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Research Article

INVESTIGATION OF SYNERGISTIC EFFECT OF GELDANAMYCIN AND CUCURBITACIN ON HUMAN COLON CANCER

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ABSTRACT. Colon cancer is one of the most common cancers observed in men and women in the world, today in many countries after cardiovascular diseases; it is second leading cause of death. In this the study we aimed to investigate the effects of use of Cucurbitacin I and together with Geldanamycin (GA) which have apoptotic and anti-proliferativeeffects on HT29. Cells were treated with GA, CU and GA+CU. Effect of GA, CU on cell viabilitywere demonstrated by MTT assay and activities of bax, caspase-3, bcl-2, GRP78, wee1, AIF and GAAD153 were performedwith ELISA method after 48 hour treatment. Our study showed that the combined use of GA and CU significantly reduced cell viability of colon cells. Also, the same dose of GA and CU used together increased the expression of proapoptotic proteins and decreased anti-apoptotic proteins more than their effects alone. As aconclusion, our study showed that GA and CU synergistically induce apoptosis on humancolon cancer.

Keywords: *Cucurbitacin, geldanamycin, colon cancer, apoptosis, synergy.*

INTRODUCTION

Cancer is an important disease whose incidence and mortality rates are highly increased in recent years. Especially colon cancer, which is the one of the most common cancer, today in many countries, takes places a second after cardiovascular diseases that cause death [1, 2]. In the USA researchers reported that approximately 150000 new cases per year were detected and one third of patients were dead related to cancer problems [3]. In our country according to Ministry of Health statics; colon cancer with 6.2% incidence rate, take fourth in all cancer types ranking [4, 5].

Herbal drugs which takes its origin from natural products, have been used extensively in cancer treatment for many years and gained importance [6,7]. Phytochemicals are important bioactive herbal compounds which reduces colon cancer incidence [8]. Cucurbitacin is one of the group of phytochemicals and exist in composition of *Ecballium elaterium* which is known as "acıkavun, cırtatan, eşekhıyarı, EbuCehilkarpuzu, acıhıyar"

in Turkish. *E. elaterium* is from *Cucurbitaceae* family and blooms between May and September in Mediterrean zone [8,13]. The first isolated Cucurbitacin from *E. elaterium* was a-Elaterin in 1831 by Morries and Hennel. In 1906, Berg affirmed that this compound has C28H3807 formula and then the certain formula and molecular weight was discovered as C32H440 by Rivett and Herbstein in 1957 [15]. In 1909, Power and Moor isolated a new Cucurbitacin from plant and entitled as 3-Elaterin(Cucurbitacin-B) [14]. After that, Rao and friends isolated this compound and proved the previous research [16]. Until today, a series of Cucurbitacin named Cucurbitacin D, I, R, L, H, G, E and derivatives were isolated from this plant [13]. Cucurbitacin has bitter taste and toxic effects and is highly oxygenated compound. As a structure, it has 19(10→9β)-abeo-10α-lanost-5-ene tetracyclic cucurbitan core skeleton and Cucurbitacin divides in to 12 subgroup (A to T) with side chain modifications and stereochemical values [10, 17]. It is used traditionally as antipyretic, analgesic, antiinflammatory, antimicrobial, antidiabetic and cancer preventor in Asian countries such as China for many years and it has free and glycosylated forms [18, 19]. Since 1952, the antitumoral effect of this plant has been known. Belkin at al. [20], found that *E. elaterium* extract are active in mouse insitu experiences. a-Elaterin whose activity is low when administered subcutaneously; shows high activity when administered intraperitoneal. However, in both administration 3-Elaterin has the highest activity rate. Also, the presence of activity was found in elateridin which is the derivative of a-Elaterin. Geldanamycin (GA), a benzoquinoidansamycin antibiotic produced by Streptomyces hygroscopicus, is a potent inhibitor of Hsp90 proteins and has anti-tumor activit [21]. Heat shock protein 90 (Hsp90) is a chaperone that binds to multiple proteins and facilitates their proper refolding. It has become an important target for cancer therapy by affecting many physiological processes such as intracellular transport, protein degradation and signal transduction [22]. Geldanamycin has been shown to block replication of viruses both in vitro and in vivo through inhibition of Hsp90 [23].

In cancer, apoptosis plays the key role in cancer development and resistance against the chemotherapeutics. The C/EBP homologous protein, also known as growth arrest and DNA damage inducing protein 153 (GADD153), is a major component of the endoplasmic reticulum stress-mediated apoptosis pathway [24]. The 78 kilo dalton glucose-regulated protein (GRP78) is a major endoplasmic reticulum (ER) molecular chaperone with antiapoptotic properties and an important regulator of the unfolded protein response (UPR). ER stress induction by GRP78 in cancer cells represents a pro-survival branch of the UPR [25]. Wee 1; When DNA damage occurs, it phosphorylates CDK1 and keeps the CDK1-cyclin B complex in an inactive form, preventing entry into mitosis [26]. AIF is a flavoprotein that promotes cell viability as a mitochondrial oxidoreductase, but can also mediate cell death through its proapoptotic nuclear activity [27]. Anti-apoptotic Bcl-2 family proteins not only protect against cell death via apoptosis, but also allow senescent cells to survive [28].

In this study, we aimed to investigate how the together administration of GA and CU on the human colon cancer cells, affects the proapoptotic protein levels and activities. It's known that GA and CU have anticancer effects. It is curious whether togetherness of GAs and CUs can potentiate each other's effects. For this purpose, we investigate the effects of GA and CU combination on the cellular apoptosis mediators in colon cancer cells.

MATERIALS AND METHODS

Cell lines and reagents

HT29 human colorectal adenocarcinoma cell line was obtained from ATCC, Geldanamycin and Cucurbitacin were purchased from Sigma (Sigma, Germany).

Cell Culture

Cells were maintained in modified McCoy's 5A Medium (10% Fetal Bovine Serum (FBS) (Hyclone)in a humidified incubator at 37 °C under 5% CO_2 atmosphere.

MTT Assay

Cells were treated with GA, CU, GA+CUat10μM concentration. Cells were maintained in modified McCoy's 5A Medium (10% Fetal Bovine Serum (FBS) (Hyclone)in a humidified incubator at 37 °C under 5% CO_2atmosphere. Cells were cultivated in a single layer in the standard cell culture 96 plates that includes densities of 10000 cells/well. Cellswere treated with GA, CU, GA+CU at 10 μM concentration. Cells were used without GA, CU as a control.

After the incubation period, 10 μl of MTT labeling reagent was added to each well. For 4 hours the microplate was incubated humid atmosphere.100 μl of the solubilization solution was added into each well. The plate was allowed to stand overnight in the incubator in a humidified atmosphere. Absorbance was measured at 595 nm using the microplate reader.

Total Protein Extraction

Cells were washed with ice-cold PBS. After 1ml radioimmunoprecipitation assay buffer [1.0% Triton X-100, 50 mM Tris/HCl (pH 8), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS)], 30μl PMSF (fenylmetane sulfonil fluoride),30μl sodium vanadate, 5μl protease inhibitor were added on 10^6cells. The culture medium from the adherent cells was slowly aspirated with a suction and Pasteur pipette. The cell suspensions were transferred to a TPX tube. Cells were centrifuged at 1500 rpm for 10 min at 4 °C and were aspirated as much supernatant as possible. After that we obtained homogenates by breaking up cells on ice with ultrasonic dissection device. Homogenates were centrifuged for 10 minutes in 14000 rpm and upper part (supernatant) was obtained; bottom part (pellets) was thrown away.

Protein Quantification Assay

For the protein quantification of cells, we used Bradford method. With using bovine serum albumin (1 μg/ml) prepared standard in concentration of serial dilution. We added 1ml Bradford solution on the standard and sample, then mixed with vortex and measured absorbance manually in 595 wavelengths in spectrophotometer. According to drawn standard curve in Prism programme, protein quantification was made in measurable form of μg/μl.

ELISA Assay (Enzyme Linked Immunosorbent Assay)

Caspase-3, bax, bcl-2, AIF, Wee 1, GRP78 and GADD153 levels were measured by ELISA. Cells were seeded in 24 plates that includes $1x10^5$. It was incubated in these

conditions:37 ^{o}C temperature, 5% CO2 in incubator. These cells were treated with 10 µM of CU, GA and GA+CU combination for 48 hour and then homogenized for protein analysis with ELISA method. Experiment protocols done according to the commercial ELISA kit (SunRedBio).

Statistical Analysis

Statistical evaluation of the data obtained from the study will be done by IBM SPSS Statistics 23 package program. Statistical significance between groups was determined by the One-way ANOVA (Tukey) test. Statistical significance was considered at a p-value of <0.05. Data is showed as means±SE.

RESULTS AND DISCUSSION

Cell viability

MTT assay confirmed the inhibitory effects of CU and GA on HT29 cells proliferation. In this study**.** HT29 cells were treatment CU (100, 200 nM), GA (1, 5 nM) and CU+GA (100, 5 nM) for 48 h. It was determined that the survival percentage of the cells treated with the CU+GA combination was significantly lower than the control cells (Figure 1).

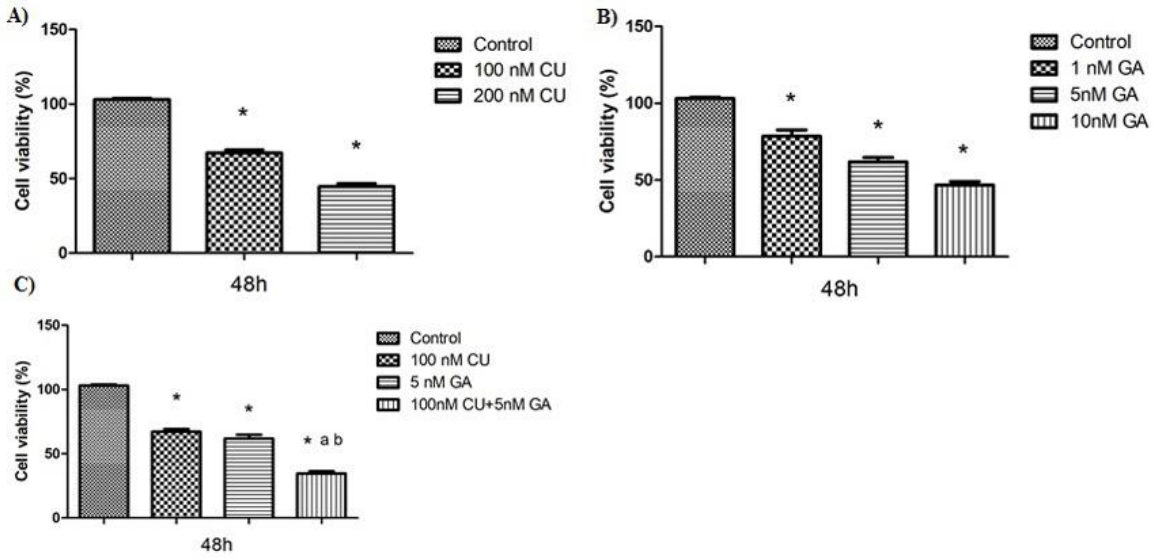

Fig. 1. *Effect of* ***A)*** *CU* ***B)*** *GA and* ***C)*** *CU+GA on the cell viability of HT29 cells using MTT assay for 48 h. Bars represent the mean±SE of three independent experiments.*

Protein Expressions

HT29 cells were treatment Cu (100 nM), GA (5 nM) and CU+GA (100, 5 nM) for 48 hours. Our study shows that CU+GA combination significantly decreased the expression of anti-apoptotic protein bcl-2 (figure 2) more than single administration of CU and GA. In the other hand CU+GA significantly increased the expression of Bax, Caspase 3 and AIF, GAADD153, Wee 1 and GRP78 more than single administration of CU and GA.

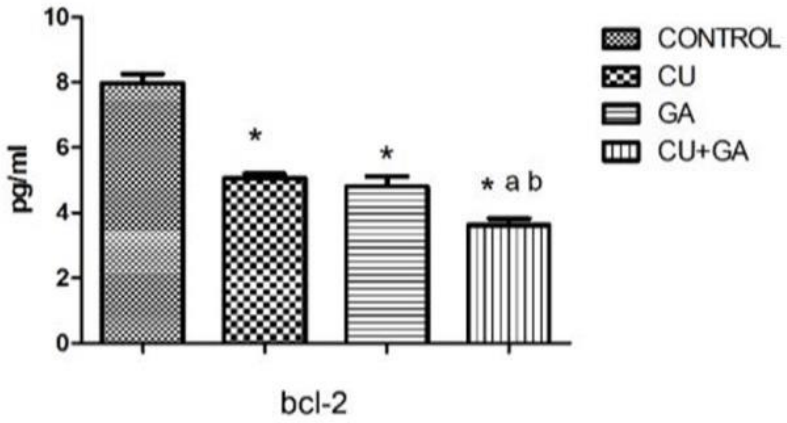

Fig. 2. *Effect of Cu and GA onBcl-2 expression in HT29 cells. Data presented as mean±SE.*

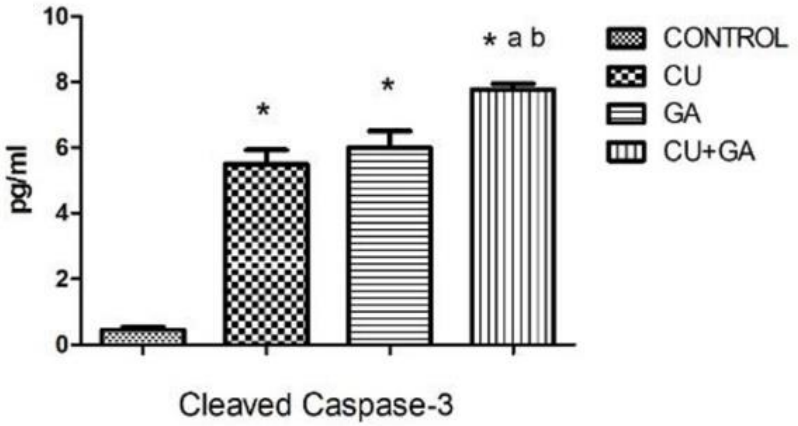

Fig. 3. *Effect of Cu and GA on Caspase 3 expression in HT29 cells. Data presented as mean±SE.*

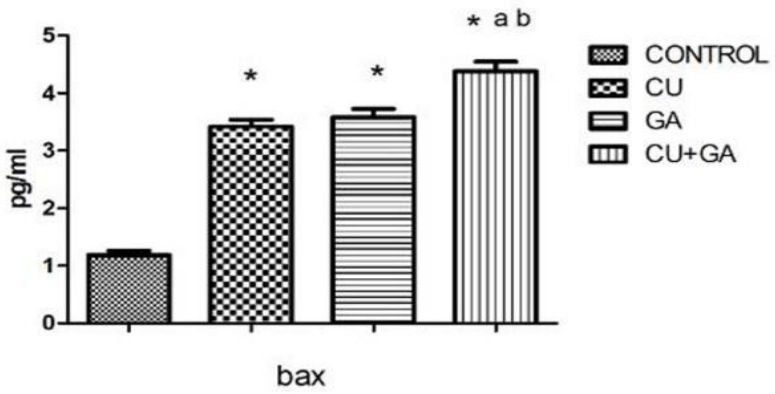

Fig. 4. *Effect of Cu and GA on Bax expression in HT29 cells. Data presented as mean±SE.*

Table 1. *Effects of CU and GA on expression of Wee1 and GADD153, AIF and dual function GRP78 proteins.*

	CONTROL (pg/ml)	CU (pg/ml)	GA (pg/ml)	CU+GA (pg/ml)
wee 1	0.402±0.072	1.070±0.070*	1.083±0.098*	1.450±0.043*[ab]
AIF	0.350± 0.049	1.097±0.058*	1.533±0.099*	2.083±0.048*[ab]
gadd153	0.172±0.020	0.912±0.074*	1.097±0.053*	1.340±0.051*[ab]
grp78	0.458±0.048	0.892±0.192*	1.052±0.206*	1.717±0.070*[ab]

The difference between values in the same column is statistically significant. Data are given as mean±SE. Statistical analysis: Student t test. P<0.05.

In the study, cell viability which related to proliferation was measured. According to the graphics; CU+GA remarkably reduced cell viability.

In this study, Cu+GA significantly decreased the amount of Bcl-2, an antiapoptotic protein while significantly increased the concentration of proapoptotic proteins (Bax, Caspase 3 and AIF), mitotic-growth arrest proteins (Wee 1 and GADD153) and dual function protein GRP78 in human colorectal adenocarcinoma cells.

Mitochondria is important with ability to regulate metabolism and on cell viability. Apoptosis has several functions: during the embryogenesis elimination of unwanted tissues; re-shaping of tissues; playing role in aging, homeostasis, development; elimination of defected, expired and infected tissues. Both Bcl-2 and caspases are main mediators in apoptotic pathway [28]. Activation of extrinsic pathway caspase-8 and 10 causes formation of death inductive signal complex and activation of extrinsic pathway caspase-9 causes formation of apoptosome complex, both of them induces caspase-3 [29]. After that, caspase-3 causes the DNA fragmentation. Bcl-2 family has pro-apoptotic and anti-apoptotic members. Proapoptotic or antiapoptotic signals affects the mitochondria against the balance between Bcl-2 proteins. If the apoptotic signals been dominant, cytochrome -C leave from the mitocondria to generate apoptosome complex. Bax protein regulates and then activates the apoptosis and observed that it was increased [30]. As observed this study, of mitochondrial pathways and related pathways make a response to both drugs, GA+CU, cells go through the cell death. So that the observed data show the synergistic relationship between drugs. Apoptosis causes important defects in developmental, autoimmune, neurodegenerative diseases and cancer. GA has already anti-cancer effects and Cucurbitacin has also biochemical activity and functions in diseases such as cancer. The additional biological effect of CU on GA was observed and that effect may give a hope to cancer research [20].

We observed the synergistic effect of CU+GA combination on colon cancer through the complicated proportion of each compounds. Herbal drugs involve several compounds which are essential in health sciences. This study shows that diversity of herbal compounds may be a part of healing. Among the cancer phases, apoptotic induction is important particularly at a molecular level.CU+GA combination effect size is bigger than single administration of each compound. We obtain outputs as a result of investigating

the protein levels. Protein levels may indicate the progression of cancer. The balance between protein levels also affects the apoptosis and it has been seen complicatedly in cancer progress. In colon cancer, numbers of data show the presence of synergism in our study. The activation of apoptotic pathways and on the other hand inhibition of anti-apoptotic pathways may cause logically apoptotic induction. While anti-apoptotic protein bcl-2 was decreased in that study; other apoptotic proteins were increased. Eventually results are logically expectable. The togetherness of GU and CU create a significant effect than single administration. Even though we did not use mainly chemotherapeutic drug, the anti-cancer effect could occur. We could see again the evidence of herbal drug importance. The synergism may cause by the total role of signal transduction pathways, apoptotic pathways, drug pharmacodynamics, patient's immune capacity. They may alter the drug response and progress. In this study, we observed the existence of synergism but further studies will demonstrate the more about this synergism and detailed information.

CONCLUSION

In conclusion; our study showed that GA and CU have ameliorative effects on cancer therapy. GA and CU show synergistic effects and used together regulates apoptosis on human colon cancer via apoptotic and proapoptotic markers. However, the use of two active substances in different studies will provide a better understanding of their efficacy.

Conflict of Interest. "The authors declared that there is no conflict of interest."

Authorship Contributions. Concept: M.C., M.G. Design: A.A., H.M.K., Data Collection or Processing: H.M.K., G.E., Analysis or Interpretation: G.E., A.A., Literature Search:M.C., A.A., M.G., Writing: M.C., A.A., M.G.

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