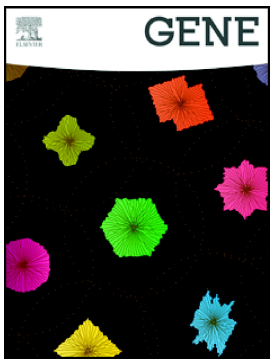

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Zeynali M. Shima, Mohammadian Mahshid, Kheradmand Fatemeh, Fathi-Azarbayjani Anahita, Rasmi Yousef, Esna-ashari Omid, Malekinejad Hassan

PII: S0378-1119(18)31050-3
DOI: doi:10.1016/j.gene.2018.10.016
Reference: GENE 43272

To appear in: *Gene*

Received date: 29 July 2018
Revised date: 2 October 2018
Accepted date: 9 October 2018

Please cite this article as: Zeynali M. Shima, Mohammadian Mahshid, Kheradmand Fatemeh, Fathi-Azarbayjani Anahita, Rasmi Yousef, Esna-ashari Omid, Malekinejad Hassan , A molecular basis for the synergy between 17'allylamino'17'demethoxy geldanamycin with Capecitabine and Irinotecan in human colorectal cancer cells through VEFG and MMP-9 gene expression. Gene (2018), doi:10.1016/j.gene.2018.10.016

A molecular basis for the synergy between 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan in human colorectal cancer cells through VEFG and MMP-9 gene expression

Zeynali M. Shima[1], Mohammadian Mahshid[2], Kheradmand Fatemeh[3,*], Fathi-Azarbayjani Anahita[4], Rasmi Yousef [5], Esna-ashari Omid[6], Malekinejad Hassan [7]

[1] *Department of Clinical Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran*

[2] *Department of Clinical Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran*

[3] *Department of Clinical Biochemistry, Cellular Molecular and Solid Tumor Research Center, Urmia University of Medical Science, Urmia, Iran*

[4] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran*

[5] *Department of Clinical Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran*

[6] *Radiotherapy Center, Omid Hospital, Urmia, Iran*

[7] *Department of pharmacology and toxicology, Faculty of Pharmacy, Urmia University of Medical sciences, Urmia, Iran*

Running Title: Combination therapy in Colorectal Cancer treatment

*Corresponding Author:

Kheradmand Fatemeh, *Department of Clinical Biochemistry, Cellular Molecular and Solid Tumor Research Center, Urmia University of Medical Science, Urmia, Iran*

fkheradmand@yahoo.com

Abstract:

Anti-proliferative, anti-metastatic and anti-angiogenic effects of 17-allylamino-17-demethoxy geldanamycin (17-AAG) were studied alone and in combination with Capecitabine (Cap) and/or Irinotecan (IR) on HT-29 human colorectal carcinoma cells. Expression of MMP-9 (matrix metalloproteinase-9) and VEGF (vascular endothelial growth factor) mRNA was analyzed by real-time PCR method. The study was further followed by wound scratch assay for migration assessment. Nitric oxide content, MDA generation and total anti-oxidant capacity were also assessed. Results showed significant differences between mono- and double therapy ($p < 0.05$). Combination of 17-AAG with IR or Cap resulted in synergistic effect (Combination Index <1). Among double combination groups only Cap/17-AAG showed significant differences in MMP-9 and VEGF genes expression and wound healing assay. Moreover, a significant decrease of wound area in our triple combination group was obtained, indicating the antagonistic effect. IR/17-AAG and IR/Cap double combination groups resulted in down-regulation of MMP-9 and VEGF mRNA expression, respectively. Significant generation of MDA and decrease in TAC values have been observed in all our tested groups, however, the IR/17-AAG combination was the only group that could elevate NO concentration, significantly. Our findings demonstrated potent anti-angiogenesis and anti-metastatic effects for 17-AAG when it is provided in double combination especially with Cap, suggesting a new protocol in colorectal cancer combination therapy. These findings may indicate that down-regulation of VEGF and MMP-9 genes is directly related to angiogenesis and metastasis.

Key Words: Anti-cancer; Anti-angiogenesis; Anti-metastasis; Oxidative stress; Wound healing

1. Introduction

Colorectal cancer is the third and fifth most common cancer of Iranian women and men, respectively (1, 2). Risk factors for colorectal cancer include obesity, meat and fat-rich diet, smoking, alcohol consumption and hyperinsulinemia (3, 4). Standard clinical practice for patients with colorectal cancer are chemotherapy, radiation therapy and cryosurgery post-surgical resection (3). Common chemotherapeutic regimens include FOLFOX (Fluorouracil/Leucovorin and Oxaliplatin) and FOLFIRI [Fluorouracil/Leucovorin and Irinotecan (IR)] (5). Capecitabine (Cap) is an oral chemotherapeutic agent that is converted to fluorouracil by thymidine phosphorylase (6). A randomized phase III study showed that Cap had more activity, favorable toxicity profile and greater response rate as compared to fluorouracil/leucovorin against colorectal cancer (7). IR (competothecin-11) is a chemotherapy agent that is converted to its active metabolite, (SN-38, an inhibitor of topoisomerase I) via carboxyl esterase (8, 9). It is mainly used in metastatic colorectal cancer patients (10).

Despite great improvements in cancer chemotherapy, in some cases treatments are inadequate and require novel drug regimens to be added (11). Recent approaches include the application of specific molecular targets such as proteins involved in the tumor angiogenesis and metastasis (12, 13). A newly developed anticancer agent, 17-allylamino-17-demethoxy geldanamycin (17-AAG) has shown therapeutic effects on HER2-positive metastatic breast cancer and renal carcinomas. 17-AAG is a heat shock protein 90 (HSP90) inhibitor, which binds to HSP90 and disrupts its function (13). An investigation on MDA-MB-453 human breast cancer cell line indicated that HSP90 interacts with matrix metalopeptidase (MMP) -2 and -9 (14). MMPs are key molecules involved in the degradation of extracellular matrix

during metastasis. Previous findings have demonstrated an enhanced expression of gelatinase (MMP-9) and its tissue activity in colorectal cancer (15).

Vascular endothelial growth factor (VEGF) on the other hand, is a homodimeric glycoprotein, which elevates blood vessel growth from near capillaries and stimulates angiogenesis (16). Previous studies have confirmed the role of VEGF in tumor angiogenesis of colorectal cancer patients. It has been well documented that MMP-9 secretion from tumor cells could activate VEGF and promote the angiogenesis (17, 18). Studies have shown that Cap and IR prevent angiogenesis by targeting VEGF and 17-AAG inhibits metastasis by suppressing MMP-9 expression and secretion (18, 19). Besides one of the toxicity mechanism of IR and 17-AAG has been proposed to be oxidative stress induction (20, 21). Reactive oxygen species production has been proven in mice treated with IR (22).

Hence, considering the effect of 17-AAG on HSP90 and its interaction with MMP-9, we aimed to evaluate the effect of new agent; 17-AAG alone and in double and triple combination with standard regimens Cap and/or IR on VEGF and MMP-9 genes expression, cell viability, antioxidant status and cell migration capacity in human colorectal adenocarcinoma cell line (HT-29). The outcome of proposed assays elucidated the cytotoxicity of tested compounds against colorectal cancer cells and their capability in the prevention of cancer cells migration (an index of metastases) and down-regulation of genes that are directly involved in angiogenesis and metastases.

2. Materials and methods

2.1. Materials

Cap was obtained from Sigma-Aldrich (St. Louis, Missouri, USA), IR and 17-AAG were obtained from LC Laboratories (Woburn, MA, USA). Ethylene diaminetetraacetic acid, sulphanilamide, N-(1-naphthyl) ethylenediamine -2HCl, tetramethylbenzidine, 2, 4, 6-tri-2-

pyridyl-1,3,5-triazin were purchased from Merck (Darmstadt, Germany). Stock solutions (10 mM for Cap and IR and 50 μM for 17-AAG) were prepared in distilled water and kept at -80 °C.

2.2. Cell culture

Human colorectal adenocarcinoma cell line, HT-29, was obtained from Iranian Biological Resource Center (Tehran, Iran). HT-29 cells were cultured in high glucose DMEM medium (Biowest, France), supplemented with 10% fetal bovine serum (Biowest, France) and 1% penicilline-streptomycine (Biowest, France). Cells were grown at 37 °C and humid incubator with 5% CO_2 atmosphere. Third and fifth passages were used for study purposes.

2.3. Cell Viability assay

Cells were seeded in 96-well micro-plate. After 24 h incubation, the cells were exposed to various drugs concentrations; 17-AAG (80, 40, 20 and10 nM), IR (8, 4, 2, 1 and 0.5 μM) and Cap (8, 4, 2 and 1μM).

The cytotoxic effect of drugs on HT-29 was tested by Premix WST-1Cell Proliferation Assay System (TAKARA BIO Inc., Japan). In brief, 24 h after exposure against the test compounds at 37°C, cells were further incubated with WST-1 (10 μL WST-1 reagent) for 3 h at 37°C. Absorbance was read at 450 nm with a reference wavelength > 650nm on Awareness Technology Stat Fax 2100 micro-plate reader (SAN DIEGO, CA, USA). Optical density (OD) values were obtained for control and treated cells. The inhibitory rate (%) was calculated using the following formula:

%viable cells = OD value of treated well / OD value of control well

The IC_{50} value of treatment groups relative to the control were calculated by Compusyn software (Combosyn, Inc., Paramus, USA). Cells were treated with $1\times IC_{50}$, $0.5\times IC_{50}$ and

$0.25\times IC_{50}$ in double and with $0.5\times IC_{50}$ and $0.25\times IC_{50}$ in triple combination groups. Cap and IR in 0.25, 0.5, 1, 2, 4, 8 µM and 17-AAG in 10, 20, 40, 80 nM were used. All experiments were carried out in quintuple.

To evaluate the cytotoxic effects of various drug combinations, the combination index (CI) was calculated according to Chou and Talalay method on Compusyn software (Combosyn, Inc., Paramus, US). The CI<1, =1 and >1 indicate synergism, additive effect, and antagonism, respectively (23).

Fraction affected (f_a) values indicate fraction of cells that have been affected by the drug at IC_{50} value and DRI (Dose reduction index) as an indicator, which determine the level of dose reduction for each drug when given in a synergistic combination compared to individual drug therapy were also calculated (12).

2.4. Nitric Oxide measurement

The total nitrite/nitrate production as an indicator of Nitric Oxide (NO) content was determined in cell culture supernatant according to the Griess reaction (24). In the Griess reaction, NO is rapidly converted into the more stable nitrite, which in an acidic environment is further converted into HNO_2. In reaction with sulphanilamide, HNO_2 forms a diazonium salt, which reacts with N-(1-naphthyl) ethylenediamine-2HCl to form an azo dye. The absorbance of formed dye was measured at a wavelength of 540 nm and was compared to the standard sodium nitrate curve.

2.5. Lipid peroxidation assay

The effect of various anticancer agents on cell membrane damage was determined by measuring the concentration of lipid peroxidation end-product malondialdehyde (MDA). To

determine the lipid peroxidation rate, the MDA content of collected supernatants was measured using the thiobarbituric acid (TBA) reaction as described previously (25). In short, 0.5 ml of the supernatant was mixed with 3 ml ortophosphoric acid (1% v/v) followed by vortex mixing. Subsequently 2 ml of 6.7 g L^{-1} TBA was added to the samples. The samples were heated at 100 °C for 45 min and chilled on ice. Finally, a 3 ml N-butanol was added and the samples were further centrifuged at 3,000 × *g* for 10 min. The absorbance of supernatant was measured spectrophotometerically at 532 nm and the MDA concentrations were calculated according to the simultaneously prepared calibration curves using MDA standards. The MDA level was expressed as nMol per 1×10^6 cells.

2.6. Total anti-oxidant capacity assay

TAC was measured in the supernatant of cell culture based on ferric reduction antioxidant power (FRAP) assay (26). Briefly, at low pH, which was provided using acetate buffer (300 mM, pH 3.6), reduction of Fe^{3+}-TPTZ (2, 4, 6-tri-2-pyridyl-1,3,5-triazin, Merck, Darmstadt, Germany) complex to the ferrous form, produces an intensive blue color that could be measured at 593 nm. The intensity of complex color following addition of the appropriate volume of the collected supernatants from treated and non-treated cells to reducible solution of Fe^{3+}-TPTZ is directly related to total reducing power of the electron donating antioxidant. Aqueous solution of Fe^{2+} ($FeSO_4.7H_2O$) was used as standard solutions.

2.7. Wound healing assay

Confluent HT-29 monolayers were seeded in 6-well plates (1×10^6 cells/well). Wound healing assay was performed 24 h after incubation as described previously (27). Briefly, wounds

were created in HT-29 monolayer by scratching with a 200 µl pipette tip across the entire diameter of each single well. Thereafter, detached cells were washed out with PBS and the cells were incubated with medium containing various drug compounds for 24 h. Immediatelyafter scratching (0 h) and 24 h after scratching, phase contrast images were taken. Wound widths were calculated using Image J Software (US National Institutes of Health).

2.8. RNA extraction and cDNA synthesis

Total RNA extraction of treated and untreated cells was performed with GeneAll Hybrid-R^{TM}RNA extraction kit (Seoul, South Korea). Integrity of extracted RNA was assessed with agarose gel electrophoresis and its purity was examined by measuring the ratio of OD at 260nm/280nm. Two µg RNA was reverse-transcribed into cDNA with random hexamer primers using Revert First strand cDNA synthesis kit. Gene All Hyperscript™ first strand synthesis kit (Seoul, South Korea) was used for cDNA synthesis.

2.9. Real-time PCR

Primer pairs shown in Table1 were designed according to melting temperature and primer-dimmer formation and were Blast-ed in ncbi/primer-blast (http://blast.ncbi.nlm.nih.gov/blast.cgi). Primers were checked for primer dimer and hairpin formation again with Generunner software (http://www.generunner.net). Real-time PCR was performed using Real Q Plus 2x Master Mix Green (Amplicon, Denmark) and Bio Molecular Systems MicqPCR cycler (QLD, Australia). The PCR conditions were as follow: 15 min at 95°C, then 40 cycles of 95°C for 20 sec; 58°C for 60 sec and 72°C for 5 minutes. Expression levels of MMP-9 and VEGF were normalized in accordance to the expression levels of β-actin as the housekeeping gene. Results were calculated by $2^{-\Delta\Delta Ct}$ method.

2.10. Statistical analysis

Data were analyzed by using SPSS 16 software (SPSS Inc., Chicago, IL, USA) and P-value<0.05 was considered statistically significant. Data were expressed as mean ± SD and were analyzed using one-way ANOVA.

3. Results

3.1. Cytotoxic effects of Cap, IR and 17-AAG and their combinations

HT-29 cell viability was declined by Cap, IR and 17-AAG in a dose-dependent manner after 24 h exposure (Fig.1). The IC_{50} values of Cap, IR and 17-AAG were determined by WST-1 data as 3.4 μM, 6.9 μM and 60 nM, respectively. A synergistic interaction was observed in viability test of all double treatment groups ($1\times IC_{50}$, $0.5\times IC_{50}$ and $0.25\times IC_{50}$) when compared to 17-AAG, IR and Cap monotherapy ($p\leq 0.05$). In 17-AAG/IR/Cap triple treatment groups at $0.5\times IC_{50}$ and $0.25\times IC_{50}$ concentrations, no significant differences was observed in comparison with monotherapy ($p \leq 0.05$). Moreover, we observed statistically significant differences among 17-AAG/IR/Cap triple treatment group when compared to Cap/IR ($0.25\times IC_{50}$) or Cap/IR ($1\times IC_{50}$) groups. These findings indicate that inclusion of 17-AAG, in Cap and IR drug regimen could decline cell viability more effectively than mono-treatment or triple treatment groups. Based on the CI values represented in Table 2, double combination groups of 17-AAG, IR or Cap show synergistic effects, whereas the triple combination group (17-AAG/Cap/IR) indicates a clear antagonistic effect. The DRI values shown in Table 3 indicate the number of reduction in drug concentration of double treatment groups with same IC_{50} value but lower toxicity as compared to monotherapy groups.

3.2. The combination of three test compounds failed to increase the NO content

The results of NO determination revealed that only Cap single treatment was able to reduce slightly the NO generation. At the same time, when a combination treatment was performed, we found IR and 17-AAG combination could elevate the NO concentration significantly ($p<0.05$). We failed to demonstrate any additive or synergistic effects, when the cells were exposed to the combination of three test compounds (Figure 3).

3.3. The IR treatment in individual form resulted in the highest lipid peroxidation in HT-29 Cells

Both Cap and IR in single treatment and in combination with 17-AAG and not in combination with each other elevated the lipid peroxidation rate in HT-29 cells. Results on the other hand showed that when cells were treated with triple combination, MDA content significantly elevated. The highest MDA concentration was found in the supernatant of cells, which exposed to IR alone (Figure 4).

3.4. TAC values were lowered in all tested groups

Total antioxidant capacity in cells exposed to all compounds either in single or combination treatments, was declined significantly ($p < 0.05$). Although there were non-significant differences between the test groups but the 17-AAG treatment showed the highest potency in the reduction of TAC. Neither double nor triple combination resulted in extra reduction of TAC (Figure 5).

3.5. Combination of CAP with 17-AAG prevented from cellular migration

Analysis of wound area in various groups indicated that 24 h after wound creating and exposing to growth medium (control group), wound area was reduced significantly ($p < 0.05$). The mentioned area reduction was found non-significant in the cells were exposed to 17-AGG alone or to the combination of IR/CAP and significant when the cells were exposed to IR/17-AGG. By contrary, exposing the cells against the combination of Cap/17-AGG resulted in a significant ($p < 0.05$) expansion of wound area. An interesting finding of wound healing assay was a remarkable reduction of wound area in the group of cells, which was treated with a combination of all three test agents of CAP/IR/17-AGG (Figure 6).

3.6. Effects of single, double and triple treatments by Cap, IR and 17-AAG on VEGF and MMP-9 mRNA expression in HT-29 cell line

As shown in Figure 7-A, the expression of VEGF mRNA down regulated significantly in Cap/17-AAG, IR/Cap double treatment groups and in Cap/IR/17-AAG triple treatment in comparison to control group ($p < 0.05$). Down-regulation of VEGF mRNA expression was not statistically significant at IC_{50} level of 17-AAG, Cap and IR single treatments and IR/17-AAG double treatment groups. According to Figure 7-B, MMP-9 mRNA down-regulation at IC_{50} level of 17-AAG, Cap and IR single treatments and 17-AAG/Cap and 17-AAG/IR double treatments were significant, whereas the IR/Cap double treatment and 17-AAG/Cap/IR triple treatment showed no significant effect on MMP-9 mRNA expression. The difference on the MMP-9 mRNA expression down-regulation was considered statistically significant at 17-AAG mono-treatment and Cap/17-AAG double, IR and Cap/IR/17-AAG triple, Cap/17-AAG double and Cap/IR/17-AAG triple treatment groups ($p < 0.05$).

4. Discussion

Various clinical trial studies indicated that 17-AAG is a suitable therapeutic drug in a wide range of cancers including breast (28, 29) and prostate cancer (30) as well as multiple myeloma (31, 32). According to the results of previous studies that have shown high potency and low toxicity of combination therapy, in this study we combined 17-AAG as the new chemotherapy agent with routine agents, Cap and IR. In the present study single treatment with 17-AAG, Cap and IR decreased cell viability compared to control group in HT-29 cells. Co-treatment with 17-AAG and IR or Cap showed significantly higher cytotoxicity. Combination of 17-AAG with one drug presented synergistic effect on cell viability, whereas co-treatment with two drugs combination showed antagonistic effect. In accordance to our results, reduced cell viability with mono-treatment of Cap, IR and 17-AAG in HT-29,HGUE-C-1 and SKOV3 cancer cell lines has been proven (33). Previous studies documented that the synergistic effect of Cap and IR in nude mice bearing human colon carcinomas of HCT-116 and HT-29, dictates the significance of double drug therapy compared to monotherapy (34). A phase I dose-escalation study demonstsrated that 17-AAG/IR co-treatment showed admitted cytotoxicity in colorectal, non-small cell lung and pancreatic adenocarcinoma solid tumors (35). On the conarary to our results a triple combination of 17-AAG with paclitaxel and rapamycin on MDA-MB-231 cells showed synergistic toxic effect on cell proliferation (36). This discrepancy may be due to difference in the cell line and drugs that we tested.

It has been well known that the cancer cells are susceptible to oxidative damage stimulated by exogenous ROS (reactive oxigen species) inducers like anti-cancer drugs. According to the positive correlation between TAC, MDA and viability test results, the oxidative stress of our tested drugs on HT-29 cells seems to contribute to the test compounds-induced oxidative

and not nitrosative stress. A significant increase in MDA and NO levels has also been demonstrated on HCT-116, CaCo-2, MDA-MB-231 and MCF-7 cell lines by 5-fluorouracil (5-FU) treatment (37). However a cytotoxic effect of our tested drugs might not be due to nitrosative stress, as NO level did not change significantly. Previous studies indicated that 5-FU enhances the ROS production in human gastric cancer MKN45 cells, suggesting a major role of oxidative stress involvment in cytotoxic mechanism of 5-FU (38). The only case, which was able to significantly elevate NO content, was the combination of IR/17-AAG, suggesting either a weak role of nitrastive stress in the cytotoxicity of the test compounds or less sensitivity of nitrosative stress inducing pathways in the selected cell lines. Study on rats showed that IR increases MPO (Myeloproxidase) activity, MDA and nitrogen species production and decreases antioxidants that finally resulting in oxidative damages (21, 39). A non-significant induction of nitrosative stress can be a good advantage of tested drugs and their less negative impact on normal cells through chemotherapy with these drugs.

In this study, we did not investigate the mechanisms involved in oxidative stress but the results of previous studies indicated that the effects of 17-AAG on ROS production that could inactivate kinase activity of non-degraded BRAF (V600E), have been proven in HT-29 cells (20). The capability of IR in combination with acetamide derivatives in the induction of ROS production and JNK(c-JunN-terminalkinase) activation have been reported in NSCLC (Non-small-cell lung cancer) cells (40). The antitumor activity of IR in triple combination with Diphenyl-difluoroketone and melatonin in mice with colon cancer have been explained by the reduction of oncogenic ROS and elevation of oncosuppressive ROS production in this combination (22). In this study we also examined if the synergetic effect of chemotherapy drugs may relate to the expression of two important genes involved in metastasis and angiogenesis.

Previous studies indicated that Cap in addition of cell viability reduction, down-regulated the expression of VEGF, MMP-9, ICAM-1 and CXCR4 protein in HT-29, HCT-116 and CaCo-2 human colorectal cancer cells (41). In the present study, we assessed VEGF and MMP-9 mRNA expression in our tested groups and data sowed a clear down-regulation of MMP-9 and VEGF in HT-29 cells. Our results confirmed the findings of Manu and co-workers western Blot, as theirimmunohistochemical analysis on gastric cancer cell lines proved the down-regulation of MMP-9 and VEGF by Cap (42). There is some evidence for VEGF down-regulation by 17-AAG in CCRCC (43) and by IR in HT-29 cell line (44). In contrast to our results, PCR analyses of another studies indicated that IR at cocentration equal to IC_{50} value showed a slight and non-significant increase in VEGF mRNA expression after 144 h, however the cited study demonstrated that the secretion of VEGF protein significantly decreased in HT-29 cell line. This mismatch with our results could be due to differences in treatment duration as our treatments were carried out for 24 h (45). As VEGF down-regulation was significant in our double treatment groups these combinations seem to have appropriate anti-angiogenic effect. There are reports supporting our results which indicate that a combination of 17-AAG/rapamycin showe synergistically significant effects on inhibition of angiogenesis and expression of apoptosis-related proteins like caspase-8, 9 in multiple myeloma cells (46).

17-AAG mono-treatment in U251MG glioma cells showed a significant decrease in MMP-9 mRNA expression by inhibition of focal adhesion kinase (FAK) phosphorylation that is corroborated with our findings. A reduction in hyaluronic acid content after FAK phosphorylation inhibition by 17-AAG might indicate the anti-metastatic effect of this agent (47). However 17-AAG when used in combination with FAK inhibitor PF-573228, significantly reduced cancer metastasis by FAK activity silencing in H460 cell compared to the monotherapy (48). Additionally, 17-AAG co-treatment with TRAIL (Tumor necrosing

factor related apoptosis inducing ligand) in 13 colorectal cancer cell lines demonstrated synergistic or addiditive effects and significantlly stimulated apoptosis by decresing ERBB2, AKT, IKKα and XIAP survival proteins activity (49). It may be concluded that 17-AAG when used in combination is more effective than monotherapy in metastatic colorectal cancer patients. The result of our study showed remarkably more decrease in the expression of MMP-9 mRNA in double treatment groups when compared to monotherapy.

Another endpoint which may help in the evaluation of anti-cancer potency of compounds could be cell migration capacity in wound healing assay. In line with our findings, wound healing assay results of the previous reports demonstrated that IR mono-therapy failes to decrease SW620 cells migration but when IR used in double combination with Carfilzomib significantly decreases cell migration (50). Contrary to our results, another study showed that 17-AAG single treatment significantly decreased MDA-MB-231 cells migration (51).

We also found that Cap/17-AAG double combination not only reduced MMP-9 mRNA expression, but also significantly decreased HT-29 cells migration, suggesting a positive correlation between MMP-9 mRNA down-regulation and HT-29 cells migration which are inhibited by the combination of Cap/17-AAG. Double treatments of 17-AAG with IR and especially Cap seems to be effective as an anti-metastatic treatments.

Our triple combination therapy group showed a subtle decline in cell viability down-regulated VEGF mRNA level significantly, however down-regulation of MMP-9 in this group was not significant. In addition this combination promote cell migration and could not be a suitable anti-metastatic treatment. Previous studies reported that triple combination of 17-AAG, PFT-μ and other chemotherapeutic agents such as cisplatin, doctaxel and gemcitabine in bladder cancer showed significant suppresion of Akt and Bad expression and apoposis (52).

Taken together, our findings indicate that 17-AAG alone and in particular in double combination with IR and Cap could be considered as an applicable combination therapy

against colerectal cancers. This anticancer synergy may be attributed to the down-regulation of MMP-9 and the prevention of migration. The further studies to highlight more details in the protein level and molecular events in triple therapy are waranted.

Aknowledgements: The authors are thankful to the grasious support of Cellular & Molecular research center of Urmia University of Medical Sciences.

Conflict of Interests: There is no conflict of interests between authors.

Foundings: This work was supported by the Urmia University of Medical Sciences and grant number of 95-01-32-2259.

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Figures Legends:

Figure 1. Cytotoxic effects of individual drugs on HT-29 cells: HT-29 cells were treated with different concentrations of 17-AAG (80, 40, 20 and 10 n, IR (8, 4, 2, 1 and 0.5 µM) and Cap (8, 4, 2 and 1µM) for 24 h. Data represent as mean ± SD from 3 independent experiments performed in quintuples.

Figure 2. Effects of double and triple combination therapy of 17-AAG, Cap and IR on cell viability of HT-29 cell line; Cells were treated with 1×IC50, 0.5×IC50 and 0.25×IC50 of each drug in double treatment groups and 0.5×IC50 and 0.25×IC50 of each drug in triple treatment group. Cell viability was assessed with WST-1 cell viability assay 24 h after treatment.

* indicates significant differences between mono-treatment and double combination treatments.

$^{\gamma}$ represents significant differences between mono-treatment and triple combination treatments.

$^{\&}$ represents significant differences between double treatments and triple treatments.

Data represent mean ±SD from 3 independent experiments performed in quintuples.

Figure 3. The effects of Capecitabine, Irinotecan and 17-AAG alone and in combination on the NO generation in HT-29 cell line. 1×10^{6}cells were incubated with IC50 levels in mono-treatment, 0.5×IC50 concentrations in double treatment and 0.25×IC50 levels in triple treatment for 24 h. Data expressed as mean ± SD of three independent experiments.

Figure 4. Effects of Capecitabine, Irinotecan and 17-AAG alone and in combination on the MDA generation in HT-29 cells. 1×10^6 cells were incubated with IC50 levels in mono-treatment, 0.5×IC50 concentrations in double treatment and 0.25×IC50 levels in triple treatment for 24 h. Data expressed as mean ± SD of three independent experiments. * indicates significant test MDA production in comparison to control ($p < 0.05$).

Figure 5. Effects of Capecitabine, Irinotecan and 17-AAG alone and in combination on TAC values in HT-29 cells.

Figure 6. Effects of Capecitabine, Irinotecan and 17-AAG alone and in combination on HT-29 cells migration; (A) Microphotograph of HT-29 cells immediately and 24 h after wound creation (in all pictures scale bar = 0.14 μm) and (B) the quantified wound area by using Image J software. * indicates significant reduction of wound area ($p < 0.05$).

Figure 7. Effects of Capecitabine, Irinotecan and 17-AAG alone and in combination on VEGF and MMP-9 mRNA expression in HT-29 cells; HT-29 cells were incubated with IC50 levels in mono-treatment, 0.5×IC50 concentrations in double treatment and 0.25×IC50 levels in triple treatment for 24 h. Expression of the VEGF (A) and MMP-9 (B) mRNA which has been normalized to β-actin mRNA expression, are presented. Data expressed as mean ± SD of three independent experiments. * indicates significant test genes mRNA down-regulation in comparison to control ($p < 0.05$).

Tables:

Table1.VEGF, MMP-9 and β-actin primer sequences that were used to evaluate gene expression in Real-time PCR

Table 2. Combination Index for combination treatment groups

Table3. Fraction affected (f_a) and DRI (Dose Reduction Index) for combination treatment groups; *affected fraction (f_a) values indicate fraction of cells in the setting, which have been affected by the drugs. **DRI values represent reduced levels of each drug dose in a synergistic combination treatment to obtain effects analogous to single treatments. DRI > 1and < 1 show favorable and unfavorable dose reduction, respectively, that leads to reduced toxicity levels in therapeutic applications.

Tables:

Table 1.

Primer name	Primer sequence	Product size (bp)
VEGF Forward	5' AGGAGGAGGGCAGAATCATC 3'	144
VEGF Reverse	5' GGCACACAGGATGGCTTGAA 3'	
MMP-9 Forward	5' GATGCGTGGAGAGTCGAAA 3'	192
MMP-9 Reverse	5' TAGGTGATGTTGTGGTGGTG 3'	
β-actin Forward	5' CTGGAACGGTGAAGGTGACA 3'	161
β-actin Reverse	5' TGGGGTGGCTTTTAGGATGG 3'	

Table 2.

Combined treatment	1×IC50 every single drug	0.5×IC50 every single drug	0.25×IC50 every single drug	General pattern
17-AAG/IR	0.101	0.085	0.034	Synergism
17-AAG/Cap	0.192	0.106	0.039	Synergism
IR/Cap	0.185	0.168	0.095	Synergism
17-AAG/Cap/IR	-	1.412	1.783	Antagonism

Table 3.

combination	f_a*	DRI**	
		Cap	17-AAG
Cap/17-AAG	0.72±0.03	11.44±6.3, etc.	70.88±47.6, etc.
		IR	17-AAG
IR/17-AAG	0.71±0.02	23.4±12.2, etc.	72.93±32.2, etc.
		IR	Cap
IR/Cap	0.71±0.03	18.22±6, etc.	13.03±4.9, etc.

List of abbreviations:

VEGF vascular endothelial growth factor

MMP-9 matrix metalloproteinase-9

17-AAG 17-allylamino-17-demethoxy geldanamycin

NO Nitric oxide

MDA malondialdehyde

TAC total anti-oxidant capacity

FOLFOX Fluorouracil/Leucovorin and Oxaliplatin

FOLFIRI Fluorouracil/Leucovorin and Irinotecan

ROS reactive oxygen species

JNK c-JunN-terminalkinase

ICAM-1 Intercellular Adhesion Molecule 1

CXCR4 C-X-C chemokine receptor type 4

FAK focal adhesion kinase

TRAIL Tumor necrosing factor related apoptosis inducing ligand

Highlights:

1- The synergistic effect of combination therapy by using 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan on colorectal cancer cells was highlighted;
2- The combination therapy by using 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan resulted in down-regulation of MMP-9 and VEGF mRNA level;
3- The combination therapy by using 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan expanded wound area in healing assay suggesting the anti-metastatic outcome of proposed combination regimen;
4- The combination therapy by using 17-allylamino-17-demethoxy geldanamycin with Capecitabine and Irinotecan elevated lipid peroxidation and reduced total antioxidant capacity in HT-29 cells.

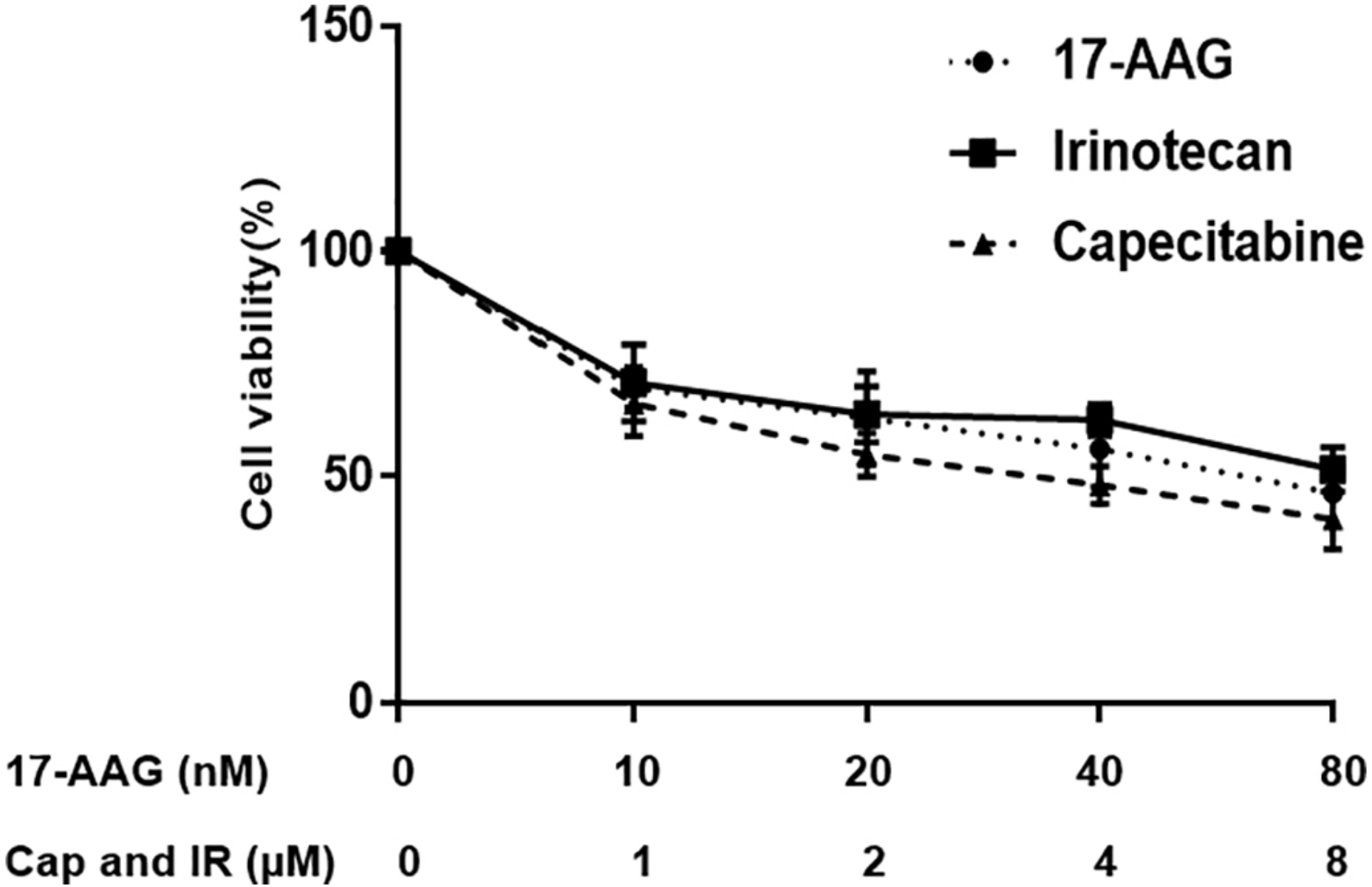
150
100
50
0
Cell viability(%)
17-AAG
Irinotecan
Capecitabine
17-AAG (nM) 0 10 20 40 80
Cap and IR (μM) 0 1 2 4 8

Figure 1

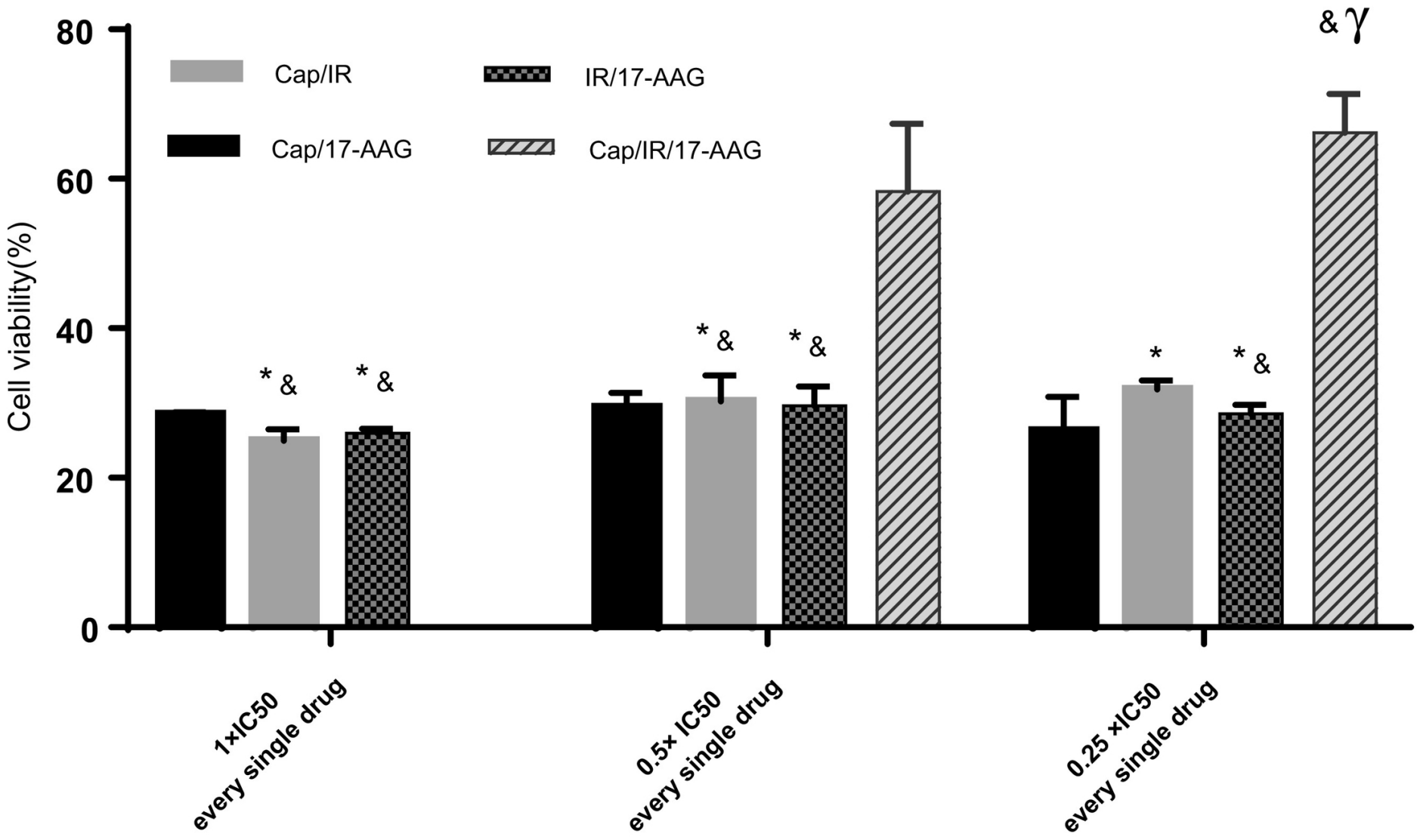
80
60
40
20
0
Cell viability(%)
Cap/IR
IR/17-AAG
Cap/17-AAG
Cap/IR/17-AAG
* &
* &
* &
* &
* &
& γ
1×IC50
every single drug
0.5× IC50
every single drug
0.25 ×IC50
every single drug

Figure 2

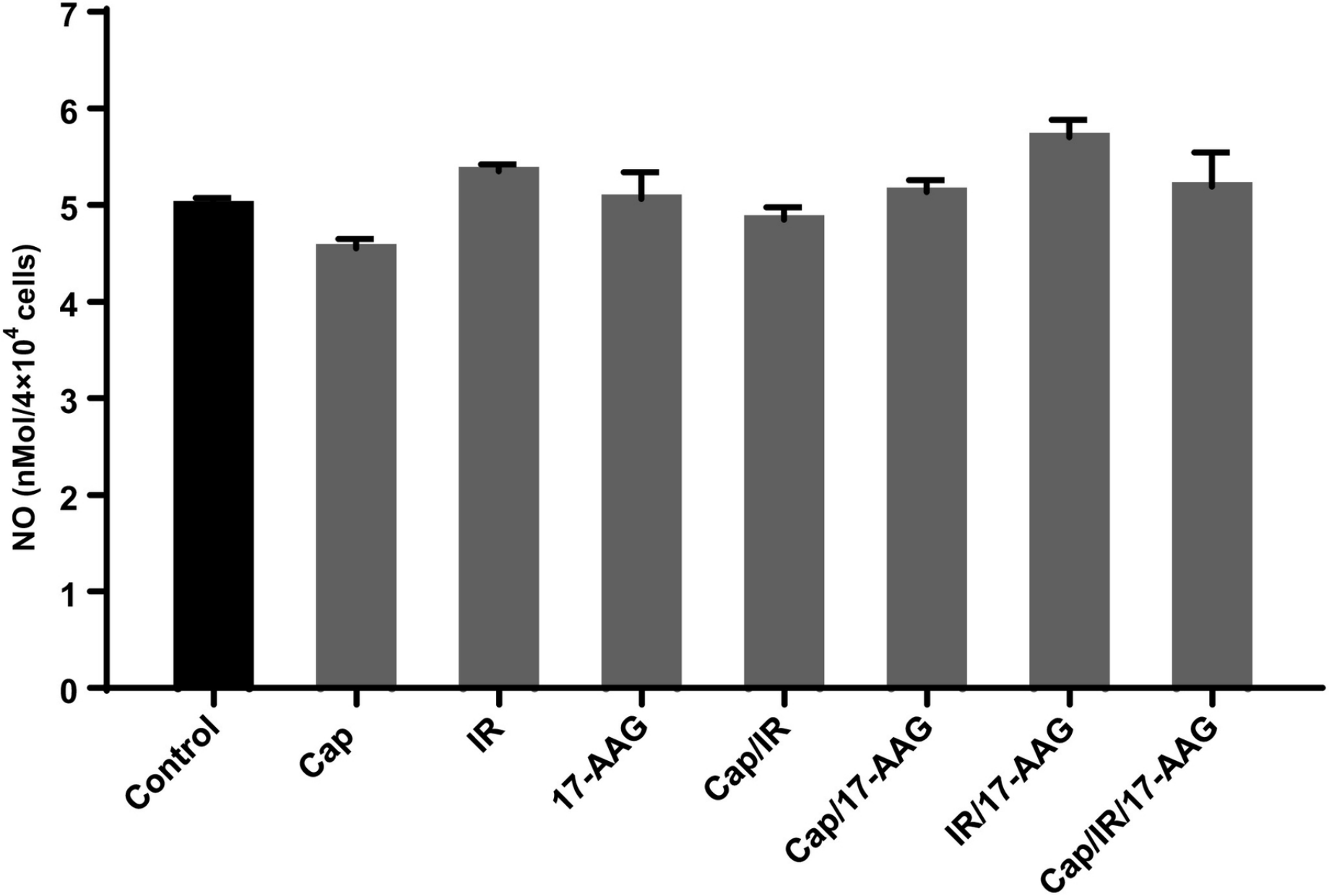
NO (nMol/4×10^4 cells)
0
1
2
3
4
5
6
7
Control
Cap
IR
17-AAG
Cap/IR
Cap/17-AAG
IR/17-AAG
Cap/IR/17-AAG

Figure 3

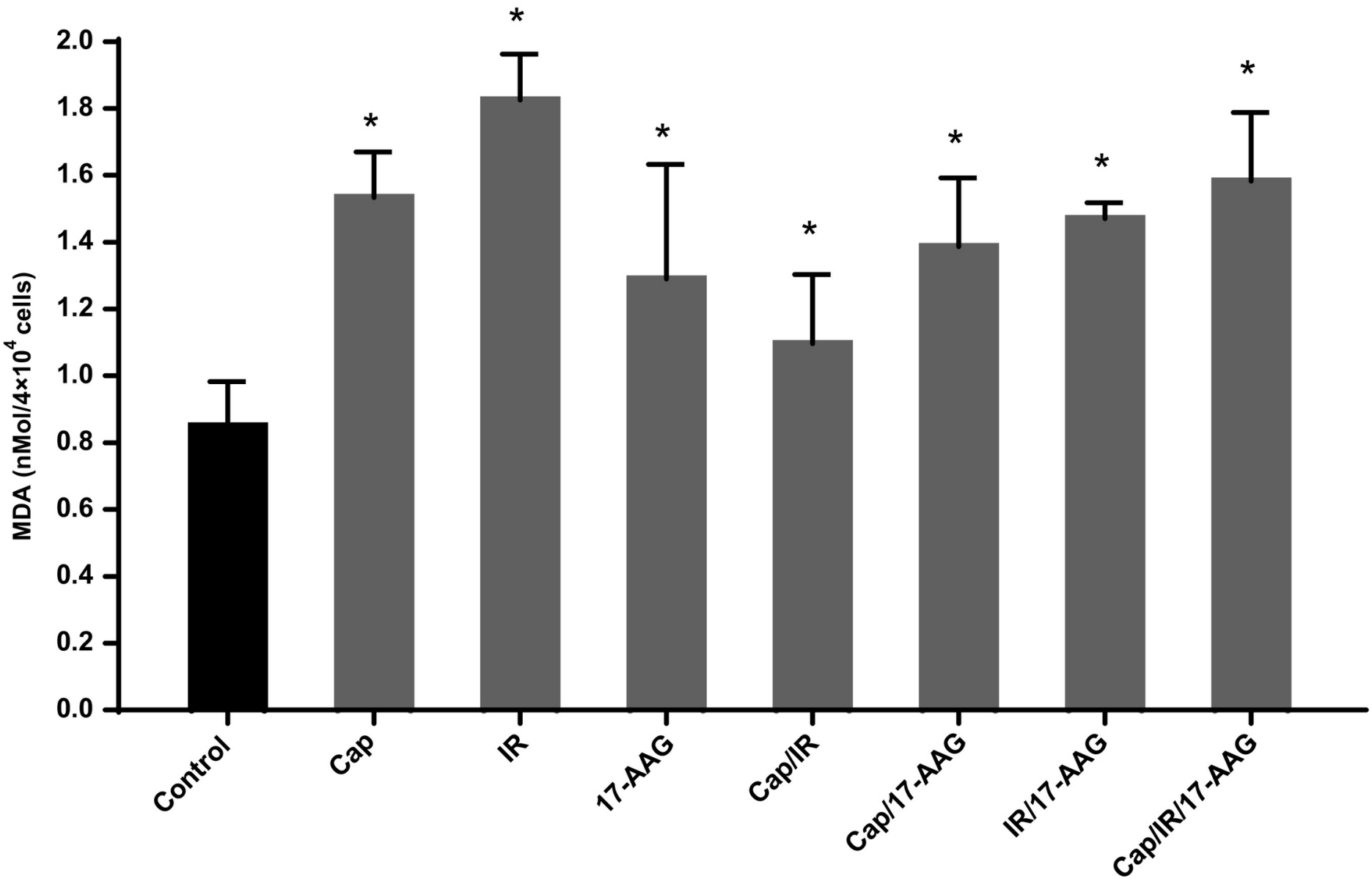
MDA (nMol/4×10^4 cells)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Cap
IR
17-AAG
Cap/IR
Cap/17-AAG
IR/17-AAG
Cap/IR/17-AAG

Figure 4

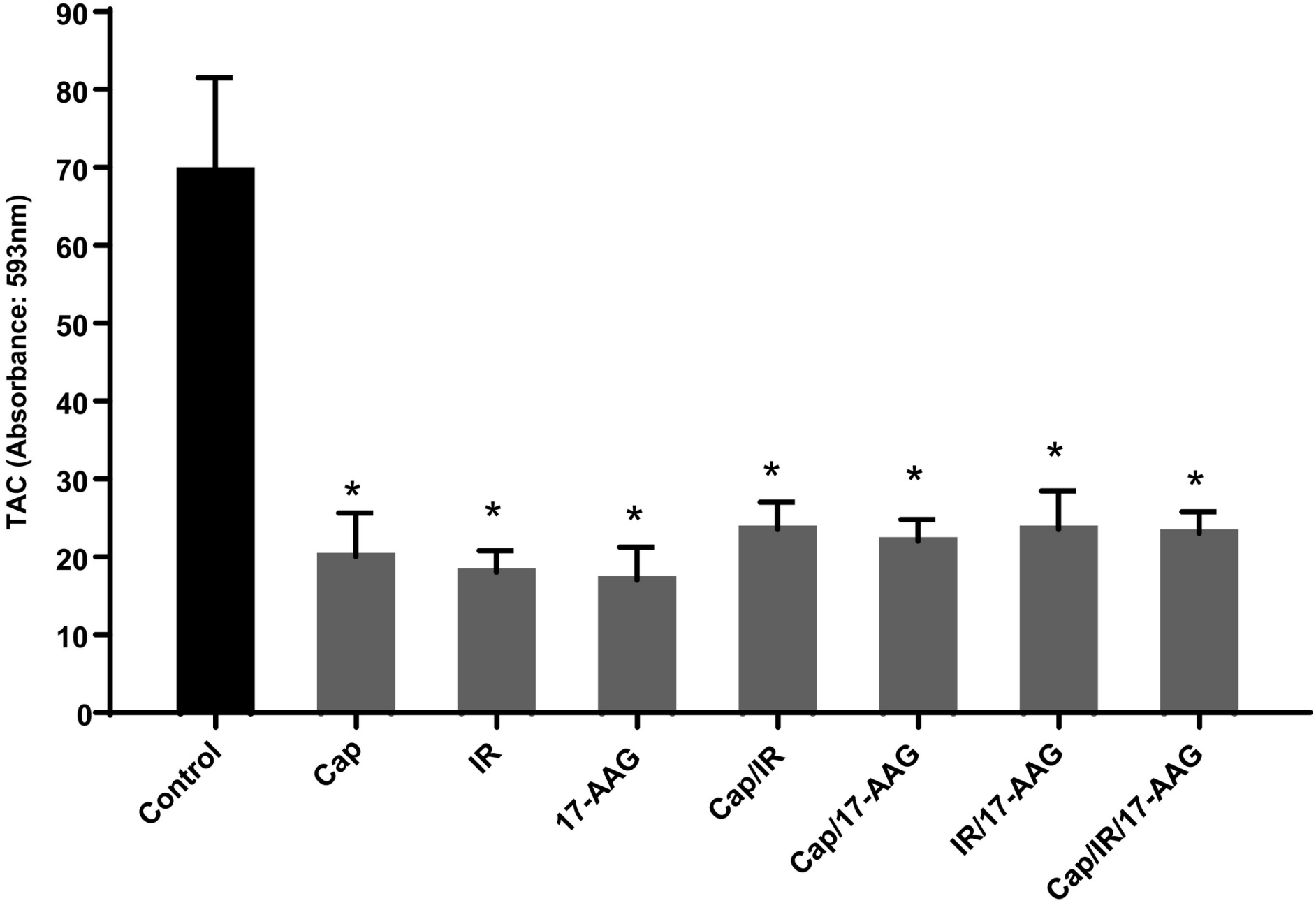
TAC (Absorbance: 593nm)
90
80
70
60
50
40
30
20
10
0
*
*
*
*
*
*
*
Control
Cap
IR
17-AAG
Cap/IR
Cap/17-AAG
IR/17-AAG
Cap/IR/17-AAG

Figure 5

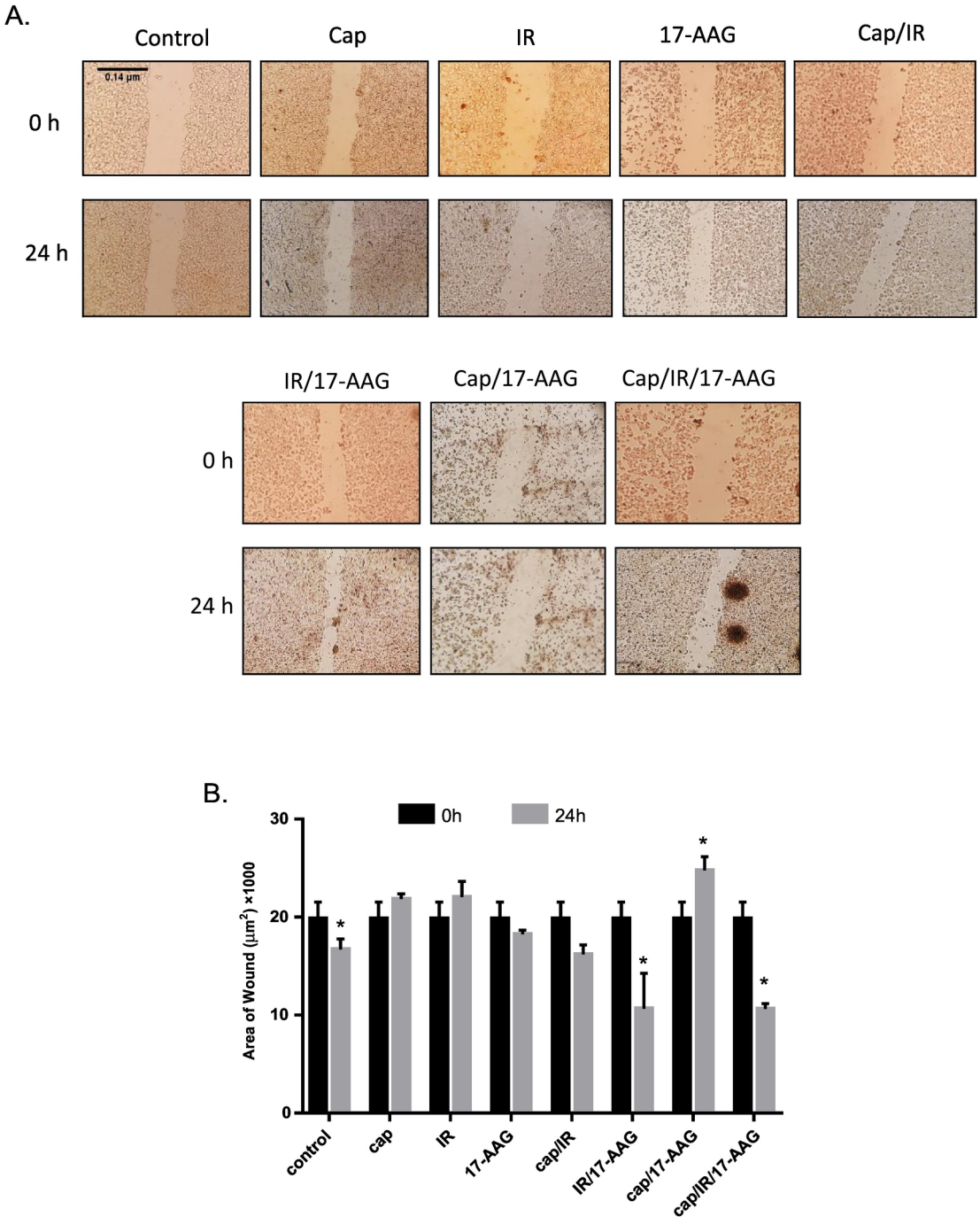
A.
Control
Cap
IR
17-AAG
Cap/IR
0.14 μm
0 h
24 h
IR/17-AAG
Cap/17-AAG
Cap/IR/17-AAG
0 h
24 h
B.
0h
24h
Area of Wound (μm²) ×1000
30
20
10
0
control
cap
IR
17-AAG
cap/IR
IR/17-AAG
cap/17-AAG
cap/IR/17-AAG

Figure 6

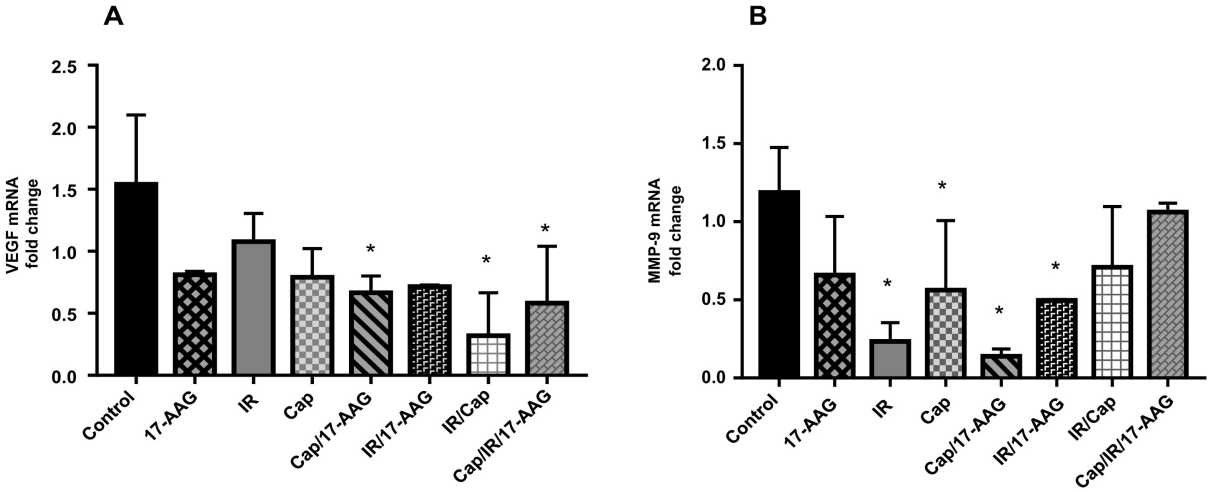
A
VEGF mRNA fold change
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
17-AAG
IR
Cap
Cap/17-AAG
IR/17-AAG
IR/Cap
Cap/IR/17-AAG
B
MMP-9 mRNA fold change
2.0
1.5
1.0
0.5
0.0
*
*
*
*
Control
17-AAG
IR
Cap
Cap/17-AAG
IR/17-AAG
IR/Cap
Cap/IR/17-AAG

Figure 7