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ORIGINAL ARTICLE

Ethacrynic acid and cinnamic acid combination exhibits selective anticancer effects on K562 chronic myeloid leukemia cells

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Abstract

Background Despite the recent advances in chemotherapy, the outcomes and the success of these treatments still remain insufficient. Novel combination treatments and treatment strategies need to be developed in order to achieve more effective treatment. This study was designed to investigate the combined effect of ethacrynic acid and cinnamic acid on cancer cell lines.

Methods The anti-proliferative effect of ethacrynic acid and cinnamic acid was investigated by MTT cell viability assay in three different cancer cell lines. Combination indexes were calculated using CompuSyn software. Apoptosis was assessed by flow cytometric Annexin V-FITC/PI double-staining. The effect of the inhibitors on cell cycle distribution was measured by propidium iodide staining.

Results The combination treatment of ethacrynic acid and cinnamic acid decreased cell proliferation significantly, by 63%, 75% and 70% for K562, HepG2 and TFK-1 cells, respectively. A 5.5-fold increase in the apoptotic cell population was observed after combination treatment of K562 cells. The population of apoptotic cells increased by 9.3 and 0.4% in HepG2 and TFK-1 cells, respectively. Furthermore, cell cycle analysis shows significant cell cycle arrest in S and G2/M phase for K562 cells and non-significant accumulation in G0/G1 phase for TFK-1 and HepG2 cells.

Conclusions Although there is a need for further investigation, our results suggest that the inhibitors used in this study cause a decrease in cellular proliferation, induce apoptosis and cause cell cycle arrest.

Keywords Cancer · Ethacrynic acid · Cinnamic acid · Combination therapy · Apoptosis · Cell cycle

Background

Cancer is the name of a group of diseases caused by abnormal cell division. Cancer cells grow without control and spread to other parts of the body through the blood circulation. There are different types of cancer treatment. Depending on the cancer type and how advanced it is, the type of the treatment may change. The most common cancer treatment is chemotherapy, in which chemical drugs are used to cure disease. These drugs may consist of synthetic compounds or active ingredients of natural products.

Ethacrynic acid (EA) is known as a diuretic substance [1]. EA has also been found to be a glutathione S-transferase P1-1 (GSTP1-1) [2] and WNT inhibitor [3]. GSTP1-1 is overexpressed in tumor cells and this results in enhanced detoxification of chemotherapeutic drugs [4]. The inhibition of GSTP1-1 by EA results in cancer cells becoming more sensitive to chemotherapy [5]. Anticancer potency has also been reported against other cancer cell lines of solid tumors and hematological malignancies [6]. EA induces apoptosis and inhibits cell growth in different cancer cell lines at high μM concentrations [7]. EA structurally contains an α,β-unsaturated ketone that can react with the cysteine residues in the cell via Michael addition [8].

Cinnamic acid (CA), also known as 3-phenylpropenoic acid, is an alpha-beta unsaturated carboxylic acid containing an aromatic ring (Fig. 1). CA is a naturally occurring

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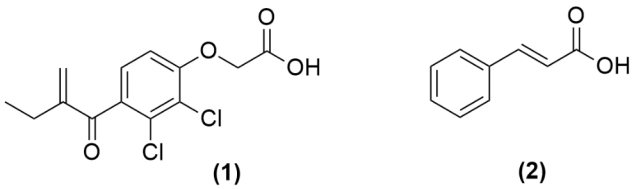

Fig. 1 Chemical structures of ethacrynic acid (1) and cinnamic acid (2)

compound that can be obtained from many natural sources such as olives, various fruits, coffee beans and vegetables. It has various biological activities including anti-inflammatory, antioxidant, antifungal, antibacterial, and anticancer properties [9–11]. In terms of anticancer activity, CA is effective against different cancer cell lines [12]. Niero and Machado-Santelli demonstrated that cinnamic acid induces apoptotic cell death and cytoskeleton disruption in human melanoma cells [13]. It was also found that CA is effective against colon cancer xenograft in nude mice and its mechanism of action is the inhibition of histone deacetylase.

Combination therapy is the method in which two or more chemotherapeutic agents are used in the treatment of cancer. The main purpose of this strategy is to enhance the efficacy compared to monotherapy by targeting the pathways synergistically or in an additive manner [14].

In the present study, we report the anticancer activities of EA, CA and their combinations against the cancer cell lines: TFK-1 (human cholangiocarcinoma), HepG2 (human hepatocellular carcinoma), and K562 (human chronic myeloid leukemia). The results of this study indicate that the combination of EA and CA has a profound synergistic antiproliferative effect on K562 cells. Moreover, this combination resulted in an elevated abundance of apoptotic cells in the K562 line compared to the TFK-1 and HepG2 lines. The results of this study may open a novel way to a more effective cancer treatment.

Methods

Chemicals

MTT reagent, ethacrynic acid and cinnamic acid were purchased from Sigma-Aldrich. The inhibitors were prepared as 5 mM EA and 10 mM CA stock solutions in DMSO (dimethylsulfoxide) according to the recommendations of the supplier and the main stocks were stored at -20ºC. RPMI-1640, fetal bovine serum (FBS), penicillin/streptomycin, and phosphate buffered saline (PBS) were obtained from Euro Clone, Biological Industries, Euro Clone and Gibco, respectively. RNAse and propidium iodide used for cell cycle analysis were obtained from Sigma-Aldrich. Annexin V/FITC was obtained from Biolegend.

Cell lines and maintenance

Human cholangiocarcinoma (CCA) cell line, TFK-1, human hepatocellular carcinoma (HCC) cell line, HepG2, and human chronic myeloid leukemia cell line (CML), K562, were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 100 U/mL penicillin/streptomycin and the cells were maintained in 5% CO_2 incubator at 37ºC.

Cell proliferation assay

Antiproliferative effects of ethacrynic acid, cinnamic acid and their combinations were determined by MTT cell viability assay on the indicated cell lines. This test is based on the principle that metabolically active cells convert the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) molecule into insoluble formazan salts and the resulting color is measured at 570 nm. Briefly, 96-well plates were seeded with 10,000 cells/well containing 200 µl of growth medium in the absence or presence of increasing concentrations of only EA, CA and their combinations. After 48 h of incubation, 10 µl of MTT reagent was added to each well and incubated for 3 h. Then, the plates were centrifuged at 1800 rpm for 10 min and the formed formazan crystals were dissolved in 100 µl DMSO. Formazan absorption intensities were measured at 570 nm by Varioskan™ LUX multimode microplate reader (Thermo Scientific™). Proliferation graphs were plotted and the the IC_{50} and IC_{30} (drug concentration that inhibits cell growth by 50% and by 30%) concentrations were calculated for both EA and CA.

Isobologram analysis for median dose effect or combination index (CI) analysis

Increasing concentrations of CA were combined with constant IC_{30} or IC_{50} concentration of EA and the cytotoxicity of the combination was evaluated by MTT assay. We used CompuSyn for Windows (CompuSyn software, Biosoft, Cambridge, UK) for isobologram analysis [15]. Experimental data points represented by dots located below, on, or above the line, indicate synergism, additivity, and antagonism, respectively. The combination index (CI) is an analysis of the combined effects of two drugs using a median effect plot analysis. A CI value < 1 indicates a synergistic effect (0.1–0.5 strong synergism; <0.1 very strong synergism); a CI value of 1 indicates an additive effect; and a CI

value > 1 an antagonistic effect (3.3–10 strong antagonism; >10 very strong antagonism).

Apoptosis assay

K562, TFK-1 and HepG2 cells were treated with either EA or CA alone or in combination. The cells were treated with EA at its IC_{50} in combination with 200 µM of CA using DMSO as a control. Apoptosis was measured by flow cytometric detection of exposed phosphatidylserine using the Annexin V-FITC apoptosis detection kit. Briefly, 1×10^6 cells were seeded in a 6-well plate and incubated with DMSO, IC_{50} of EA only (61.1 µM), 200 µM CA only or the combination of both for 48 h. Then, the cells were washed with cold PBS twice, 200 µl of Annexin binding solution was added onto the cells and mixed. Afterwards, 2 µl of Annexin V and 5 µl of propidium iodide was added to this mixture and incubated for 15 min at room temperature in the dark. Finally, cells were analyzed on the BD FACSAria III flow cytometer.

Cell cycle analysis

1×10^6 cells were treated with inhibitors alone or in combination, using DMSO as a control, for 48 h. After the incubation, the cells were washed with 1 ml of cold PBS (pH = 7.4) and centrifuged. Then, 4 ml of 70% ethanol was added to the cells and the cells were kept at -20°C for at least 24 h. Later, samples were centrifuged, supernatant was discarded and then the cell pellet was suspended in 5 ml cold PBS and then centrifuged. Then, PBS/Triton X-100 was added, followed by the addition of 100 µl of RNase-A and incubated at 37°C for 30 min. Finally, 100 µl of propidium iodide was added and left at room temperature for 10 min. Cell cycle analysis was performed by flow cytometry.

Statistical analysis

The results are presented as mean ± standard deviation (SD). The statistical significance was calculated using a one-way analysis of variance (ANOVA) for Dunnett's assay compared to the untreated controls. A value of $p < 0.05$ was considered to be statistically significant and a value of $p < 0.0001$ was considered to be highly statistically significant. Statistical analysis was performed using the GraphPad Prism 8.0.2 program.

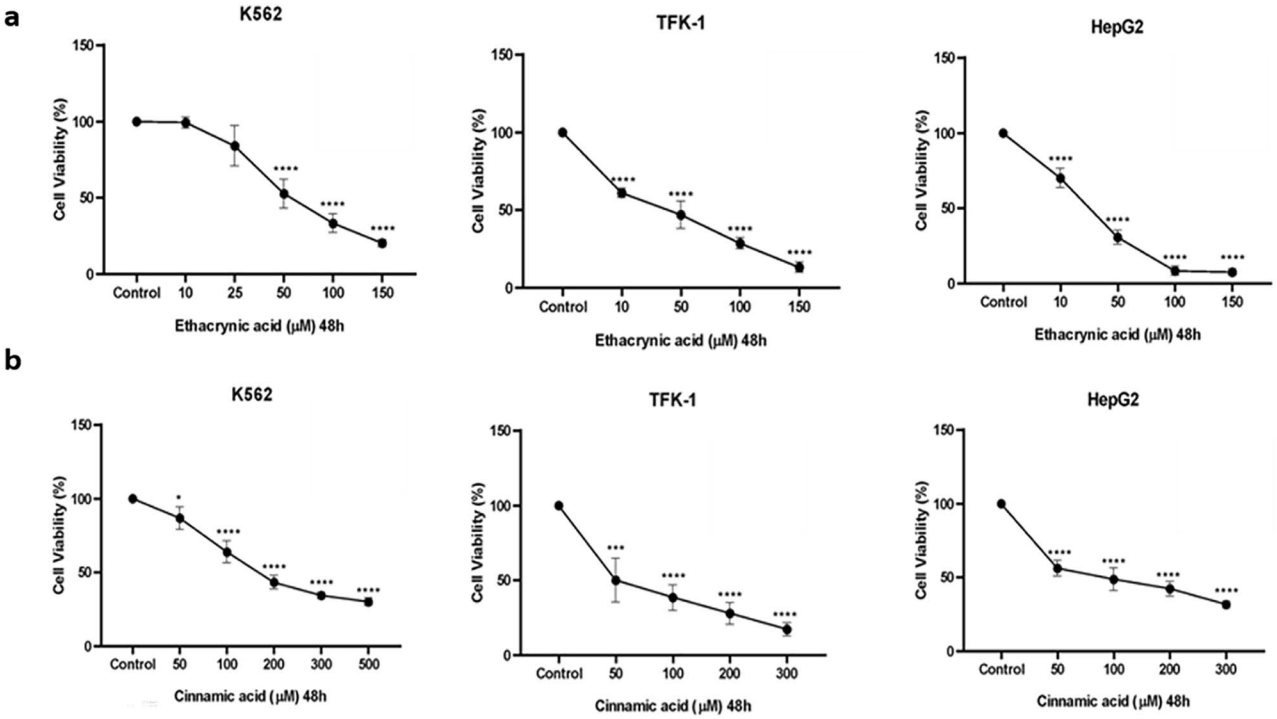

Fig. 2 The cytotoxic effect of increasing concentrations of **a** EA and **b** CA on K562, TFK-1 and HepG2 cells for 48 h. The standard deviation was calculated on the number of replicates. These results represent data from samples in triplicate across three independent experiments (n = 3) (* = P < 0.05, *** = P < 0.001, **** = P < 0.0001)

Results

Inhibitory effect of ethacrynic acid and cinnamic acid on cancer cell growth

In order to investigate the cytotoxic effects, cell lines belonging to different cancer types, K562, TFK-1 and HepG2, were treated with increasing concentrations of EA and CA for 48 h. Cytotoxicity was subsequently assessed by MTT cell viability assay. As shown in Fig. 2, EA and CA decreased the proliferation of cells in a dose dependent manner. From this dose response curve, the IC_{50} and IC_{30} values were calculated for each cell line. For K562, TFK-1 and HepG2 cells, the IC_{50} value of EA was calculated as 61.1 µM, 27.25 µM and 21.41 µM, and the IC_{30} value was calculated as 36.42 µM, 9.35 µM and 10.79 µM, respectively. The IC_{50}/IC_{30} values of CA for K562, HepG2, TFK-1 were calculated as 181.2 µM/26.02 µM, 94.47 µM/25.23 µM and 55.44 µM/20.63 µM, respectively. These concentrations were used in further experiments.

Combined cytotoxic effect of ethacrynic acid and cinnamic acid on cancer cells

Next, we aimed to determine the combined effect of EA and CA on inhibition of cell proliferation. The cells were treated with the IC_{30} of EA together with increasing concentrations of cinnamic acid varying from 50 µM to 500 µM. The results indicate that the combination of EA and CA yields a significant decrease in cell proliferation when compared to the control cells (DMSO treated) (Fig. 3). In Fig. 3, it is shown that the cell viability decreased significantly not only when compared to the control cells, but also when compared to the cells in which only IC_{30} of EA was applied. The cell viability was decreased by 63%, 70% and 75% in K562, TFK-1 and HepG2, respectively, when the cells were treated with 300 µM CA and IC_{30} of EA (Fig. 3a). Among these three cell lines, the combined treatment only showed synergistic effect on K562 cells (Fig. 3b). When compared to the single treatment of EA or CA, the combined treatment of both inhibitors resulted an increased growth-inhibitory effect on K562 cells compared to other cell lines. However, in TFK-1 and HepG2, the combined treatment is not any better than the single treatments (Fig. 3b). When the IC_{50} value of EA was combined with increasing concentrations of CA, the results demonstrated a similar pattern. (data not shown).

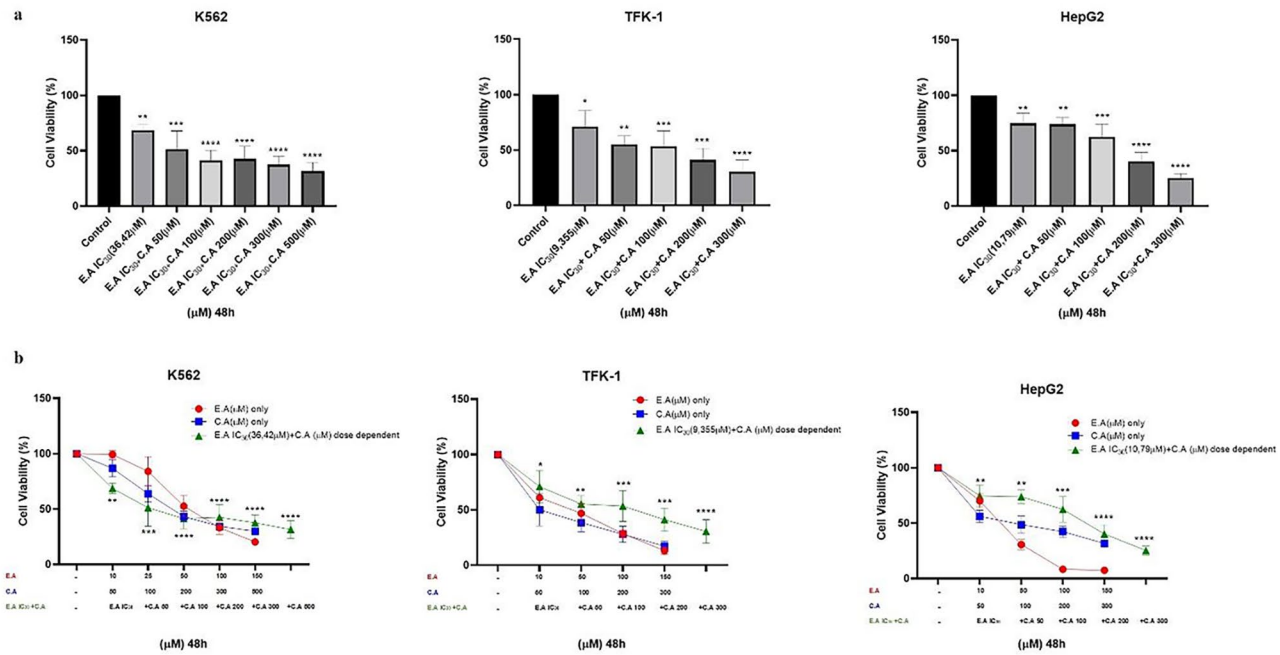

Fig. 3 The combinatorial cytotoxic effects of EA and CA on K562, TFK-1 and HepG2 cells. In panel **a**, the bar graph of the combinations is demonstrated. In panel **b**, the comparison of single (EA only, CA only with circle and square lines, respectively) and combinational treatments (triangle lines) is demonstrated. The standard deviation was calculated on the number of replicates. These results represent data from triplicate samples across three independent experiments (n = 3) (* = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001)

Isobologram test and determination of combinatorial cytotoxic effects of ethacrynic acid and cinnamic acid

Subsequently, in order to determine the combined synergistic effect of EA and CA, an isobologram test was performed (Chou and Talalay, 1984). In Table 1, the CI value for IC_{30} and IC_{50} values of EA in combination with CA are listed for K562, TFK-1 and HepG2. The results demonstrate a synergistic cytotoxic effect when IC_{30} or IC_{50} of EA is administered in combination with CA with a concentration of ≤ 100 µM for K562 (Table 1). EA and CA combination did not show any synergistic effect on TFK-1 cells. Interestingly, for HepG2 cells combination of IC_{30} and IC_{50} of EA in combination with a high CA concentration, 300 µM, indicated a synergistic effect. Based on these results, the isobologram analysis revealed that low dose combination of CA with EA synergistically inhibited cell growth for K562 cells.

Induction of apoptosis in response to combination treatment

To further confirm the impact of EA and CA, and decipher the mechanism of the inhibition of cell proliferation by EA and CA, Annexin V-FITC/PI double staining was performed to assay apoptosis. The cells were incubated with the IC_{50} concentration of EA and 200 µM CA. The results show that the combination of EA and CA significantly induced apoptosis in K562 cells. In K562 cells, single treatment of EA and CA increased the apoptotic cell population by 9.8% and 5% (Fig. 4a, left panel), respectively, and the combination treatment caused a significant 5.5-fold increase in the apoptotic cell population when compared to untreated control cells (Fig. 4a). For TFK and HepG2 cells the EA and CA combination treatment induced apoptosis up to 1.3-fold and 1.9-fold when compared to the untreated control. The combination of EA and CA induced late apoptosis and early apoptosis for TFK-1 and HepG2 cells, respectively (Fig. 4b and c). Collectively, the data suggest that the inhibitors increased cellular apoptosis when used in combination, but caused a marked apoptosis in K562 cells.

Table 1 Combined index (CI) of EA and CA on K562, TFK-1 and HepG2 cells

	K562	TFK-1	HepG2
Drug	CI Value	CI Value	CI Value
EA(IC_{30})	-	-	-
EA(IC_{30}) + CA(50uM)	0.93292	1.62187	3.93396
EA(IC_{30}) + CA(100uM)	0.78682	2.59858	3.04222
EA(IC_{30}) + CA(200uM)	1.06775	2.75703	1.46027
EA(IC_{30}) + CA(300uM)	1.30603	2.32777	0.66814
EA(IC_{30}) + CA(500uM)	1.692	-	-
EA(IC_{50})	-	-	-
EA(IC_{50}) + CA(50uM)	0.56485	2.96097	1.64421
EA(IC_{50}) + CA(100uM)	0.83443	1.86504	2.20072
EA(IC_{50}) + CA(200uM)	1.28212	1.39363	1.94673
EA(IC_{50}) + CA(300uM)	1.49929	2.61713	0.81072
EA(IC_{50}) + CA(500uM)	2.5556	-	-

The effect of combination treatment on cell cycle distribution

To evaluate the effect of EA and CA on cell cycle, the cells were treated with the inhibitors in complete medium for 48 h at the IC_{50} of EA and 200 µM CA. Flow cytometry analysis demonstrated that the combination treatment of EA and CA caused a 36% decrease in cell population in K562 in G0/G1 phase and a significant increase in G2/M phase and S phase by 3-fold and 1.8-fold, respectively (Fig. 5a). For TFK-1 cells, the cell population arrested in G0/G1 increased by 14% when compared to the untreated control cells. The percentage in S phase and G2/M phase, however, was decreased significantly (Fig. 5b). It was demonstrated that HepG2 cells accumulated in G2/M phase in response to the inhibitor treatment regardless of single or combination treatment (Fig. 5c). For HepG2 cells the populations in S phase and G0/G1 phase were decreased by 8.1 and 5.1% respectively. These data imply that the EA and CA combination caused G2/M cell cycle arrest for K562 cells and G0/G1 arrest in TFK-1 and HepG2 cells.

Discussion

It is estimated that in 2020, there were 19.3 million new cases and 10 million cancer deaths worldwide. Moreover, in 2040, the global cancer burden is expected to be 28.4 million cases which is 47% more when compared to 2020 [17]. Chemotherapy is the most efficient way of treating cancer. The purpose of these chemotherapeutic agents is to stop the cancer cells from spreading and growing in the body. In certain cases, combination therapy was found to be more effective than single therapy. Importantly, in combination therapy, lower doses of each drug could be used and thus the maximum tolerated dose of the drugs would not be exceeded [18, 19]. Regarding the potential of combination therapy, in this study, we studied ethacrynic acid and cinnamic acid combination treatment on cancer cell lines.

CA is a very well-known naturally occurring alpha-beta unsaturated acid. CA was shown to demonstrate cytostatic effects on cancer cells [20]. It was demonstrated that CA causes microfilament disorganization in a dose dependent manner and induces apoptosis of melanoma cells [13]. Another study shows that CA and its derivatives exhibited antineoplastic effects on different breast cancer cell lines.

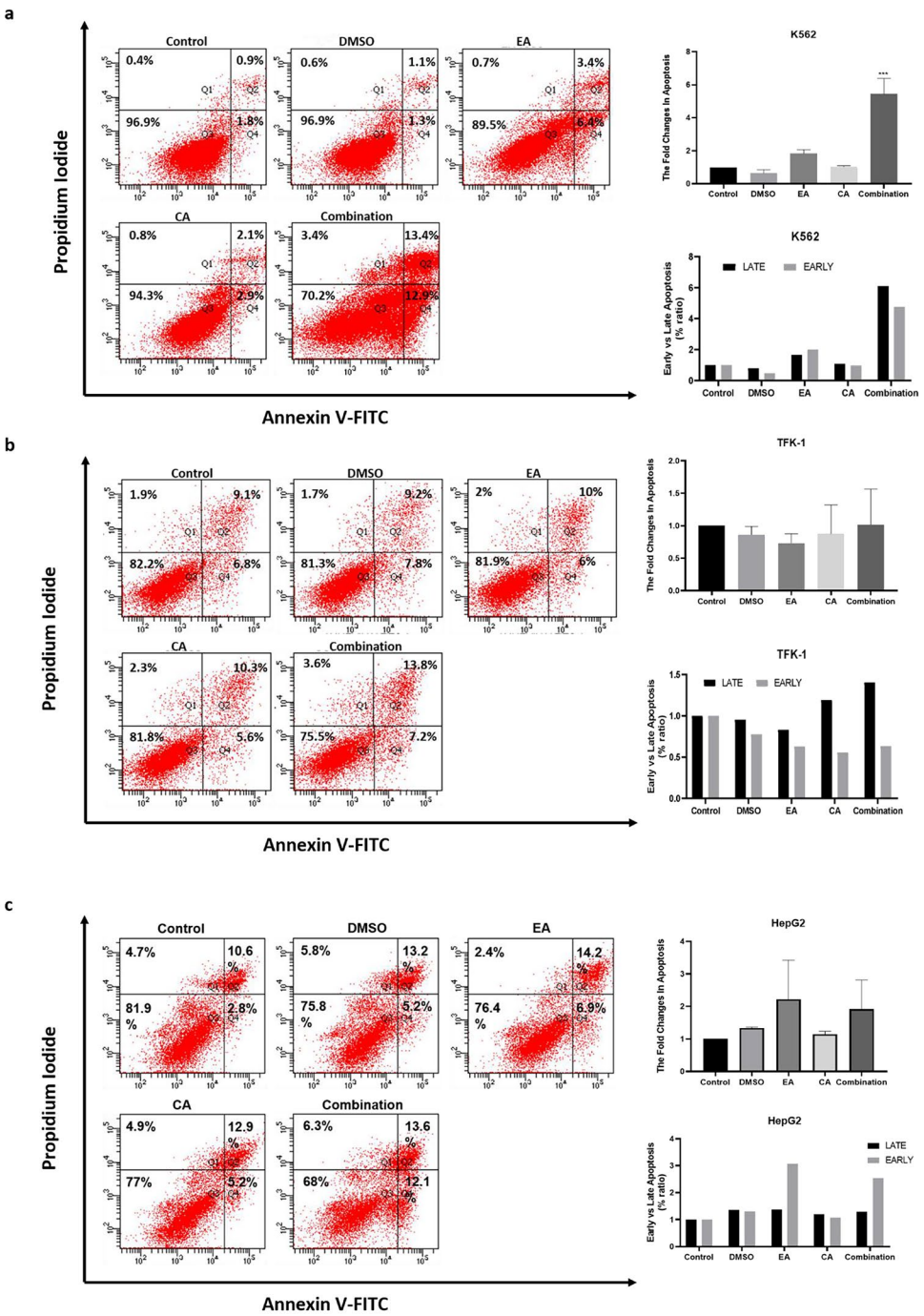

Fig. 4 Cellular apoptosis was determined by Annexin V-FITC/PI double staining for **a** K562, **b** TFK-1 and **c** HepG2 cells when IC_{50} of EA and 200 µM CA were administered. The stained cells were subjected to flow cytometry to analyze the percentage of apoptotic cells. Representative histograms are shown. In these histograms, the cells in the right lower (Q4; Annexin V-FITC$^+$/PI$^-$) and right upper (Q2; Annexin V-FITC$^+$/PI$^+$) quadrants indicate early and late apoptosis, respectively. The graphs on the right panel indicate the percentages of the apoptotic cells. The upper graph shows the total number of apoptotic cells (Q2 + Q4), and the lower graph shows the early (Q4) and late (Q2) apoptotic cell population separately. Two independent experiments were performed, combined and analyzed (*** = $P < 0.001$)

The compounds inhibited the cell growth and colony formation capability of the cells [21]. The impact of CA on triple negative breast cancer cell lines was studied and CA induced apoptosis via the TNF alpha-TNFR1 mediated extrinsic apoptotic pathway was demonstrated [22]. In colon cancer xenograft in nude mice, trans-cinnamic acid demonstrated anti-tumor activity [23]. The investigation of naturally occurring cinnamic acid derivatives on colon and cervical cancer cells shows that CA and derivatives induce cell death through inhibition of histone deacetylases which are responsible for epigenetic regulation of gene expression [24]. CA derivatives also demonstrated chemo-sensitizing effects for lung cancer cells treated with doxorubicin [25]. The anticancer effect of EA was investigated in prostate cancer and it was identified as a STAT3 inhibitor through modulation of phosphatases, like SHP2 and PTP1B. Moreover, EA treatment inhibited tumor growth in mice xenografted with DU145 cells [26].

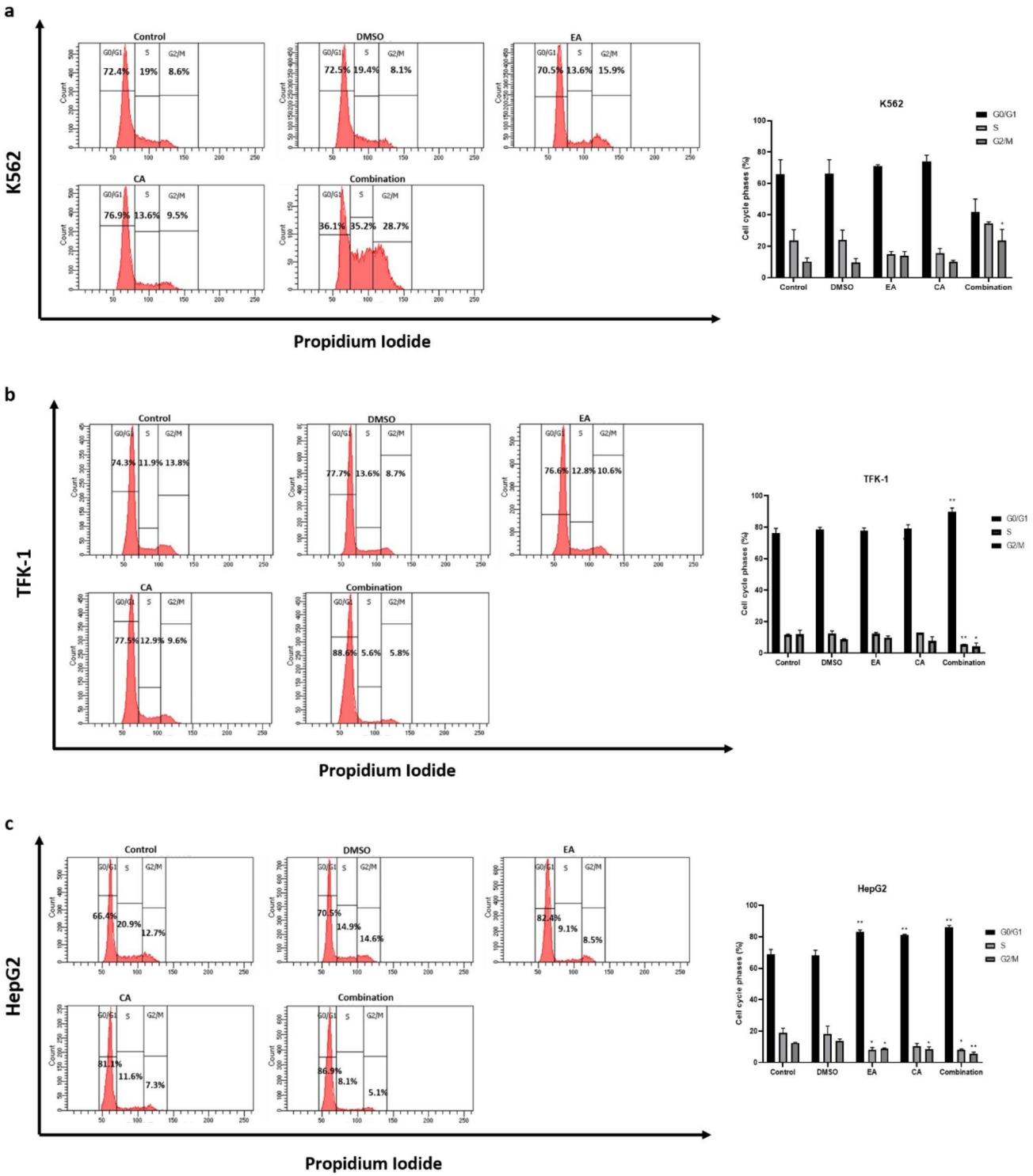

Fig. 5 The effect of EA and CA on cell cycle progression. **a** K562, **b** TFK-1 and **c** HepG2 cells were treated for 48 h with either EA and CA alone or the combination of both inhibitors. The representative histograms were shown. The cells were harvested, fixed, and stained with propidium iodide and then analyzed by flow cytometry. Two independent experiments were performed, combined and analyzed (* = $P < 0.05$, ** = $P < 0.01$)

In the current study, we investigated the cytotoxic, apoptotic and cytostatic effects of EA, CA and their

combination on different cancer cell lines for the first time. First, we analyzed the cytotoxic effects of inhibitors alone or in combination on cancer cells. Single treatment of EA and CA inhibited cell growth of all three cell lines. Although the combination treatment of cancer cells with EA and CA caused a decrease in cell growth for all cell lines, and for K562 cells only, in low concentrations, we observed a synergistic effect. For TFK-1 and HepG2 cells, the combination of these drugs caused an antagonistic effect. According to the literature, EA demonstrated cytotoxic effects on different types of cancers through its function as a GST-1 inhibitor [27]. In various lymphoma and myeloma cell lines, the cell viability and induction of apoptosis of single and combination treatments of EA and ciclopiroxolamine was assessed compared to PBMCs collected from healthy donors. It was demonstrated that there is a significant selectivity towards lymphoma and myeloma cells without effecting the cell viability of PBMCs [28]. The effect of EA on epithelial mesenchymal transition (EMT) was investigated using lung cancer cells. It was shown that EA inhibited the sphingosylphosphorylcholine induced WNT signaling, migration and EMT [29]. We next checked the effect of inhibitors alone or in combination on the induction of apoptosis and cell cycle arrest. In our study, the apoptotic effects of the inhibitors varied depending on the cell line. Simultaneous exposure of K562 cells to EA and CA augmented the early and late apoptotic cell population 5.5-fold when compared to control cells. We clearly observed a drastic increase in both early and late apoptotic cell population in K562 cells. However, for TFK-1 and HepG2, induction of apoptosis was slight and we only observed minor differences between the treated and untreated cells. Necrotic cell death was not observed in any of the cell lines that were treated with the drug combination. The effect of EA was studied in combination with tyrosine kinase inhibitors (TKIs) and it was demonstrated that EA, when combined with TKIs, induced apoptosis and cell cycle arrest [30, 31]. Finally, the combination of EA with arsenic trioxide, which is an agent used for acute promyelocytic leukemia therapy, caused induction of apoptosis [32].

In K562 CML cells, hydroxy cinnamic acid derivatives caused cell cycle arrest in the G0/G1 phase, which supports our results [33]. In our study, when K562 cells were treated with CA only, the G0/G1 cell population increased by 4% which is in line with the results in literature. Our results demonstrated that CA or EA alone did not cause any changes in cell cycle phases; however, the combination caused significant G2/M cell cycle arrest. This result may be due to the regulation of different cell cycle checkpoints, and may also be dependent on cell type and cancer type. The G2/M cell cycle arrests may be due to microtubule disorganizations [34] and thus as follow up for our study, further investigations could be performed on cytoskeletal organization upon drug treatment. As pointed out by Sova and colleagues, CA derivatives arrest cell cycle in carcinoma cells [35] which is in accordance with the current study.

Collectively, our results show profound differences between different types of cell lines. The findings of this study indicate that EA and CA combination treatment was more effective on the K562 cell line. Consistently, the effect of combination treatment demonstrated synergistic inhibitory effect on cell growth, induction of apoptosis and G2/M cell cycle arrest for K562 cells. The clinical use of EA as an anticancer drug is limited by its potent loop diuretic activity [36]. More potent derivatives of EA with anti-cancer properties could be achieved by modification of the inhibitor [37, 38]. Conjugates of EA have been developed and tested against tumor cells and this non-diuretic conjugate demonstrated a potent anti-proliferative effect [37].

Conclusions

In conclusion, our study demonstrated that EA and CA combination leads to an antiproliferative effect against cancer cell lines used in this work: K562, TFK-1 and HepG2. This combination caused a significant apoptotic cell death in K562 cells. In addition, K562 cells were arrested in the G2/M phase upon co-administration of EA and CA. Collectively, our results show that using EA and CA cause a decrease in cell proliferation, induce apoptotic cell death and cell cycle arrest. Clearly, it is important to evaluate the effect of the inhibitors mechanistically. The focus of future work might be the identification of apoptotic cell death mechanisms and investigation of the mechanism by which the inhibitors achieve their effect. Afterwards, it is important to perform further functional assays to validate the impact of such molecules or combinations. Although there is a need for follow-up studies and further investigation, our results suggest that administration of EA and CA could be potential therapeutic targets for treating cancer cells. Considering the antiproliferative properties of EA and CA, one of the important findings in this study is that both of the molecules have relatively low potency against cancer cell lines when they are used alone. It was reported that they are mainly used as an adjuvant molecule in most of the studies. Herein, we also reported that these two molecules which show low toxicity alone, could be a potential treatment option when applied in combination, especially at low concentrations in the K562 cell line.

List of abbreviations

EA	Ethacrynic acid.
CA	Cinnamic acid.

FACS	fluorescence-activated cell sorting.
EMT	Epithelial mesenchymal transition.
CML	Chronic myeloid leukemia.
CCA	human cholangiocarcinoma.
HCC	human hepatocellular carcinoma.
PBMC	peripheral blood mononuclear.

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Authors' contributions Emel Başak Gencer Akçok and İsmail Akçok contributed to the study of conception and design. Material preparation, data collection were performed by Münevver Yenigül. Analysis and interpretation were performed by Münevver Yenigül, Emel Başak Gencer Akçok and İsmail Akçok. The first draft of the manuscript was written by Emel Başak Gencer Akçok and İsmail Akçok. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

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