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Original Research

HSP90 inhibitor enhances anti-proliferative and apoptotic effects of celecoxib on HT-29 colorectal cancer cells via increasing BAX/BCL-2 ratio

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Abstract: Due to the high prevalence and mortality rate of colorectal cancer (CRC), new treatment approaches like combination therapy seem to be necessary. The relationship between chronic inflammation and colorectal cancer development and progression has been shown to be important. Celecoxib, a selective COX-2 inhibitor, is the only non-steroidal anti-inflammatory drug (NSAID) that has been approved for cancer therapy and prevention. Because of cardiovascular side effects of COX-2 inhibitors, combination therapy may improve the therapeutic profile. 17-Demethoxy-17-allylamino geldanamycin (17-AAG), a heat shock protein 90 (HSP90) inhibitor, shows anti-inflammatory effects via down-regulation of the key mediators of inflammation such as Nuclear Factor κB (NF-kB), JAK/Signal Transducer and Activator of Transcription (JAK/STAT). Thus, we studied the effect(s) of combination of 17-AAG and celecoxib on HT-29 cells viability and apoptosis induction. Based on MTT results, we showed an increase in the inhibitory effect of celecoxib when combined with 17-AAG, especially at low a concentration of celecoxib. Flow cytometry analysis demonstrated that apoptosis induction is probably the mechanism of additive inhibitory effects of 17-AAG and celecoxib combination. To explore the possible mechanism of apoptosis induction by 17-AAG and celecoxib combination, levels of BAX and BCL-2 proteins were determined by western blotting. The BAX/BCL-2 ratio in the combination group was increased compared to 17-AAG or celecoxib alone, mainly via decreasing BCL-2 levels. In conclusion, 17-AAG, increased inhibitory effects of celecoxib on HT-29 cells, probably by induction of apoptosis.

Key words: HSP90, celecoxib, colorectal cancer, apoptosis, BAX; BCL-2.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, it is the fourth most common cancer and the third most common cancer in men and women, respectively (1). It is the second cause of death from cancer; however, as a result of a new combination therapy and using novel molecular targeting agents, considerable improvement in overall survival rate has been observed (2). Thus today, interest in colorectal cancer treatment has shifted from single agent therapy to combination therapy and molecular targeting agents (3).

Correlation between inflammation and cancer development is well established (4). Inflammation has a major role in development of several hallmarks of cancer, such as promoting tumor cell proliferation, angiogenesis and escape from apoptosis, so that some researchers have introduced inflammation as the seventh hallmark of cancer (5). Taken together, inflammation is a major target of cancer treatment and prevention (6). Several lines of evidence have shown a decrease in colon cancer risk following long term use of anti-inflammatory agents like aspirin and Non-steroidal anti-inflammatory drugs (NSAIDs) (7).

Overexpression of cyclooxygenase-2 (COX-2) has been shown in several solid tumors like colon, lung and breast cancers, which are associated with poor survival rates (8,9). Celecoxib, a selective cox-2 inhibitor, is the only NSAID that has the Food and Drug Administration (FDA) approval for cancer prevention and treatment (10,11). However, researches have shown low efficiency of selective cox-2 inhibitors when used as a mono-therapy agent in cancer treatment (11). In addition, several lines of evidences have shown cardiovascular toxicity of selective cox-2 inhibitors (12). Thus, combination therapy may be a good strategy for increasing efficiency of selective cox-2 inhibitors and reducing their cardiovascular toxicity.

Recently, the molecular chaperone, heat shock protein 90 (HSP90), has gained special attention as a target in cancer treatment because of its role in promoting stability and active conformation of several oncogenic proteins (13,14). HSP90 is induced by several stressful conditions like inflammation, hypoxia and anti-cancer chemotherapy (15). Several studies have shown the regulatory function of HSP90 on inflammatory signaling pathway, thus the term "chaperokine" is used for HSP90 because of its dual function as a chaperone and as a cytokine. Among the proteins affected by HSP90 are the important components of the inflammatory signaling pathway like Nuclear Factor κB (NF-kB), JAK/Signal Transducer and Activator of Transcription (JAK/STAT),

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Toll LikeReceptor-4 (TLR-4) and NF-κB Inhibitor of IκB Kinase (IKK)) signaling pathways (15,16). Following HSP90 inhibition, these proteins are degraded in the proteasome (17). The notable subject is the potent effect of HSP90 inhibitors like 17-AAG on several cancer cells at nanomolar concentrations (18).

Because of anti-inflammatory effects of HSP90 inhibitors and selective COX-2 inhibitors, as well as a strong correlation between cancer treatment and inhibition of inflammation, in this study we decided to evaluated the effect of combination of HSP90 inhibitor, 17-Demethoxy-17-allylamino geldanamycin (17-AAG) and celecoxib on HT-29 colorectal cancer cells. We also evaluated the effect of combination of celecoxib and 17-AAG on apoptosis induction and BAX and BCL-2 protein levels.

Materials and Methods

Material and antibodies

RPMI 1640 and FBS were purchased from Gibco (USA), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) , penicillin and streptomycin were from Sigma (Germany). Celecoxib, 17-AAG, Bicinchoninic Acid (BCA) Protein Assay kit, BCL-2 antibody, BAX antibody, β-actin antibody and goat anti-mouse IgG-HRP were all purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Cell culture

Colorectal cancer cell line HT-29 was obtained from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2 (19). The culture medium was replaced every 48 h. All experiments were performed when cells were in the logarithmic phase of growth and more than 95% viability. Cell viability was checked by MTT assay and Trypan blue staining.

Cell viability assay

To evaluate the anti-proliferative activity of drugs, alone, and in combination, MTT assay was performed. Viable cells are able to reduce MTT to the water-insoluble formazan dye. 5×10^3 HT-29 cells were seeded in each well of 96-well plates and after 24h, cells were treated with drugs for indicated times and concentrations. After incubation for 72 h, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL in phosphate-buffered saline (PBS)) was added and cells were incubated at 37 ºC for 4h in a humidified atmosphere of 5% CO_2. Then MTT and media solutions were carefully removed and the tetrazolium crystal was solubilized by adding 100 µL of DMSO to each well and the absorbance was measured at 540 nm in a plate reader (Synergy HT, BioTek, USA). The results were presented as a percentage of the control RPMI. The cell viability (in %) was determined using the following equation:

Cell viability (%) = (OD (treated)/OD (control) ×100

In which OD (treated) and OD (control) represent the optical densities of treated and untreated (RPMI) cells, respectively (20). The IC_{50} (concentration of the drug that has 50% inhibitory effect on the cells) was determined by using the probit analysis in SPSS software.

Determination of the percentage of apoptotic cells by flow cytometry analysis.

We used flow cytometry analysis to calculate the percentage of apoptotic cells in HT-29 cells treated with IC_{50} concentrations (based on MTT test results) of 17-AAG and celecoxib alone or in combination. HT-29 cells were treated with 17-AAG and celecoxib with indicated concentrations for 72 h and thereafter, cells were harvested by trypsinization. Then, cells were washed twice with cold-PBS. PE Annexin V Apoptosis Detection Kit I (BD, USA) was used for estimation of the percentages of apoptotic cells as in manufacturer's instructions. In brief, after washing cells with cold-PBS, cells were re-suspended in 1X Binding Buffer at a concentration of 1 x 10^6cells/mL. One hundred µL of this solution was transferred to a 5 mL cell culture tube. After adding 5 µL of PE-Annexin V and 7-AAD, cells were incubated for 15 min at room temperature in dark, then 400 µL1X binding buffer was added to cells and percent of apoptotic cells was analyzed by a FACSCalibur flow cytometer (BD, USA) within 1 h. Viable cells are negative for both PE-Annexin V and 7-AAD, early apoptotic cells are positive for PE-Annexin V and late apoptotic cells are both PE-Annexin V and 7-AAD positive.

Western blotting

HT-29 cells were treated with IC_{50} concentrations of 17-AAG and celecoxib alone and in combination for 72h. Cells were harvested by trypsinization and were washed twice with cold-PBS. Cell lysate was prepared by incubation of the cells with Radio-Immunoprecipitation Assay (RIPA) lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mMTris, pH 8.0) (Sigma/ USA) containing protease inhibitor cocktail (Sigma/Saint Louis, USA) and phenylmethanesulfonyl fluoride (PMSF) (Sigma/ USA) on ice for 15 min. Finally, cell lysate was centrifuged (12000 ×*g*, 15 min, 4°C) and supernatant was collected for the next steps. Protein concentration was determined using BCA kit (Santa Cruz, CA, USA). One hundred micrograms of protein was used for electrophoresis on 14% SDS-PAGE gels, proteins were then transferred to a PVDF membrane (Biorad). To block the nonspecific binding, PVDF membrane was blocked with 5% skim milk (Merck/Germany) in Tris buffered saline (pH=7.4) containing 0.5% v/v Tween 20 (TBST, Panreac/Spain) at room temperature for one h and was incubated with primary and secondary antibodies overnight and one hour, respectively. All washing steps were performed three times for 20 min in TBST [10 mMTris-HCl (pH 8.0), 150 mM NaCl and 1% Tween-20]. Blots were developed using Clarity Western ECL Substrate (Biorad, USA) and exposure to X-ray film. Results were analyzed with Image Software version 1.49v (NIH, USA).

Statistical analysis

Statistical analysis of data was performed by SPSS version 23 version 23 (SPSS Inc, USA), using One-way ANOVA test. All data are expressed as mean ± standard error of mean (SEM). *P* values of less than 0.05 were considered statistically significant.

Results

The inhibitory and toxicity effects of 17-AAG and celecoxib alone and in combination on HT-29 cells

To explore the inhibitory and toxicity effects of 17-AAG and celecoxib alone and in combination, we used MTT test. At first, cells were treated with 10, 20, 40, 80 and 100 µM concentrations of celecoxib and 2.5, 5, 10, 25 and 50 nM concentrations of 17-AAG for 72 h. As shown in the dose–response curve (Figures.1. A, B) and based on probit analysis, IC_{50} concentration of celecoxib and 17-AAG were64±5.5 µM and 25±0.31 nM, respectively. Percent of cell viability for HT-29 cells treated with 17-AAG was 92.53±1.07, 66.56±1.06, 48.36±2.99, 37.36±1.18 and 35.33±2.9 and 91.76±2.86, 82.1±3.55, 68.66±5.54, 40.33±6.66 and 18.33±1.45 for celecoxib. For combination therapy, we used final concentrations of 10, 20, 40 and 80 µM celecoxib in combination with each one of the 2.5, 5, 10 and 25 nM17-AAG for 72 h. As shown in figures 1. C, D, E and F, after treatment with combination of celecoxib and 17-AAG, percent of viability for HT-29 cells was decreased significantly relative to cells treated with each one of these drugs alone. Considering the side effects of celecoxib, these enhanced inhibitory effects are remarkable in lower doses of celecoxib. As shown in figure 1. C, when we treated the cells with 10 µM celecoxib, % cell viability was 91.7 ± 2.8, while when we applied 10 µM celecoxib simultaneously with concentrations of 2.5, 5, 10 and 25 nM 17-AAG, Percent of cell viability were significantly decreased to 76.66±3.94, 59.56±5.47, 35.36±3.94 and 25.36±3.41, respectively (P<0.05).

The most toxic effects were observed when cells were treated with 80 µM celecoxib in combination with 2.5, 5, 10 and 25 nM 17-AAG. After using 80 µM celecoxib, Percent of cell viability was 40.33±6.66, while in combination therapy, the percent of cell viability decreased to 14.53±2.74, 10.7±2.4, 5.95±1.58 and 6.63±2.26, respectively (P<0.05).

As previously mentioned, the IC_{50} for celecoxib was 64±5.5 µM, while when we combined the concentration of 10, 20, 40 and 80 µM of celecoxib with each of the 2.5, 5, 10 and 25 nM concentrations of 17-AAG for 72 h, the IC_{50} of celecoxib decreased significantly. For example, the IC_{50}s for celecoxib in combination with 2.5 and 5 nM concentrations, were obtained 35.2±5.1 and 21.19±5.2, respectively (P=0.019).

17-AAG and celecoxib combination increased percentage of apoptotic cells in HT-29 cells

To specify that induction of apoptosis is the mechanism for enhanced toxicity which was observed for combination of celecoxib and 17-AAG, the HT-29 cells were treated with IC_{50} concentrations (based on the MTT results) of celecoxib and 17-AAG alone and in combination for 72 h and the percentage of apoptotic

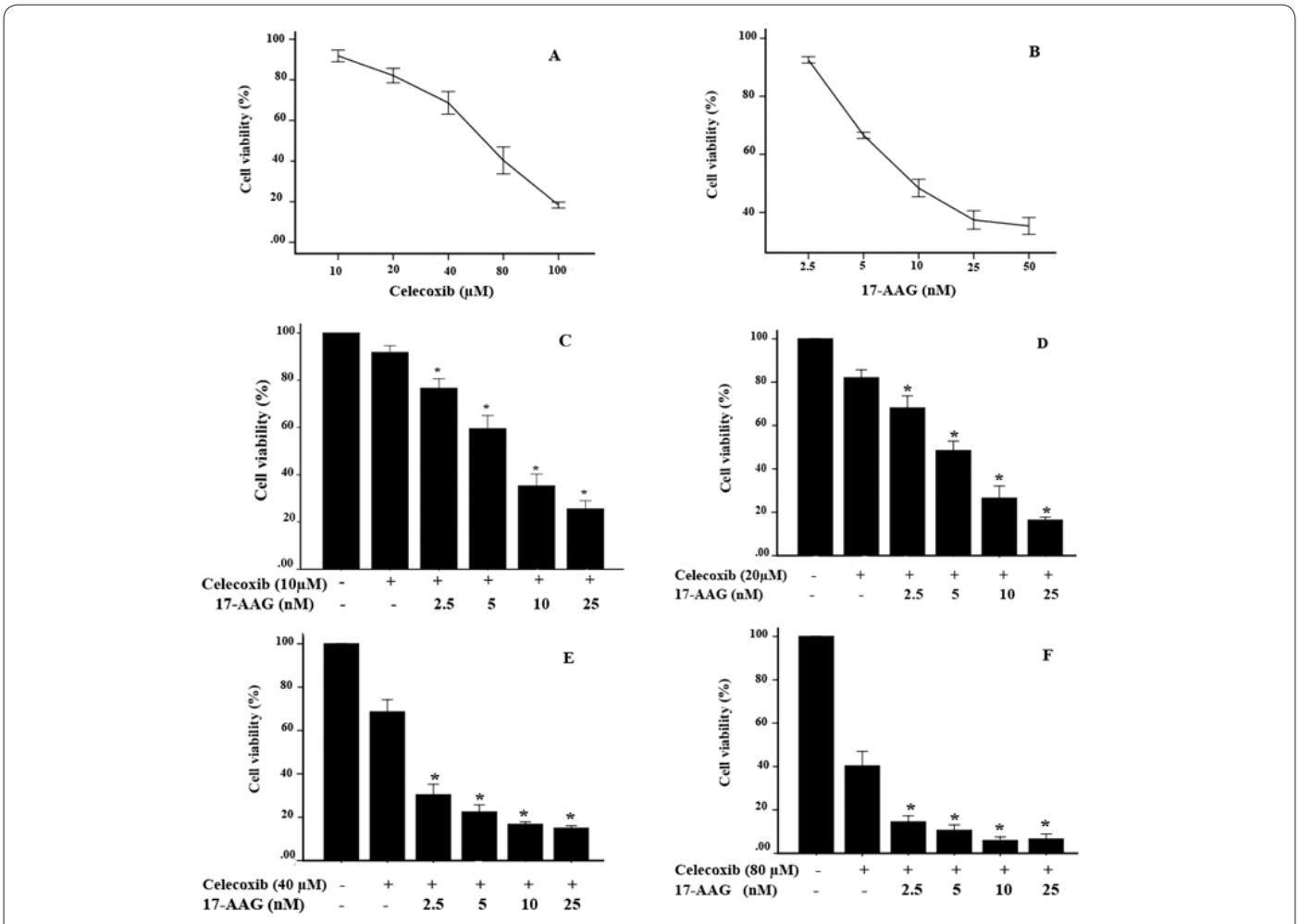

Figure 1. Effects of celecoxib and 17-AAG treatment, alone and in combination on HT-29 cells viability. HT-29 cells were treated with different concentrations of celecoxib and 17-AAG, alone and in combination for 72 h. Cell viability (%) was detected by MTT assay. Probit analysis was used for IC_{50} determination (A, B). For dual treatment 10, 20, 40 and 80 µM celecoxib and 2.5, 5, 10 and 25 nM 17-AAG were used (C, D, E and F). Data are shown as mean ± SEM from three independent experiments. *P< 0.05.

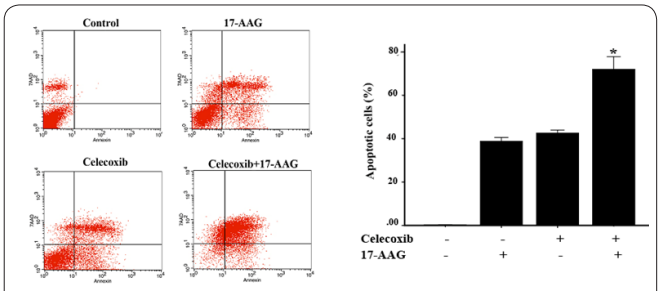

Figure 2. Induction of apoptosis by celecoxib or 17-AAG, alone and in combination in HT-29 cells. HT-29 cells were treated with the IC_{50} concentrations of celecoxib or 17-AAG, alone and in combination for 72 h. The percentages of total apoptotic cells (both early and late apoptotic cells) were estimated by flow cytometry analysis. Data are shown as mean ± SEM. **P*< 0.05.

cells was calculated, as described in the 2.3 section of materials and methods.

As shown in Figure 2 the percentage of apoptotic cells when treated with IC_{50} concentrations of celecoxib and 17-AAG were 42.6±1.38 and 38.8±1.75 respectively, while combination treatment of HT-29 cells with these concentrations increased the percentage of apoptotic cells to 71.98±5.8 (*P*<0.05).

Combination of celecoxib and 17-AAG increased BAX/BCL-2 ratio mainly due to suppressing BCL-2 level

Due to the observed enhanced apoptotic effect of combination of celecoxib and 17-AAG and in order to determine the possible mechanisms involved in the induction of apoptosis, we studied the expressed levels of BAX and BCL-2 and BAX/BCL-2 ratio in HT-29 cells. HT-29 cells were treated with 64 μM and 25 nM celecoxib and 17-AAG respectively, alone or in combination for 72 h. Protein levels for BAX and BCL-2 were determined using Western blotting. Western blotting analysis showed that the ratio of BAX/BCL-2 was significantly increased in the combination group compared to the other tree states. (100% in control group to 161.35%±8.2 in combination group: *P*= 0.002) (Figure 3). Moreover, the BAX/BCL-2 ratio in the 17-AAG group is significantly higher than the control group and significantly lower than combination group. Despite increased BAX/BCL-2 ratio, the increase in BAX levels was not statistically significant in the combination group compared with the control group, while BCL-2 in combination group was significantly reduced compared with the control group (*P*=0.016).

Discussion

Our results showed an additional inhibitory effect of celecoxib and 17-AAG combination on HT-29 cells. The results of Flow cytometry indicated that this additional inhibitory effect may occur due to induction of apoptosis. In addition, we showed that increasing BAX / BCL-2 ratio may be a cause of apoptosis induction in response to celecoxib and 17-AAG combination.

Epidemiological studies have shown a reduction in the risk of colorectal cancer with regular use of NSAIDs and suggested that there might be a relationship between the incidence of this cancer and chronic inflammation (7). Celecoxib is the only non-steroidal anti-inflammatory medication that could receive the FDA approve as adjuvant therapy in patients with familial adenomatous polyposis and clinical trials for its use in the treatment of sporadic colorectal cancer are also ongoing (10). Celecoxib, as a specific inhibitor of the COX-2 and decreasing prostaglandin mediators of inflammation, is able to inhibit growth of various types of tumors, such as liver, colorectal and stomach tumors (21). Although COX-2 inhibitors have fewer side effects than NSAIDs, but studies have shown that the specific inhibitors of COX-2 increase the risk of cardiovascular events by disturbing the balance between ant-thrombin and pro-thrombin eicosanoids (22). Therefore, application of high concentrations in single-agent therapy increases the risk of cardiovascular events (23), so their combination with anticancer drugs can be a useful approach for improving the cardiovascular effects. Our results showed additional inhibitory effects of celecoxib and 17-AAG combination on the HT-29 cells growth. The important point is that the combination of 17-AAG and celecoxib in the low doses showed significant toxicity, which can reduce the side effects of high doses of celecoxib. In agreement with our results, Guruswamy and colleagues showed enhanced inhibitory effect of combination of celecoxib and lovastatin in low doses. In another study, this additional effect was observed in combination of low doses of celecoxib and Atorvastatin in inhibition of the induction of colorectal cancer in animal models (24). A body of

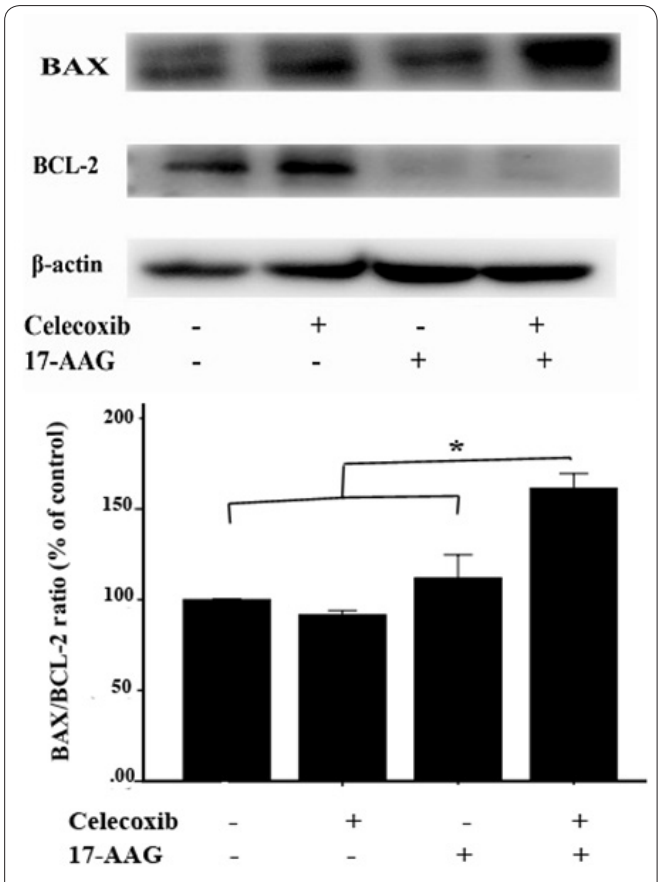

Figure 3. Effects of celecoxib or 17-AAG treatment, alone and in combination on BAX and BCL-2 levels and BAX/BCL-2 ratio. HT-29 cells were treated with the IC_{50} concentrations of celecoxib and 17-AAG, alone and in combination for 72 h. BAX and BCL-2 levels were estimated by western blotting analysis and band densities were normalized to β-actin protein expression. Data are shown as mean ± SEM from three independent experiments. **P* < 0.05.

evidences have shown that HSP90 inhibitors are able to increase sensitivity of tumor cells to drugs such as oxaliplatin, fluorouracil and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (25,26). However, additional studies should be conducted in order to investigate the effect(s) of combination of celecoxib and 17-AAG on animal models of colorectal cancer.

As for IC_{50}, the results of MTT test showed a significant reduction in IC_{50} concentration of celecoxib by combining celecoxib and 17-AAG. For example, in combination of 2.5 nM 17-AAG with various concentrations of celecoxib a 54% decrease in IC_{50} of celecoxib was observed.

Based on the analysis of the results of flow cytometry, we propose that apoptosis is the mechanism for toxic effects induced by combination of celecoxib and 17-AAG. As shown in Figure 2, in the combination group the majority of apoptotic cells are in the late phase of apoptosis, similar to 17-AAG and celecoxib groups. 17-AAG, as an inhibitor of HSP90, induces apoptosis in cancer cells through degradation of several oncogene proteins and inhibition of multiple signaling pathways(13). Celecoxib, through both a COX-2 dependent mechanism and a COX-2 independent mechanism has shown strong anti-tumor properties, and induction of apoptosis is one of its major properties (27). Based on the pro-apoptotic effects of celecoxib and 17-AAG, we hypothesize that additional inhibitory effects of celecoxib and 17-AAG combination that was observed in MTT, is probably due to the induction of apoptosis. The combination of IC_{50} concentrations of celecoxib and 17-AAG showed a significant increase in induction of apoptosis compared to either of these two drugs alone. Apoptosis is regulated by apoptotic and anti-apoptotic members of BCL-2 family proteins, and improper regulation of these proteins are observed in cancer (28). BCL-2, as a member of the anti-apoptotic proteins, through inhibition of apoptosis induced by anti-cancer agents, plays a major role in survival of tumor cells (29). HSP90 has anti-apoptotic function and some of apoptosis mediators, such as BCL-2 is its client proteins, so inhibition of HSP90 induces apoptosis (30). Powers et al, recently showed that induced cell death by 17-AAG is dependent on the induction of pro-apoptotic protein BAX (13). However, in this study, HT-29 cells treated with 17-AAG showed a non-significant induction of BAX which may be due to using low a concentration (IC_{50}) of 17-AAG. Also, our results showed an increase in BAX/BCL-2 ratio in response to combination of 17-AAG and celecoxib that is mainly because of BCL-2 reduction than BAX induction. A collection of evidences also indicated that the combination of celecoxib with anti-cancer drugs such 5-lipoxygenase inhibitor and ABT-737 showed an increase in apoptosis response by reducing the amount of BCL-2 and increasing the ratio of BAX/BCL-2 (31).

In conclusion, we showed an additional inhibitory effect for the celecoxib and 17-AAG combination on the HT-29 colorectal cancer cells. This additional effect was considerable when low doses of celecoxib were used. Another possible mechanism involved in the effect observed for the combination of the two drugs, is induction of apoptosis which occurs by increasing the BAX/BCL-2 ratio.

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