Synergistic antitumor effect of all-trans retinoic acid on regorafenib-treated human colon cancer cell lines via modulation of the AMPK/VEGF/ERK/NFKB/caspase-3 signaling axis

marium Hamada
Damanhour University Faculty of Pharmacy
Radwa Mehanna
Faculty of Medicine of Alexandria University: Alexandria University Faculty of Medicine
Mohammad Hasseb
Damanhour University Faculty of Pharmacy
Maha Houssen (mahahoussen@pharm.dmu.edu.eg)
Damanhour University Faculty of Pharmacy https://orcid.org/0000-0002-7703-2751

Research Article

Keywords: colon cancer, regorafenib, all trans retinoic acid, Angiogenesis, VEGF

Posted Date: July 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1771641/v1

Abstract

Purpose

Colon cancer is the second most common cause of cancer-related death worldwide. Angiogenesis mediated by vascular endothelial growth factor (VEGF) plays important roles in cancer progression, and activated mitogen protein kinase (AMPK) has also been shown to exert both positive and negative effects on angiogenesis and different oncogenic signaling pathways. The multikinase inhibitor regorafenib prevents the activation of numerous kinases involved in angiogenesis, proliferation, and metastasis. All-trans retinoic acid (ATRA) has also been demonstrated to inhibit the growth and the development of different types of tumors. This study aims to assess the antitumor effects of ATRA in regorafenib-treated human colon cancer cell lines and investigate the molecular mechanism mediating these effects.

Methods

The levels of VEGF, AMPK, extracellular signalregulated protein kinase 1 (ERK1), and nuclear factor kappa B (NF-κB) were analyzed using enzyme-linked immunosorbent assay, and caspase-3 activity was assessed colorimetrically.

Results

ATRA potentiated AMPK and caspase-3 activities and reduced the levels of VEGF, ERK1, and NF-κB levels in regorafenib-treated human colon cancer cell lines.

Conclusion

ATRA exerted synergistic antiproliferative, antiangiogenic, and proapoptotic effects on regorafenib-treated colorectal cancer cells by modulating the AMPK/VEFG/ERK/NF-κB/caspase-3 signaling axis.

Introduction

Colorectal cancer (CRC) is the second most common cancer in women and the third most common in men worldwide. It is also the world's second-leading cause of cancer-related death[1].

The sustained proliferation, angiogenesis, and inhibited apoptosis of tumor cells are hallmarks of malignant tumors. Studies have shown that the activated mitogen protein kinase (AMPK), nuclear factor kappa B (NF-κB), and vascular endothelial growth factor (VEGF) signaling pathways are involved in cell proliferation, apoptosis, angiogenesis, immunosuppression, medication resistance, and tumormetabolic process either directly or indirectly [2–4].

VEGF is an angiogenic protein that is activated by the PI3K/AKT signaling route, protein kinase C, and the microtubule-associated protein kinase/extracellular signal-regulated kinase(ERK) signaling pathways, which leads to the proliferation, migration, and survival of endothelial cells [5].

Many oncogenic signaling pathways implicated in cancer progression are mediated by AMPK, which is a heterotrimeric kinase that phosphorylates substrates at serine/threonine residues and is found in all eukaryotes. When adenosine monophosphate (AMP) or the ratio of AMP to adenosine triphosphate (ATP) rises during metabolic stress, AMPK is activated, and when energy is abundant, it is inhibited[6].

In September 2012, the US Food and Drug Administration approved regorafenib, a VEGF signaling inhibitor, as a salvage treatment for patients with CRCwho had previously undergone chemotherapy with or without anti-VEGF or anti–epidermal growth factor receptor (EGFR) therapy.Nonetheless, patients are growing increasingly resistant to regorafenib, which is a major impediment to effective treatment[7]. Consequently, to improve the effectiveness of CRC therapy, several anticancer drugs are being used in combination with regorafenib[8]. Numerous investigations have shown that natural medicine monomers coupled with chemotherapy medications have synergistic anticancer effects.

One of the main metabolites of vitamin A, all-trans retinoic acid (ATRA), has potential chemotherapeutic and chemopreventive effects in the prevention and treatment of cancer [9, 10]. ATRA has shown promise in the treatment of diseases such as breast cancer, glioblastoma, and head-and-neck cancer[11].

To this end, this study aimed to evaluate the anticancer effects of ATRA on human colon cancer cell lines treated with regorafenib and identify the molecular processes underlying these effects.

Materials And Methods

Chemicals and therapeutic agents

Regorafenib (BAY 73-4506) was purchased from SelleckChem (Houston, TX, USA). A 10 mM stock solution was generated in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and stored at − 20°C. Regorafenib was diluted in phosphate-buffered saline (PBS) immediately before use. All compound preparations were stored at room temperature in the dark and used the same day. ATRA (Sigma-Aldrich) was diluted directly into media prior to use. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Serva (Heidelberg, Germany). Dulbecco's modified Eagle's medium (DMEM), trypsin, PBS, and penicillin/streptomycin mixture were procured from Lonza (Basel, Switzerland).

Cell lines and culture conditions

Two human CRC cell lines, Caco-2 and HCT-116, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained, and all experiments were conducted at the Center of Excellence for Research in Regenerative Medicine and its Applications (CERRMA), Alexandria Faculty of Medicine (Alexandria, Egypt), following the instructions of the ethical committee of the faculty of

pharmacy at Damanhur University (ref No. 220PB16). Cells were cultured in DMEM–high glucose (Lonza) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific) and incubated in a humidified CO_2 incubator at 37°C and 5% CO_2.

Cell viability assay

Caco-2 and HCT-116 cells were tested for sensitivity to regorafenib and ATRA in vitro using a modified MTT assay according to methods used in previous studies[12, 13]. Exponentially growing cells were seeded in 96-well plates (Corning, NY, USA) with a seeding density of 5×10^3/well. Twenty-four hours later, regorafenib in concentrations of 1,3,5, 7, and 8 µg/mL and ATRA in concentrations of 80,90,100, 110, and 150 µg/mL were added, with each concentration of eight replicas. After 48 h, 10 µL of 5 mg/mL MTT was added to each well, and the plates were incubated for 4 h at 37°C in a CO_2 incubator.

Media were replaced with 100 µL of DMSO and gently rocked in the dark for 20 min to dissolve formazan crystals. Absorbance was measured at λmax 570 nm (A570 nm) using an automated enzyme-linked immunosorbent assay (ELISA) microplate reader (Infinite F50, Tecan,Männedorf, Switzerland). The percentages of cell viability were calculated as the ratio of treated to untreated cells (control) average absorbance regarded as 100% living cells. Cell viability (%) = (Atreated/Acontrol) ·100. Percentage cell viability was used to calculate the IC_{50} values of the two cancer cell lines after 48 h of treatment using sigmoidal dose-response curve-fitting models (GraphPad Prism Software, version 8, GraphPad Inc., La Jolla, CA, USA).

Regorafenib and ATRA combinations of $IC5_{0\%}$, $<IC_{50}$, and $>IC_{50\%}$ were performed to determine whether they had additive or synergistic effects and to choose the most convenient combination with which to complete the study.

Apoptosis assay (annexin-V-FITC/propidium iodide assay)

We studied the induction of apoptosis by regorafenib solution, ATRA, or their combination using Annexin-V assay via flow cytometry. The investigated samples were regorafenib solution, ATRA, and regorafenib/ATRA combination. Briefly, cells were incubated at a density (of 3×10^5)in a six-well plate (Corning) and allowed to adherein an incubator for 24 h at 37°C. Next, cells were treated with$IC_{50}$of regorafenib (8µg/mL for Caco cells and 7 µg/mL for HCT cells), 110 µg/mL ATRA ($IC_{50\%}$) or a combination of sub-IC_{50} dose (100 × 5) for 48 h.Afterwards, cells were then trypsinized, collected via centrifugation at 2000 rpm, and stained with Annexin V-FITC and propidium iodide as per the manufacturer's protocol. Analysis of apoptotic cells was conducted by 20,000 cells gating via flow cytometer (BD FACSCalibur flow cytometer; BD Biosciences, San Jose, CA, USA). The experiment was conducted in triplicate (n = 3).

Proliferation marker; KI67

Cells exposed to IC_{50} of regorafenib, IC_{50} of ATRA, and a combination of sub-IC_{50} (100 × 5) were trypsinized and turned into a single-cell suspension for intracellular staining with the anti-ki67 antibody. First, cells were fixed with 4% paraformaldehyde at room temperature for 10 min followed by permeabilization for 30 min using 1% Triton-X. Blocking was performed using 2% bovine serum albumin for an additional 30 min to avoid nonspecific binding. The cells were finally stained with a specific Ki-67 conjugated antibody (cat No. 11882S, Cell Signaling Technology, Danvers, MA, USA) and incubated at 4°C for 60 min in the dark. The fluorescent intensity of the labeled cells was analyzed using Becton Dickinson (Franklin Lakes, NJ, USA), FACS caliber flow cytometer operated with Cell Quest software.

ELISA techniques

The different proteins were determined in both cell lines according to the manufacturer's instructions. For NF-κb, AMPK, and pERK, we used the My Biosource Assay Kit (MBS2514316 m, MBS2514316, MBS2511875; San Diego, CA, USA), whereas for VEGF, we used the CUSABIO, CSB-E11718h (Houston, USA). This assay uses the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human NF-κB, AMPK, pERK, and VEGF was precoated onto a microplate. Samples were pipetted into the wells, and the measured human biomarkers present in the solutions were bound by the immobilized antibody. A yellow color was developed, which was proportional to the amount of the bound NF-κB, AMPK, pERK, or VEGF. The intensity of the color was measured at 450 nm.

Caspase-3 activity

We evaluated caspase activity on the basis of the spectrophotometric detection of the chromophore p-nitroaniline (pNA) at 405 nm after cleavage from its labeled substrate DVD-pNA. The protein concentration of the samples was analyzed and normalized in lysis buffer to equal the protein concentrations. The colorimetric assay (caspase-3-C, Sigma Aldrich) was used according to the manufacturer's instructions.

Statistical analysis of the data

Data were fed to the computer and analyzed using the IBM SPSS software package version 20.0(IBM Corp, Armonk, NY, USA). The Shapiro–Wilk test was used to verify the normality of the distribution. Quantitative data were described using the mean and standard deviation. The significance of the obtained results was judged at the 5% level. An *F*-test (analysis of variance) was used for normally distributed quantitative variables to compare between more than two groups, and the post hoc Tukey test was used for pairwise comparisons.

Results

Cell viability

Compared with the control group, there was a significant decrease in cell viability ($p < 0.05$) with regorafenib as compared with ATRA-treated HCT cells (Fig. 1A,B). Additionally, a significant decrease in

cell viability was detected in regorafenib-treated Caco cells as compared with ATRA-treated Caco cells ($p \leq 0.0001$; Fig. 2A,B).

Effect of Regorafenib and/or ATRA on ki67

As compared with the control group, cell proliferation was significantly ($p < 0.05$) decreased by the regorafenib- and ATRA-treated groups (Fig. 1A,B). However, the regorafenib/ATRA sub-IC_{50} combination showed a significant decrease in cell proliferation as compared with the control group and with the regorafenib- and ATRA-treated groups ($p \leq 0.0001$;Figure 2A,B).

Effect of regorafenib and/or ATRA on pAMPK level

The level of pAMPK was significantly ($p < 0.05$) increased in the regorafenib-treated groups in comparison with the control groups. Although ATRA treatment (IC_{50}) significantly ($p < 0.05$) increased the phosphorylation of AMPK in only the HCT-116 cell line (Fig. 3B), the sub-IC_{50} of the combined treatment significantly increased the pAMPK level as compared with cells treated with regorafenib or ATRA alone in both cell lines ($p \leq 0.0001$;Figure 3A,B).

Effect of regorafenib and/or ATRA on cell apoptosis using AnnexinV

Compared with the control group, cell apoptosis was significantly ($p < 0.05$) increased by the regorafenib-treated groups in both cell lines (Fig. 4A,B). However, in the ATRA IC_{50}–treated group, apoptosis was significantly ($p < 0.05$) higher than in the control group only in the HCT-116 cell line (Fig. 4B). Regorafenib/ATRA sub-IC_{50} combination showed a significant increase in cell apoptosis compared with the control group and with the regorafenib- and ATRA IC_{50}–treated groups ($p \leq 0.0001$;Figure 4A,B).

Effect of Regorafenib and/or ATRA on VEGF levels

Both Caco and HCT-116 cells showed a significant ($p < 0.05$) reduction in VEGF level, in cells treated with IC_{50} of regorafenib and ATRA, or their sub-IC_{50} combination in comparison with the untreated control cells (Fig. 5A,B). However, sub-IC_{50} combined treatment showed significantly lower levels of VEGF than either regorafenib or ATRA alone ($p \leq 0.0001$;Figure 5A,B).

Effect of regorafenib and/or ATRA on caspase-3 activity

The result showed that the in vitro activity of caspase was significantly ($p < 0.05$) enhanced by IC_{50} of ATRA or/and regorafenib treatment in both cell types compared with control (Fig. 6A,B**)**. The caspase activity in sub-IC_{50} combined treatment was significantly greater than that in IC_{50} ATRA–or regorafenib–treated groups in both cell lines ($p \leq 0.0001$;Figure 6A,B**)**.

Effect of regorafenib and/or ATRA on pERKlevel

In comparison with the untreated control group, both Caco and HCT-116 cells showed a significant ($p < 0.05$) decrease in pERK level in cells treated with IC_{50} regorafenib and ATRA or their sub-IC_{50} combination (Fig. 7A,B**)**. However, sub-IC_{50} combined treatment showed significantly lower levels of pERK than either regorafenib or ATRA alone ($p \leq 0.0001$;Figure 7A,B**)**.

Effect of regorafenib and/or ATRA on NF-κB levels

In both cell lines, there was a significant reduction in NF-κB level ($p < 0.05$) in cells treated with IC_{50} of regorafenib and ATRA or their sub-IC_{50} combination(Fig. 8A,B**)**. Nevertheless, combined treatment showed significantly lower levels of NF-κB than either regorafenib or ATRA alone ($p \leq 0.0001$;Figure 8A,B).

Discussion

CRC has the second-highest cancer-related mortality rate worldwide. The fact that most patients with CRC have definitive diagnoses at the terminal stages means that their life expectancy is usually less than 1 year[1] .

Chemotherapy based on regorafenib has been shown to be effective in treating patients with cancer; nonetheless, resistance to regorafenib is the most significant barrier to a positive prognosis. Consequently, there is an unmet medical need for effective medicines that can stabilize and/or reduce the progression of metastatic CRC[8] .

Numerous anticancer studies have shown that ATRA, a vitamin A derivative, inhibits cell multiplication markers such as cyclin D1 and human telomerase switch transcriptase, as well as growth factors such as EGFR and VEGF.

To that end, this study aimed to determine the synergistic chemotherapeutic effect of ATRA (vitamin A) and regorafenib in colon cancer cell lines and investigate the molecular mechanisms mediating these effects by elucidating the impact of their combination on AMPK and VEGF signaling [14, 15].

To our knowledge, this is the first study to investigate the anticancer properties of ATRA as an AMPK activator on regorafenib-treated human colon cancer cell lines.

In the present investigation, we discovered that the combination of ATRA and regorafenib increased the cytotoxicity of tumor cells. This was consistent with prior research that found ATRA to have a strong cytotoxic effect in sorafenib-treated HCC cell lines. This effect was attributed to the overexpression of Bax and mitochondrial translocation, which resulted in mitochondria-mediated intrinsic apoptosis [16].

In the present study, the combination of ATRA and regorafenib resulted in a considerable increase in AMPK activity. This increase in AMPK activity mediated by ATRA was consistent with the findings of

Shijima et al in 2015[17], who reported that downregulation of glycolytic genes by ATRA lowered ATP synthesis and promoted AMPK activation and hence improved the efficacy of chemotherapy by controlling a metabolic pathway critical for cancer cell survival.

In the combination of ATRA and regorafenib, there was a considerable decrease in VEGF, a marker of angiogenesis. This suppression can be attributed in part to ATRA-induced AMPK activation, as increased AMPK activity leads to mammalian target of rapamycin (mTOR) inhibition. The inhibition of mTOR decreases the expression of transcription factor hypoxia-induced factor–1 (HIF-1), which controls VEGF synthesis[18]. Conversely, ATRA may inhibit the transcription of the VEGF gene via a direct repeat 1 element situated at the transcription initiation site and modulates VEGF expression via the regulation of HIF-1 [19, 20].

Our findings revealed that ATRA-induced AMPK activation in the combination of regorafenib and ATRA inhibits ERK activity. ATRA has also been shown to directly alter ERK activity by stimulating the retinoic acid receptor [21].

The present study also revealed a considerable decrease in NF-κB levels as a final RAS/ERK signaling substrate. This inhibition is due to the inhibitory effects of AMPK on NF-κB signaling, which are mediated by multiple downstream targets such as SIRT1, PGC-1alpha and p53[22].

The present study showed that ATRA, both directly and as an AMPK activator, inhibits proliferation and promotes apoptosis, as seen by KI67 suppression and caspase-3 activation. This finding is in line with previous studies linking these effects to AMPK's role in preventing cell-cycle progression via the regulation of the phosphorylation and stability of p27kip, a cell-cycle inhibitor. Phosphorylation of p53, which causes cell death, results in apoptotic cell death rather than increasing survival[23].

Furthermore, retinoic acid–induced gene products may activate upstream caspases or caspase-3 by some other as-yet-unidentified pathway, or another mitochondrial intermembrane protein such as AIF may directlyactivate caspase-3[24] .

From this study, we conclude that ATRA exhibits a potential antitumor effect via the modulation of different AMPK signaling cascades. Further studies in the future are warranted to elucidate the impact of ATRA on different oncogenic signaling mediating the progression of CRC.

Declarations

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Conflict of interest

All authors declare that they have no conflict of interest

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.The study was approved by the Ethical Committee of the Faculty of Pharmacy, Damanhour University.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from (mahahoussen@pharm.dmu.edu.eg,)

All authors as follows:

Mariam Hamada, Radwa Ali Mehana, Mohammad M. Abd-Al haseeb, Maha Houssen

CRediT authorship contribution statement

Marium Hamada: conducting all the practical experiments,collecting review of the literature. **Radwa Mehanna**: Main supervisor for the practical work, revising all paper drafts. **Mohammad hasseb:** sharing in supervising the practical, conducting statistical analysis revising all paper drafts **Maha Houssen**: Design research idea and construct the research plan, revising the research results, writing the first and all paper drafts and authoring all paper drafts, revising all paper drafts, collecting review of literature, supervise all work, corresponding author

Data availability statement

Data archiving is not mandated but data will be made available on reasonable request

References

1. Marley AR, Nan H. Epidemiology of colorectal cancer. Int J Mol Epidemiol Genet. 2016;7(3):105.
2. Alhurry AMAH, et al., *A review of the incidence of colorectal cancer in the Middle East.* Iranian Journal of Colorectal Research, 2017. **5**(3–4).
3. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. Cancer Epidemiol Prev Biomarkers. 2009;18(6):1688–94.

4. Jemal A, et al. Global patterns ofcancer incidence and mortality rates and trends. Cancer Epidemiol Prev Biomarkers. 2010;19(8):1893–907.
5. Bruns CJ, et al. Vascular endothelial growth factor is an in vivo survival factor for tumor endothelium in a murine model ofcolorectal carcinoma liver metastases. Cancer. 2000;89(3):488–99.
6. Kuhajda F. AMP-activated protein kinase and human cancer: cancer metabolism revisited. Int J Obes. 2008;32(4):S36–41.
7. Grothey A, et al. Regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT): an international, multicentre, randomised, placebo-controlled, phase 3 trial. The Lancet. 2013;381(9863):303–12.
8. Network N, *Clinical practice guidelines in oncologycolon cancer*. 2017.
9. Liu W, et al. The protective role of all-transretinoic acid (ATRA) against colorectal cancer development is achieved via increasing miR-3666 expression and decreasing E2F7 expression. Biomed Pharmacother. 2018;104:94–101.
10. Huang S, et al., *Targeting pin1 by all-trans retinoic acid (ATRA) overcomes tamoxifen resistance in breast cancer via multifactorial mechanisms*. Frontiers in cell and developmental biology, 2019: p. 322.
11. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer. 2001;1(3):181–93.
12. Tolosa L, Donato MT, Gómez-Lechón MJ. *General cytotoxicity assessment by means of the MTT assay*, in *Protocols in in vitro hepatocyte research*. Springer; 2015. pp. 333–48.
13. Pauwels R, et al. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J Virol Methods. 1988;20(4):309–21.
14. Chen M-C, et al. Retinoic acid and cancertreatment. Biomedicine. 2014;4(4):1–6.
15. Applegate CC, Lane MA. Role of retinoids in the prevention and treatment of colorectal cancer. World J Gastrointest Oncol. 2015;7(10):184.
16. Kurosu T, et al. Sorafenib induces apoptosis specifically in cells expressing BCR/ABL by inhibiting its kinase activity to activate the intrinsic mitochondrial pathway. Cancer Res. 2009;69(9):3927–36.
17. Ishijima N, et al. Activation of AMP-activated protein kinase by retinoic acid sensitizes hepatocellular carcinoma cells to apoptosis induced by sorafenib. Cancer Sci. 2015;106(5):567–75.
18. Tadakawa M, et al., *The anti-diabetic drug metformin inhibits vascular endothelial growth factor expression via the mammalian target of rapamycin complex 1/hypoxia-inducible factor-1α signaling pathway in ELT-3 cells.* Molecular and cellular endocrinology, 2015. 399: p. 1–8.
19. Albadari N, Deng S, Li W. The transcriptional factors HIF-1 and HIF-2 and their novelinhibitors in cancer therapy. Expert Opin Drug Discov. 2019;14(7):667–82.
20. Liang C, Guo S, Yang L. Effects of all–trans retinoic acid on VEGF and HIF–1α expression in glioma cells under normoxia and hypoxia and its anti–angiogenic effect in an intracerebral glioma model. Mol Med Rep. 2014;10(5):2713–9.

21. Huo L, et al. All-trans retinoic acid modulates mitogen-activated protein kinase pathway activation in human scleral fibroblasts through retinoic acidreceptor beta. Mol Vis. 2013;19:1795.
22. Yu W-L, et al., *AMPK-SIRT1 Pathway Modulates The Apoptosis, Proliferation and Migration of AR42J Cells By Regulating p53 and NF-κB*. 2021.
23. Radu M. The role of p27 phosphorylation in mediating atRA sensitivity of ovarian carcinoma cells. Temple University; 2008.
24. Ali-Khan SE, Hales BF. Caspase-3 mediates retinoid-induced apoptosis in the organogenesis-stage mouse limb. Birth Defects Research Part A: Clinical and Molecular Teratology. 2003;67(10):848–60.

Figures

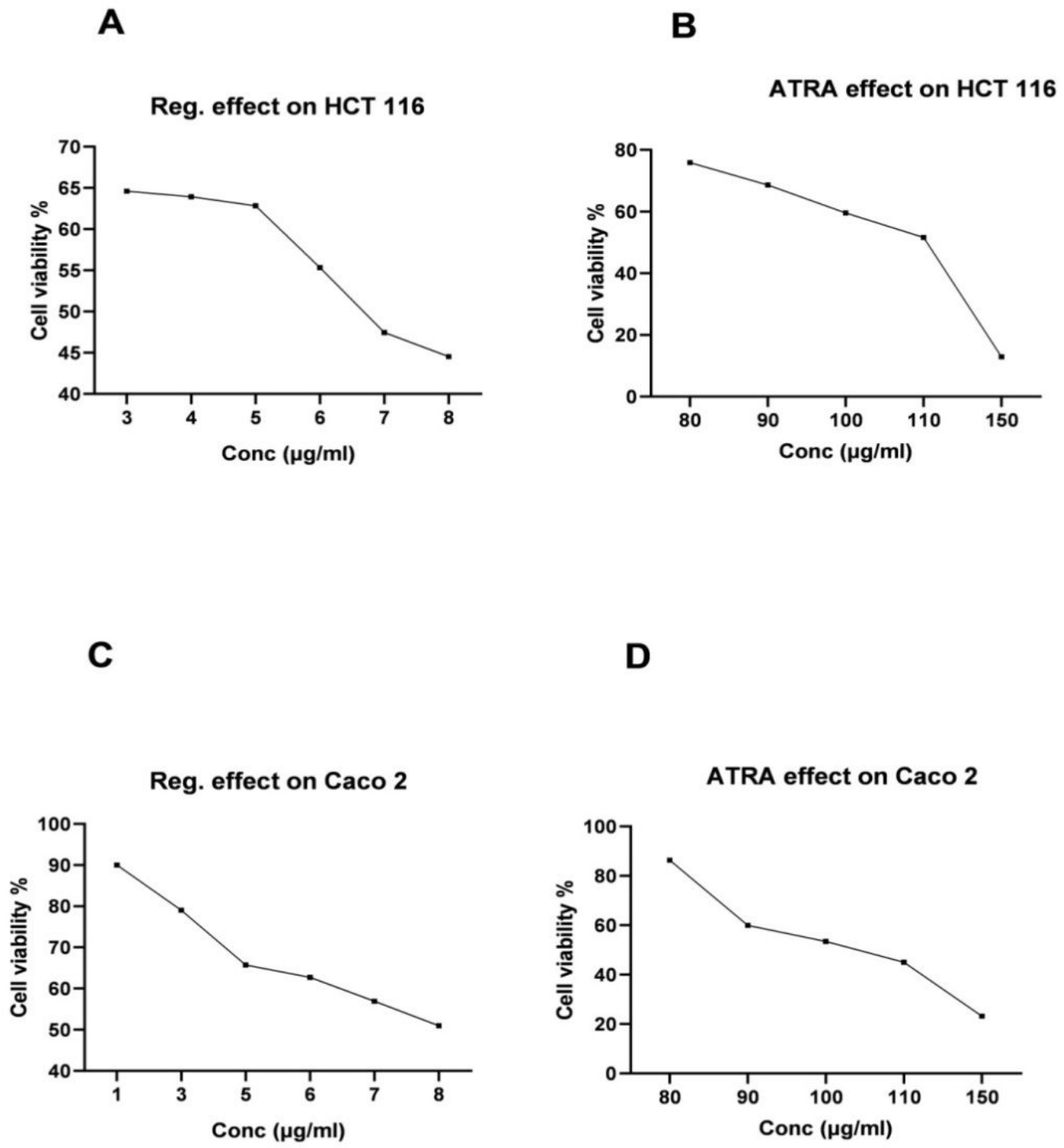

Figure 1

Cell viability assays of CRC colorectal carcinoma cells treated with regorafenib and ATRA. Cells were treated with regorafenib or ATRA alone at the indicated concentrations. Cell viabilities were determined by MTT assay and expressed as percentages of those of control (DMSO treatment). (A) HCT 116 cells were treated with various concentrations of regorafenib alone for 48 h. (B) HCT 116 cells were treated with various concentrations of ATRA alone for 48 h. (C) Caco2 cells were treated with various concentrations

of regorafenib alone for 48 h. (D) Caco2 cells were treated with various concentrations of ATRA alone for 48 h. Experiments were run in triplicate and carried out at least two times on separate occasions. ($p < 0.05$ vs. untreated control)in HCT 116 cells. ($p < 0.0001$ vs. untreated control)in Caco cells.

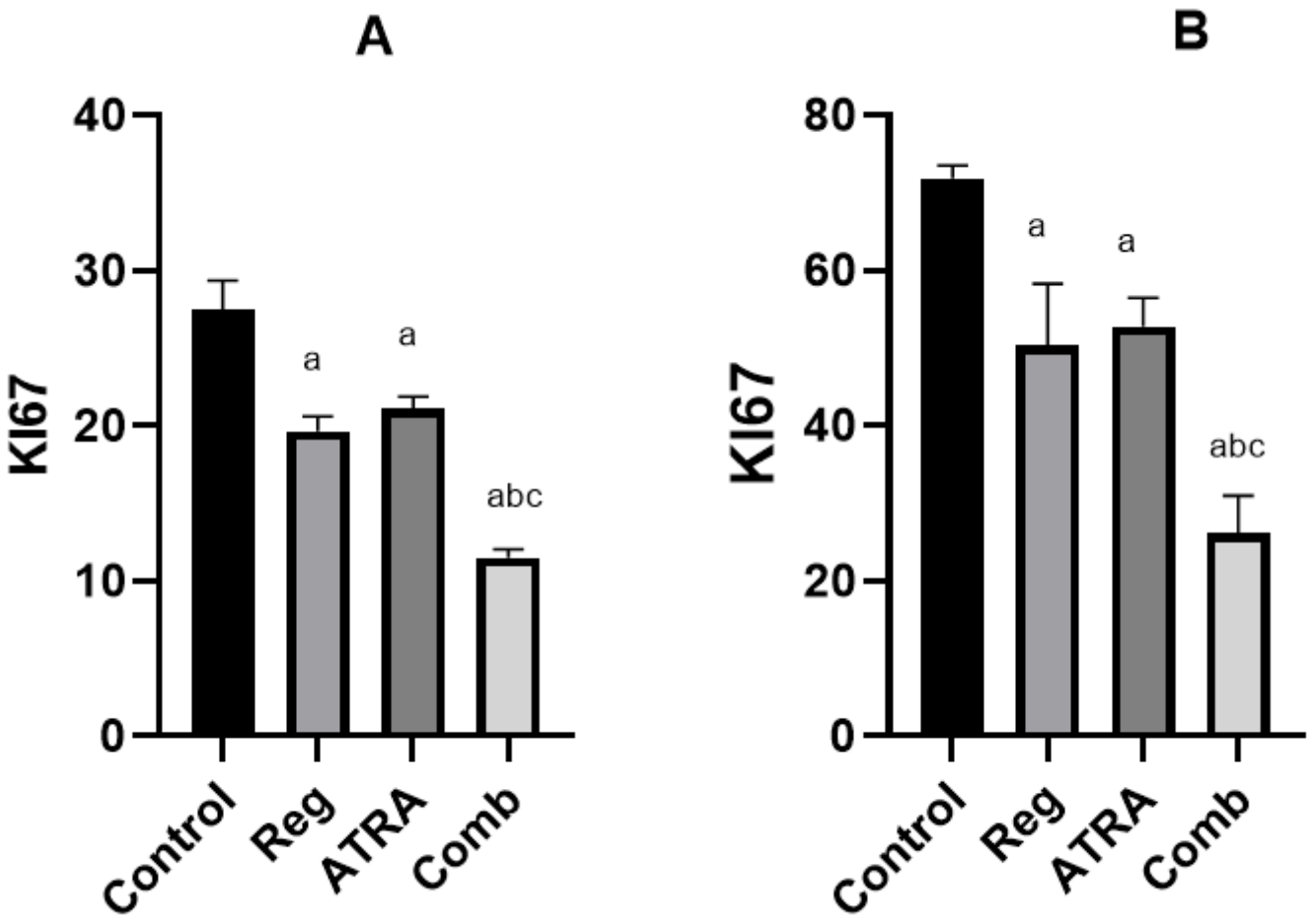

Figure 2

Effect of regorafenib and/or ATRA on KI67 level in colorectal cancer cell lines.(A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib-treated group (Reg). c, Significant in comparison with IC_{50} ATRA-treated group ($p < 0.001$). Reg, regorafenib; comb, combination of sub-IC_{50} of regorafenib and ATRA

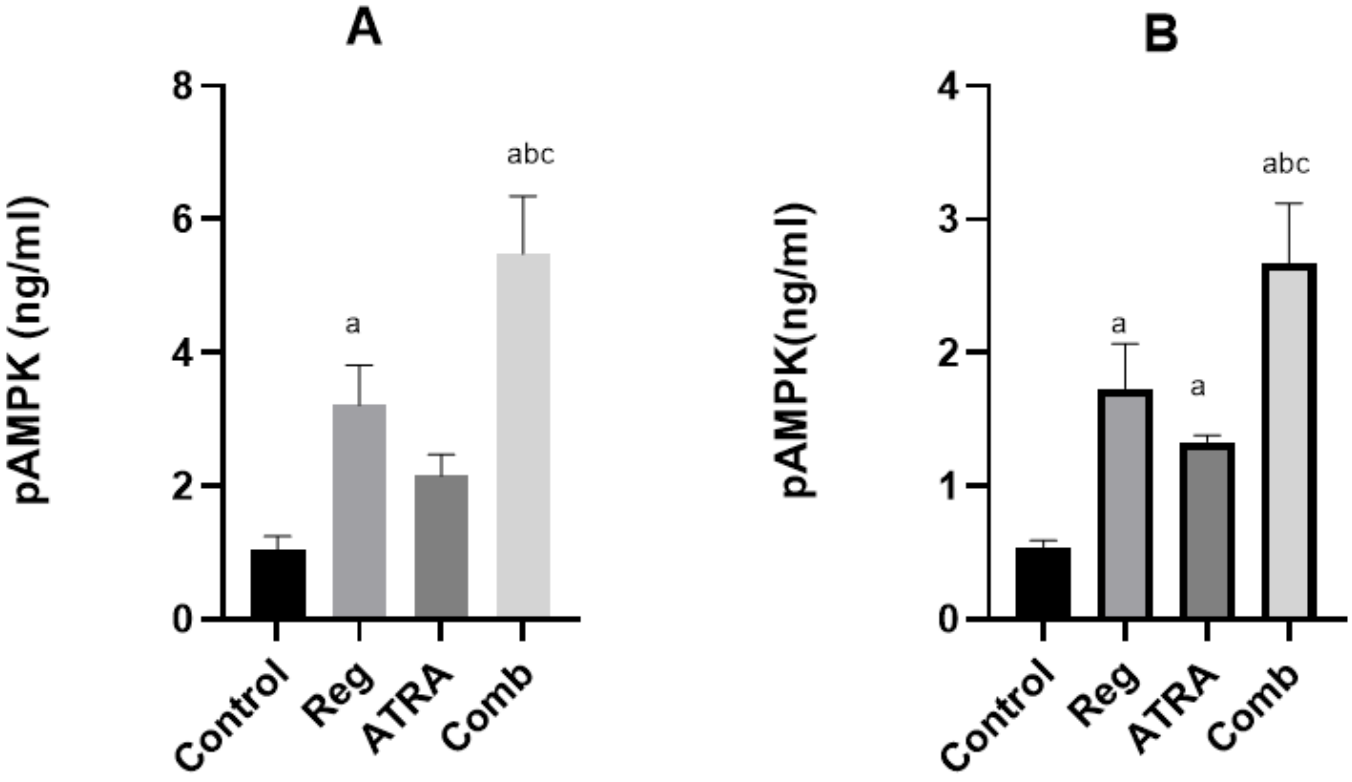

Figure 3

Effect of regorafenib and/or ATRA on pAMPK level in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0.a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib-treated group (Reg). c, Significant in comparison with IC_{50} ATRA-treated group (p< 0.001). Reg, regorafenib; comb, combination of sub-IC_{50} of regorafenib and ATRA.

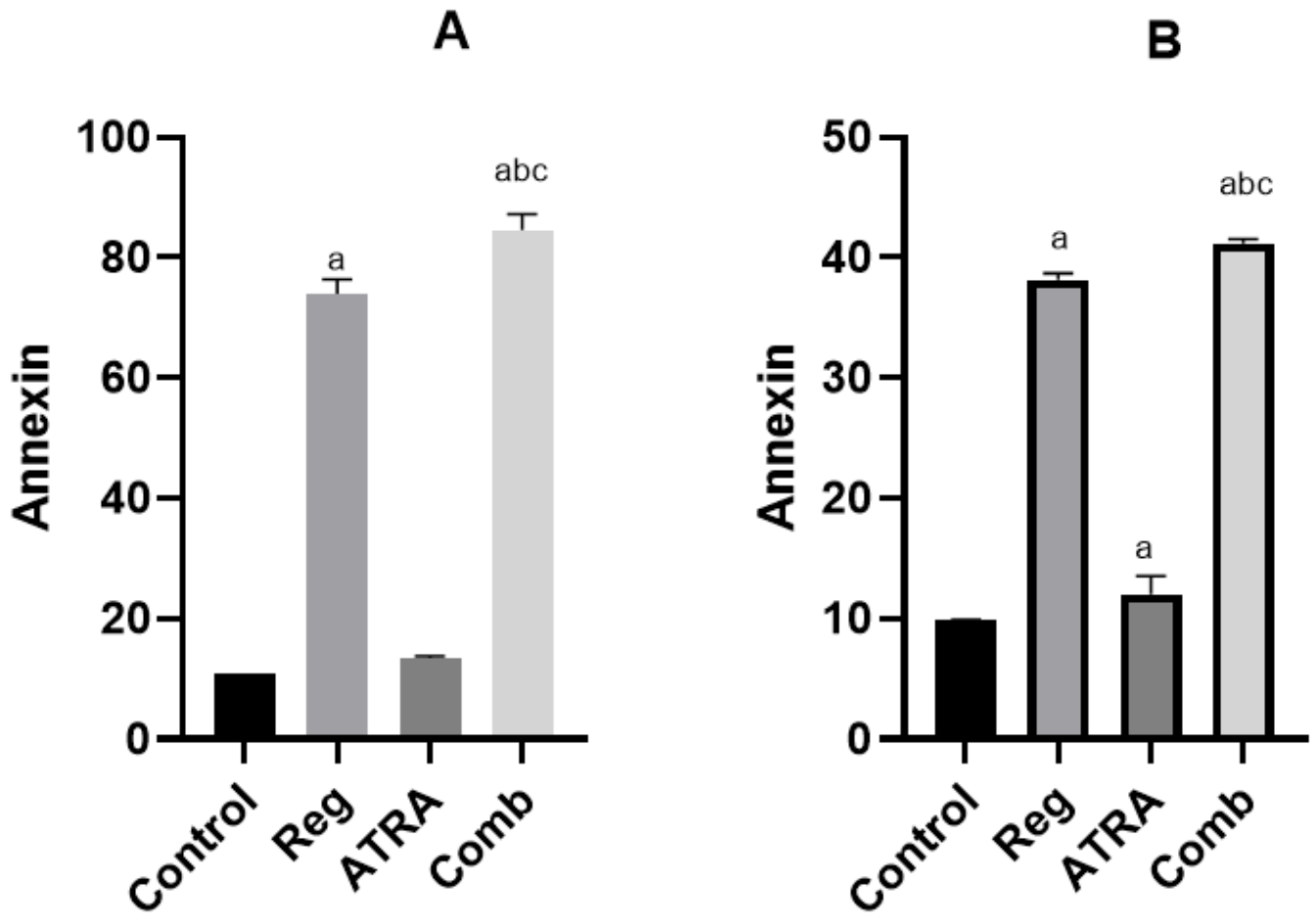

Figure 4

Effect of regorafenib and/or ATRA on the level of cell apoptosis in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib–treated group (Reg). c, Significant in comparison with IC_{50} ATRA–treated group ($p < 0.001$). Reg, regorafenib; comb, combination of sub-IC_{50} of regorafenib and ATRA.

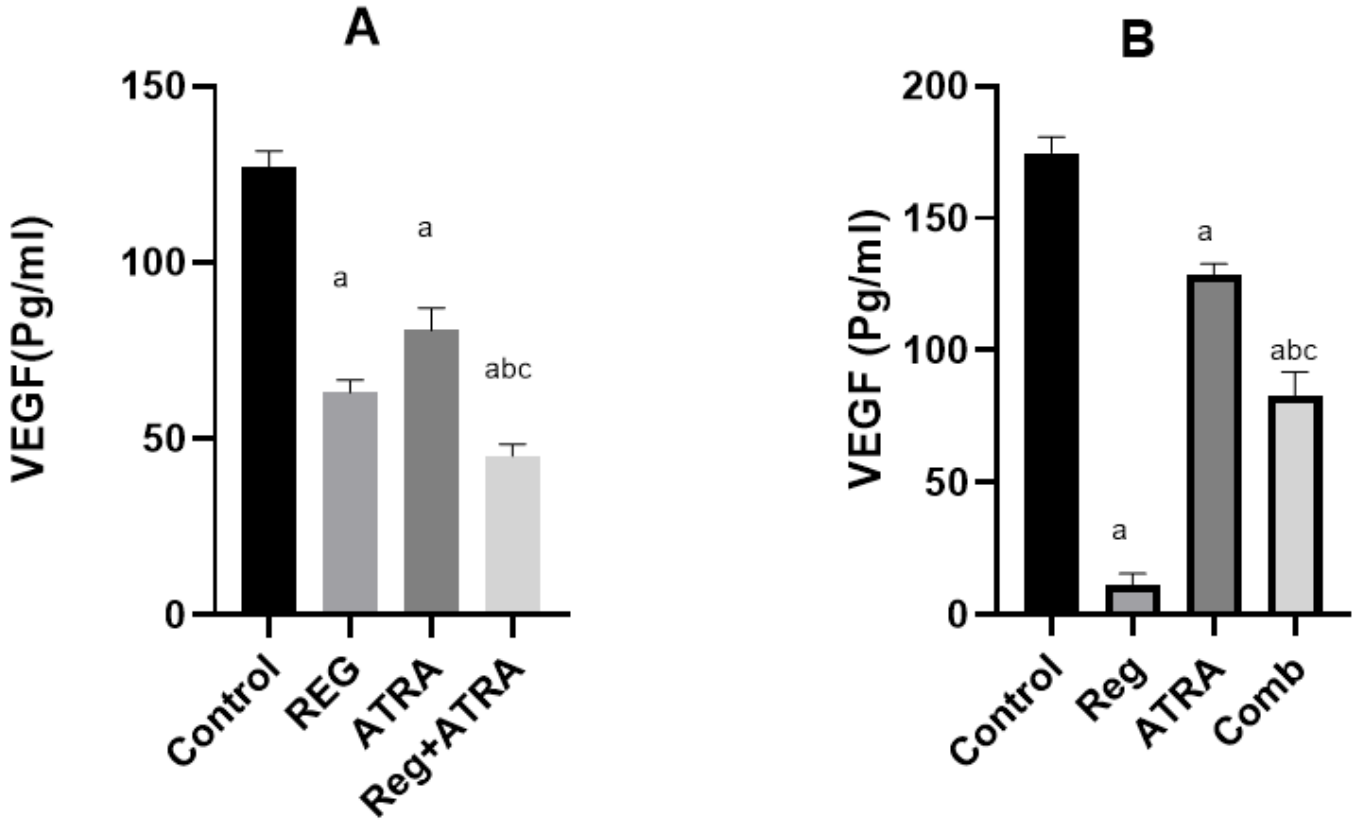

Figure 5

Effect of regorafenib and/or ATRA on VEGF level in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib–treated group (Reg). c, Significant in comparison with IC_{50} ATRA–treated group ($p < 0.001$). Reg, regorafenib; comb, combination of sub-IC_{50} of regorafenib and ATRA.

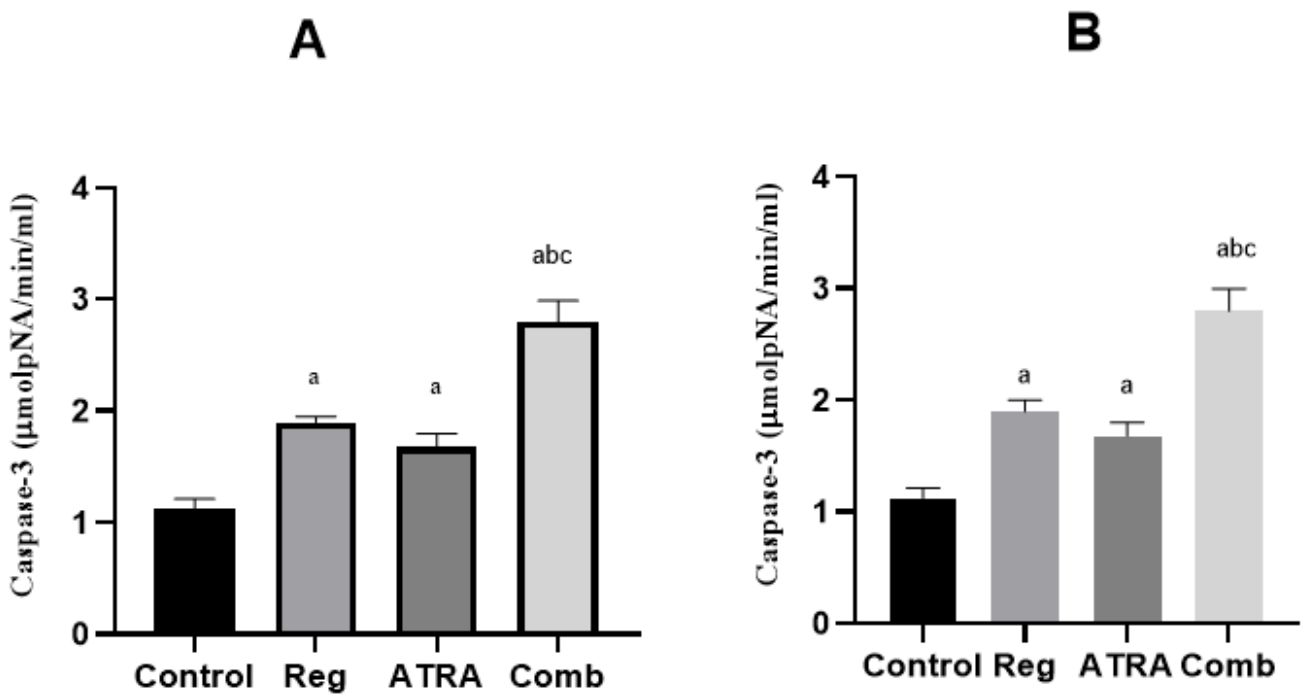

Figure 6

Effect of regorafenib and/or ATRA on caspase-3 activity level in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b,Significant in comparison with IC_{50} regorafenib–treated group (Reg). c, Significant in comparison with IC_{50} ATRA–treated group (p< 0.001). Reg, regorafenib; comb, combination of sub-IC_{50} of regorafenib and ATRA.

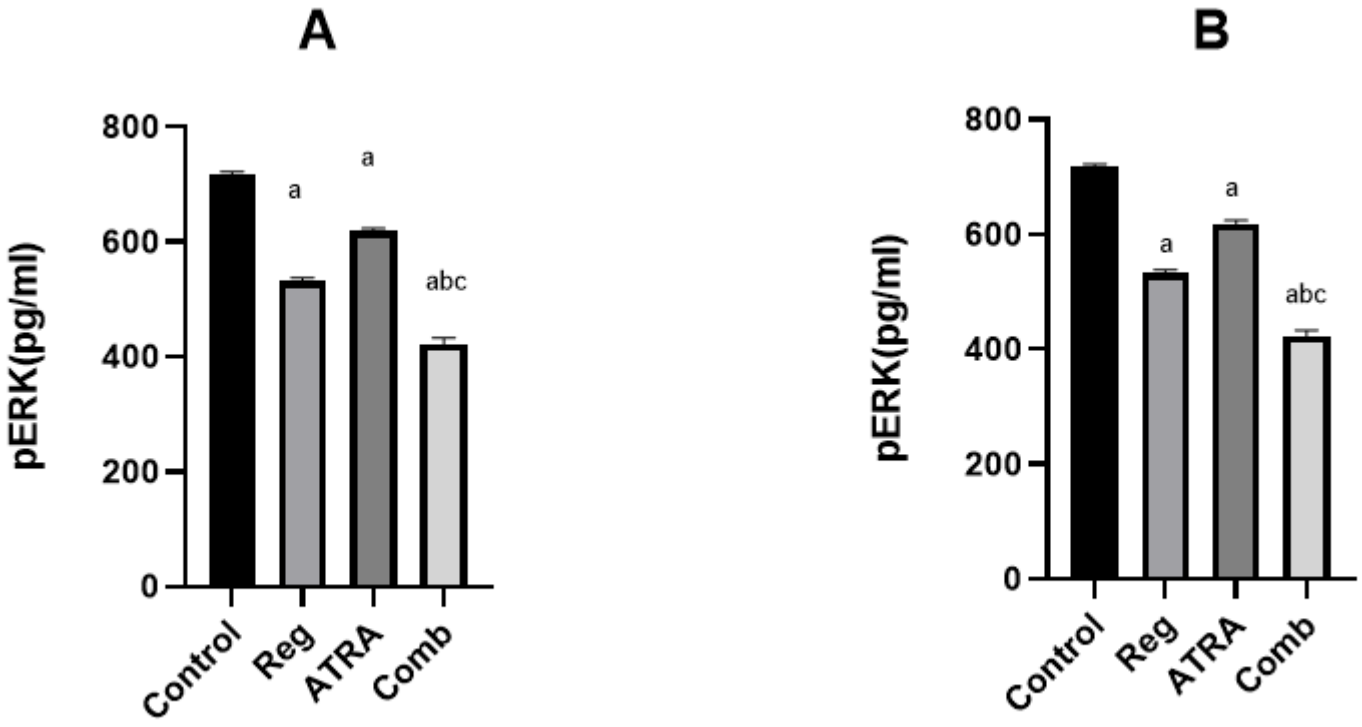

Figure 7

Effect of regorafenib and/or ATRA on pERK level in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib–treated group (Reg). c, Significant in comparison with IC_{50} ATRA–treated group ($p < 0.001$). Reg, regorafenib; Comb, combination of sub-IC_{50} of regorafenib and ATRA.

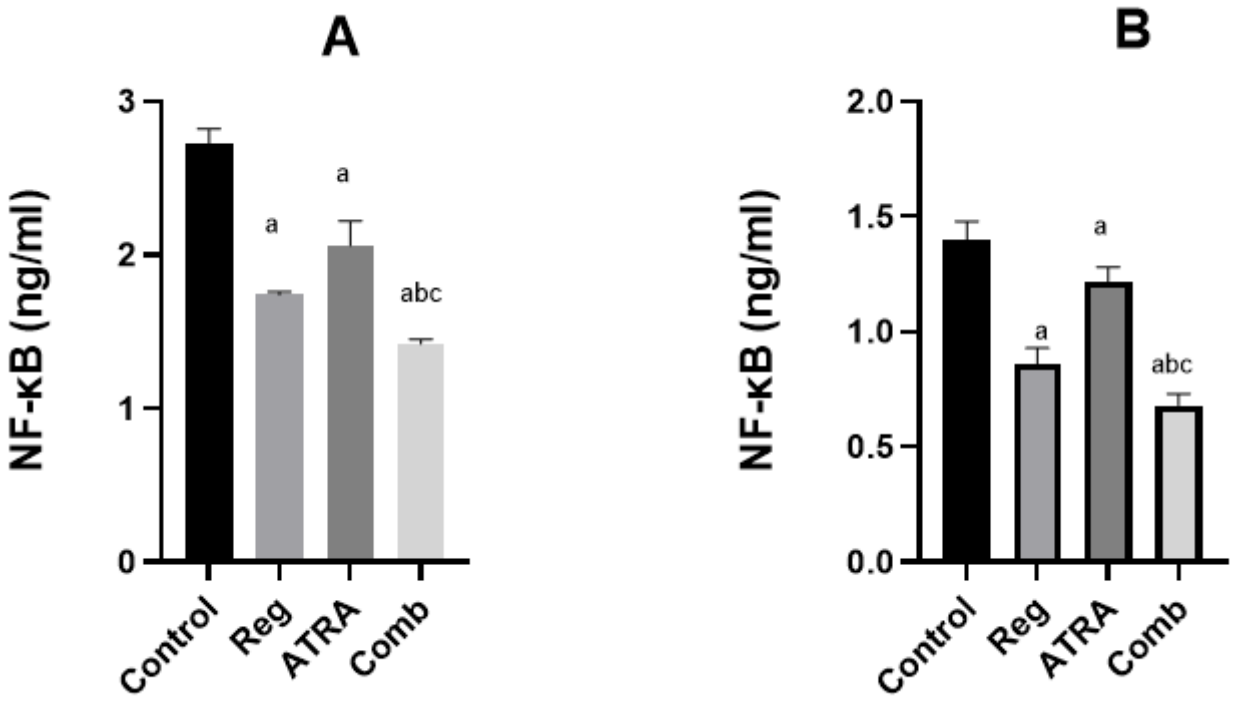

Figure 8

Effect of regorafenib and/or ATRA on NF-κB level in colorectal cancer cell lines. (A) Caco-2 cell line. (B) HCT-116 cell line. All data are expressed as mean ± standard deviation of three separate experiments conducted in triplicate. The statistical analysis was carried out using one-way ANOVA test followed by Tukey's multiple comparisons test using GraphPad Prism software v 6.0. a, Significant in comparison with control. b, Significant in comparison with IC_{50} regorafenib–treated group (Reg). c, Significant in comparison with IC_{50} ATRA–treated group ($p < 0.001$). Reg, regorafenib; Comb, combination of sub-IC_{50} of regorafenib and ATRA.