcentrifuge. The supernatant was removed and neutralized by adding 1.1 vol of Freon containing 0.5 M tri-n-octylamin. Aliquots of 100 μl were periodated by adding 30 μl of 4 M methylamine solution and 10 μl sodium periodate solution (concentration: 100 g/l). After incubation at 37˚C for 30 min, the reaction was stopped by adding 5 μl of 1 M rhamnose solution. The extracted dNTPs were measured using a Merck 'La Chrom' HPLC system equipped with L-7200 autosampler, L-7100 pump, L-7400 UV detector, and D-7000 interface. Samples were eluted with a 3.2 M ammonium phosphate buffer, pH 3.4 (pH adjusted by addition of 0.32 mol/l H_3PO_4), containing 20 mM acetonitrile using a 4.6x250 mm Partisil 10 SAX analytical column (Whatman, Kent, UK). Separation was performed at constant ambient temperature with a flow rate of 2 ml/min. The concentration of dNTPs was calculated as percent of total area under the curve for each sample. Intracellular concentrations of dNTPs in untreated control cells were 4.96, 24.52, and 7.22 μM for dCTP, dTTP, and dATP, respectively.

Incorporation of ^{14}C-labelled cytidine into DNA. To analyze the effect of KITC incubation on the activity of DNA synthesis, an assay was performed as described previously (31). Logarithmically growing HL-60 cells ($0.4x10^6$ cells per ml) were incubated with various concentrations of KITC for 24 h. After incubation, cells were counted and pulsed with ^{14}C-cytidine (0.3125 μCi, 5 nM) for 30 min at 37˚C. Afterwards, cells were collected by centrifugation and washed with PBS. Total DNA was extracted from $5x10^6$ cells and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (Perkin-Elmer, Boston, MA).

Sequential growth inhibition assay using KITC and Ara-C. Employing a sequential growth inhibition assay, HL-60 cells ($0.1x10^6$ per ml) were first incubated with different concentrations of KITC (5, 10, and 20 μM) for 24 h. Then KITC was washed out and cells were further exposed to various concentrations of Ara-C (5, 10, and 15 nM) for another 48 h, always using a constant combination ratio. After that period, cells were counted using a microcellcounter CC-108.

Statistical calculations. Dose-response curves were calculated using the Prism 4.03 software package (GraphPad, San Diego, CA, USA) and statistical significance was determined by unpaired t-test. The calculations of dose response curves and combination effects were performed using the Calcusyn software designed by Chou and Talalay (Biosoft, Ferguson, MO) (32).

Results

Effect of KITC on the growth of HL-60 human promyelocytic leukemia cells. Logarithmically growing HL-60 cells were incubated with increasing concentrations of KITC. After 48 and 72 h of incubation, KITC inhibited the growth of tumor cells with IC_{50} values of 49 and 23 μM, respectively. Results are depicted in Fig. 2.

Induction of apoptosis in HL-60 human promyelocytic leukemia cells by KITC. HL-60 cells were incubated with 12.5, 25, 50, and 100 μM KITC for 48 h. Then cells were double stained with Hoechst 33258 and propidium iodide as described in the methods section. After the incubation period, the morphology of HL-60 cells showed nuclear condensation and fragmentation (early apoptosis) as well as signs of late apoptosis with membrane damage and incorporation of propidium iodide. As seen in Fig. 3, induction of apoptosis was dose dependent with up to 39% of cells showing hallmarks of apoptosis at 100 μM KITC.

Cell-cycle distribution in HL-60 human promyelocytic leukemia cells after treatment with KITC. HL-60 cells were prepared as described in the methods section and incubated with increasing concentrations of KITC for 24 h. Up to a concentration of 400 μM, no remarkable changes of the cell cycle phase distribution were observed. Growth arrest after treatment with 400 μM KITC occurred mainly in the G0-G1 phase, increasing

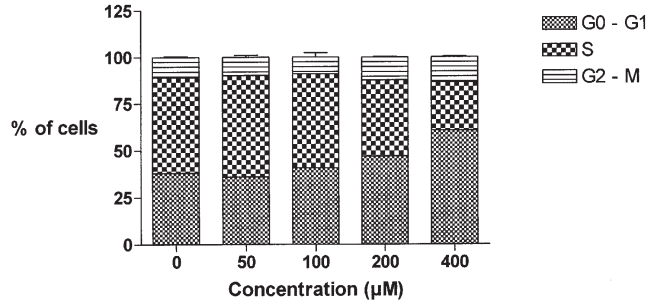

Figure 4. Cell cycle distribution in human HL-60 promyelocytic leukemia cells after incubation with KITC for 24 h. HL-60 cells ($0.4x10^6$ per ml) were seeded in 25-cm^2 Nunc tissue culture flasks and incubated with increasing concentrations of KITC at 37˚C under cell culture conditions. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was calculated with ModFit LT software (Verity Software House, Topsham, ME, USA). Data are means ± standard errors of three determinations of one representative experiment.

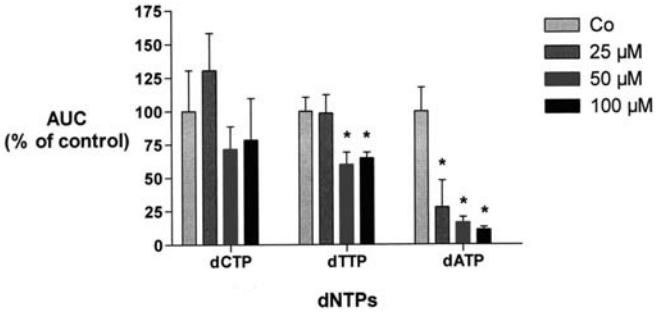

Figure 5. Concentration of deoxyribonucleoside triphosphate (dNTP) pools in human HL-60 promyelocytic leukemia cells after treatment with KITC for 24 h. Logarithmically growing HL-60 cells ($0.4x10^6$ cells per ml) were incubated with 25, 50, and 100 μM KITC for 24 h. Afterwards, $5x10^7$ cells were separated for the extraction of dNTPs. The concentration of dNTPs was calculated as percent of total area under the curve for each sample. Intracellular concentrations of dNTPs in untreated control cells were 4.96, 24.52, and 7.22 μM for dCTP, dTTP, and dATP, respectively. Data are means ± standard errors of three determinations of one representative experiment. *Values significantly ($p<0.05$) different from control.

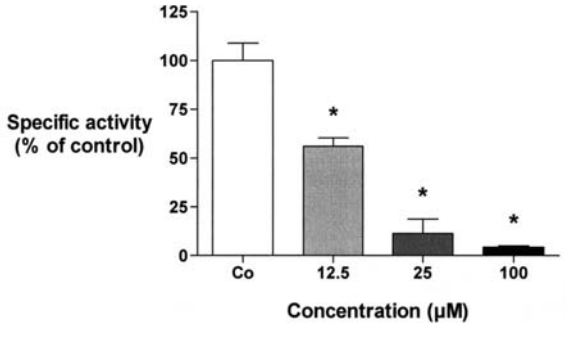

Figure 6. *In situ* measurement of ribonucleotide reductase (RR) activity in human HL-60 promyelocytic leukemia cells after incubation with KITC for 24 h. Logarithmically growing HL-60 cells ($0.4x10^6$ cells per ml) were incubated with increasing concentrations of KITC for 24 h. After the incubation period, cells were counted and pulsed with ^{14}C-cytidine (0.3125 μCi, 5 nM) for 30 min at 37˚C. Then cells were collected by centrifugation and washed with PBS. Total DNA was extracted from $5x10^6$ cells and specific radioactivity of the samples was determined using a Wallac 1414 liquid scintillation counter (Perkin-Elmer, Boston, MA). Data are means ± standard errors of two determinations of one representative experiment. *Values significantly ($p<0.05$) different from control.

the cell population from 38% to 61%. Simultaneously, cells in the S phase were decreased from 51% to 26%. Results are summarized in Fig. 4.

Effect of KITC on deoxyribonucleoside triphosphates (dNTPs) in HL-60 cells. HL-60 cells were incubated with 25, 50, and 100 μM of KITC for 24 h. Then dNTP pool sizes were determined using the HPLC method described in the methods section. KITC treatment caused a remarkable imbalance of dNTPs in HL-60 cells. Incubation of cells with 50 and 100 μM KITC for 24 h resulted in a depletion of intracellular dCTP, dTTP, and dATP pools. Treatment with 50 μM KITC significantly decreased dTTP and dATP pools to 60% and 16% of control values, respectively. In contrast, 25 μM KITC led to an increase of the dCTP pool to 130% of control values, while decreasing the dATP pool to 27% of controls. After incubation with 100 μM KITC for 24 h, dTTP and dATP pools were again significantly decreased when compared with untreated controls. All dGTP pools remained beyond the detectability of the method. Results are shown in Fig. 5.

Inhibition of incorporation of ^{14}C-cytidine into DNA. Incorporation of ^{14}C-cytidine into the DNA of HL-60 cells was determined after incubation with KITC. After treatment of HL-60 cells with 12.5, 25, and 100 μM KITC for 24 h, ^{14}C-cytidine incorporation into DNA was significantly decreased to 56%, 11.5%, and 4.3% of control values, respectively. Results are depicted in Fig. 6.

Synergistic combination effects of KITC and Ara-C on the growth of HL-60 cells. Logarithmically growing HL-60 cells were seeded at a concentration of $0.1x10^6$ per ml and incubated with increasing concentrations of drugs. Eight out of nine combinations applied (5, 10, and 20 μM KITC combined with 5, 10, and 15 nM Ara-C, respectively) yielded synergistic combination effects according to the equation of Chou and Talalay. All data are summarized in Table I.

Discussion

Increased cell proliferation and decreased cell death (by means of apoptosis) are two major processes that contribute to the progression of cancer cell growth. Consequently, new agents that can inhibit cell proliferation and/or induce apoptosis are of great therapeutic value. Resveratrol (3,4',5-trihydroxystilbene; RV) is a naturally occurring polyphenolic compound found in grapes, wine and various medical plants. Numerous studies have revealed that RV exerts remarkable antioxidant, anti-inflammatory, and anti-tumor activities as well as demonstrating a preventive effect for cancer. RV was proven to inhibit ribonucleotide reductase (RR) which is the rate-limiting enzyme for *de novo* DNA synthesis and therefore considered an excellent target for cancer chemotherapy. Balanced deoxyribonucleoside triphosphate (dNTP) concentrations are essential for DNA synthesis, especially in rapidly proliferating tumor cells. Various inhibitors of RR, such as hydroxyurea or difluorodeoxycytidine (Gemcitabine, dFdC), are commonly used for the treatment of various human malignancies.

Table I. Synergistic combination effects of KITC and Ara-C in human HL-60 promyelocytic leukemia cells employing a sequential growth inhibition assay.

Compound	Concentration (μM/nM)	Cell number (% of control)	Predicted value[a]	Combination index[b]
KITC (A)	5	88.2		
(μM)	10	57.8		
	20	58.2		
Ara-C (B)	5	76.2		
(nM)	10	67.0		
	15	60.5		
KITC	5			
+ Ara-C	5	55.2	67.2	0.525[c]
KITC	5			
+ Ara-C	10	50.6	59.1	0.608[c]
KITC	5			
+ Ara-C	15	38.1	53.4	0.417[c]
KITC	10			
+ Ara-C	5	41.3	44.2	0.465[c]
KITC	10			
+ Ara-C	10	37.7	38.8	0.483[c]
KITC	10			
+ Ara-C	15	38.1	35.1	
KITC	20			
+ Ara-C	5	38.1	44.4	0.730[c]
KITC	20			
+ Ara-C	10	35.1	39.0	0.720[c]
KITC	20			
+ Ara-C	15	33.5	35.2	0.736[c]

Cells were sequentially incubated with KITC (24 h) and Ara-C (48 h), and then cell count was determined. Data are means of three determinations of one representative experiment. All standard deviations were within 5%. [a]Predicted value: (%A x %B)/100. [b]Combination indices according to the equation of Chou and Talalay (32). [c]Synergistic combination effect.

In this context, we prepared a series of polymethoxylated RV analogs with the aim of discovering new agents with potential clinical relevance. One of these derivatives is KITC, which proved to be a potent inhibitor of RR, resulting in a significant alteration of dNTP pool balance. Due to this effect, DNA synthesis in rapidly growing cancer cells is blocked and cell cycle perturbations and/or induction of apoptosis are the consequence. After 72 h of incubation, KITC inhibited the growth of tumor cells with an IC_{50} value of 23 μM. Although RV was found to be a more potent inhibitor of RR by causing depletion of all dNTP pools in HL-60 cells (28), KITC possesses a slightly different profile based on its effects on intracellular dNTP concentrations. Incubation with 50 or 100 μM KITC caused a significant decrease in intracellular dTTP and dATP pools while the amount of dGTP remained beyond the detectability of the method. In particular, a similar depletion of dATP pool sizes could previously be observed with Gemcitabine (9,33), a mechanism mainly contributing to the antitumor properties of this clinically established anticancer drug. By causing such an imbalance on the concentration of precursors of *de novo* DNA synthesis, KITC was expected to cause programmed cell death by means of apoptosis and/or to interfere with cell cycle progression. Using a specific double staining method, we indeed found that incubation with KITC led to a dose-dependent induction of apoptosis. However, KITC seems not to exert any influence on the cell cycle distribution of HL-60 cells as a remarkable arrest in the G0/G1 phase could only be observed after treatment with 400 μM of the drug. Since RV has been identified to act in a synergistic manner when applied in combination with Ara-C (28), we finally tested whether KITC could also synergistically potentiate the cytostatic effects of Ara-C. As shown above, KITC depleted intracellular dTTP and dATP pools, while Ara-C is metabolized to Ara-CTP before being incorporated into DNA. We therefore suggested that a combined treatment with these two agents could lead to synergistic antitumor activity. Indeed, using a sequential combination of KITC and Ara-C, eight out of nine concentrations applied yielded combination indices <1,

indicating synergism according to the equation of Chou and Talalay (32). Due to these promising results, KITC may support conventional chemotherapy of human malignancies and therefore deserves further preclinical and *in vivo* testing.

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