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Article

AdipoRon, Adiponectin Receptor Agonist, Exhibits Antiproliferative and Apoptotic Effects in HT29 and HCT116 Colon Cancer Cells

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Abstract:

AdipoRon is the first synthetic analog of endogenous adiponectin, which is an adipose tissue derived hormone. As it is characterized with pharmacological properties like adiponectin through binding and activating AdipoR1 and AdipoR2 receptors this makes it a target for therapeutic treatments for a multitude of disorders. In this study AdipoRon effect on HT29 and HCT116 colon cancer cells were investigated. Cytotoxic activity of AdipoRon was observed, with a significant inhibition rate in HT29 and HCT116 cells. Moreover, there was a significant decrease in the number of HCT116 colonies by clonogenicity test. In addition, cell migration assay showed significant decrease in cell migration especially after 24 hours after treatment and more with 24 µM concentration of Adiporon. Immunofluorescence staining showed an increase of apoptotic caspase 3 and decrease of anti-apoptotic Bcl-2 in HCT11 in cells incubated with AdipoRon (6 µM) for 24 hours. Western blotting demonstrated a decrease in cell number with AdipoRon treatment, cyclin D1 was also suppressed. In addition, treatment with AdipoRon increases the phosphorylation of P27 as well as p AKT. In conclusion, our findings provide initial evidence of AdipoRon as an anticancer activity towards colon cancer cells; therefore, encouraging and designing future studies to further understand this pattern is essential.

Keywords: AdipoRon, cytotoxicity, apoptotic, colon cancer cells, antiproliferative

1. Introduction

According to the World Health Organization (WHO), colorectal cancer (CRC) is the third most prevalent cancer globally, constituting 10% of all cancer cases [1]. It is also the second major cause of death associated with cancer [1,2]. This ailment is mostly diagnosed when cancer has reached advanced stages, limiting treatment options significantly. Colon cancer can lead to severe pain [2,3] and complications and the treatment entails surgical intervention, chemotherapy, radiation

therapy, and immunotherapy for resectable CRC; whereas combinational regimens are employed for non-resectable CRC [3,4]. Cancer cells can evade the actions of chemotherapeutics in a variety of ways which signifies the importance of continued research for novel therapeutics [4].

Adiponectin is a 28 kDa protein consisting of 244 amino acids [5]. It is a cytokine released from adipose tissues and plays a vital role in various physiological functions and cellular and molecular events [5,6]. These encompass regulation of energy, insulin sensitivity, inflammation and metabolism of lipids [5]. There is an inverse relation between adiponectin levels and numerous pathologies such as diabetes, cardiovascular disorders, and cancer [5] since adiponectin exhibits anti-inflammatory, pro-apoptotic and anti-proliferative properties. Circulating levels of this cytokine are markedly reduced in obesity which indicates that adiponectin may act as a factor connecting obesity with the risk of cancer [6] including colorectal cancer [7]. AdipoRon (adiponectin receptor agonist) is a synthetic chemical that upregulates the PI3K pathway by stimulating the adiponectin receptor [8,9]. It is a hormone-like molecule which demonstrates anticancer properties and has a significant ability in controlling metabolism and inflammation [8]. The exhibited anticancer activities include those against pancreatic cancer, ovarian cancer, breast cancer, and melanoma [9]. The effects of AdipoRon have been studied by several researchers. Current evidence supports a role of adiponectin as a novel risk factor and potential diagnostic and prognostic biomarker in cancer. Furthermore, drugs that raise adiponectin levels or up-regulate signaling pathways downstream of adiponectin, or adiponectin itself, may be effective treatments for cancer. AdipoR1 and AdipoR2, the two primary receptors for adiponectin, have been shown to express themselves in a variety of cancer cell types both in vitro and in vivo [10,11].

A study provided evidence that AdipoRon raises the body's adiponectin levels and that it has the potential to limit restenosis after angioplasty by significant inhibition of platelet-derived growth factor (PDGF), induced vascular smooth muscle cell (VSMC) proliferation [10]. Additionally, some other studies indicated that AdipoRon may reduce inflammation [10, 11]. Since obesity is a major risk factor for colorectal cancer [12], potentially due to reduced adiponectin levels [6], this study aimed to investigate whether AdipoRon could suppress some of the hallmarks of cancer, particularly over-proliferation and reduced apoptosis. To our knowledge, no study has examined the effect of AdipoRon in colorectal cancer (CRC). However, a study proposed a hypothetical model in which Adiponectin (Acrp30) and AdipoRon had similar behavior in suppressing CRC cell growth [13]. In the current study, we are the first to report that AdipoRon express an antiproliferative and apoptotic effect in colon cancer cell lines.

2. Materials and methods

2.1.Cell culture

Three cancer cell lines, HT29 (human colorectal adenocarcinoma), HCT116 (human colorectal carcinoma) and MCF7 (human breast adenocarcinoma) and one diploid cell culture line, MRC5 (Normal human foetal lung fibroblast) were used in this study. All cells were obtained from the American Type Culture Collection (ATCC). The three cancer cells were sub-cultured in RPMI-1640 media (10% FBS); while MRC5 was maintained in Eagles minimum essential medium (EMEM, 10% FBS). All were kept in an incubator at 37 °C, 100 % relative humidity and supplied with 5% carbon dioxide.

2.2.Cytotoxicity and Selectivity Studies

As described in previous studies [14, 15], the MTT assay was used to assess the cytotoxicity of the AdipoRon (Medchemexpress, USA). Separately cultivated in 96-well plates (3 ×103/well), the three cell lines and one normal fibroblast were incubated at 37 °C for one night. Doxorubicin and AdipoRon, at concentrations ranging from 0 to 50 μM and diluted in 0.1% DMSO, were added to the respective wells and incubated for 24 hours. The experiment was carried out in six repeated measurements. MTT was then added to each well of the plate. After three hours of incubation, the supernatant was removed from the plates and DMSO was applied. Using a multi-plate reader (BIORAD, PR 4100, Hercules, CA, USA), absorbance was measured. The number of live cells is correlated with the optical density of the purple formazan A550. GraphPad Prism was used to calculate the compound concentration that causes 50% inhibition (IC_{50}) compared to 100% control cell growth. The selectivity index (SI) was calculated by dividing the IC_{50} value

against MRC-5 cells by the IC_{50} value against the corresponding cell line.

2.3.Clonogenic Survival Assay

As per a prior report [16], the clonogenic survival assay was employed to examine the capacity of individual cancer cells to form colonies. Six repeated experiments of HCT116 cells were planted in 6-well plates with 2 mL of medium at a low density (2 × 102). To enable adhesion, plates were incubated for the entire night at 37 °C. AdipoRon, the vehicle control, was applied to the cells at 6, 12, and 24 µM. Each compound's medium was discarded after 24 hours, and 2 millilitres of new media were added. Every two days, plates were examined under a microscope to see if the cell had formed a colony (at least 50 cells) in 14 days. After aspirating the medium, the cells were cleaned with cold PBS (phosphate- buffered saline) and fixed for five minutes in cold methanol at room temperature. Cells were then stained with 0.5% v/v methylene blue in methanol: H_2O (1:1) for 15 min. Colonies were washed with PBS and H_2O. Plates were left to dry before the final counting of colonies.

2.4.Cell migration assay

Using the previously described protocol, 90% confluent HCT116 cells were seeded at 3 × 106 in 2 mL media containing 10% FBS in wells [17]. Overnight, the plates were incubated at 37 °C. Using a sterile point, each well's cells were carefully divided into one horizontal line. AdipoRon was introduced to wells (n=6) after being diluted in a serum-free medium to achieve final concentrations of 6, 12, and 24 µM. At 37 °C, the plates were incubated. We captured pictures of the injured cells in every well at 0, 3, 6, and 24 hours of incubation. In each photograph, the distance in centimeters between the two cell borders formed by the scratch was measured. Distances reported at 0 hours following treatment were regarded as 100%, whereas migratory distances measured in subsequent time points with different doses, were converted to % compared to the 0 h distance. Curves corresponding to the distance of migration % were plotted.

2.5.Immunofluorescence Staining

The inhibition of Bcl-2 and upregulation of caspase-3 was confirmed by immunofluorescence staining. HCT116 cells (5 x 103/chamber) were treated with AdipoRon (6 µM) for 24 h, according to a previous report [18]. EVOS FL microscopy (Thermo Fisher Scientific, Waltham, MA, USA) was used for slide examination. Digital images were taken with a 40x objective.

2.6.Western Blotting

In this study, the identification of Cyclin D1, p27, AKT, and p-AKT level changes was examined. In HCT116 cells, the effects of doxorubicin (0.5 µM) and AdipoRon (6 µM) were examined in response to a prior publication [19]. In short, a 24-hour treatment was administered to HCT116 cells (1 x 106 cells/well in a 6-well plate). Total proteins were separated using the lysis buffer, and their concentration was ascertained using the Bradford method. The proteins were then electrophoresed on a polyacrylamide gel and transferred to a membrane. AKT (# 9272, 1:1000, Cell signalling), p27 (# 3686, 1:1000, Cell signalling), Cyclin D1 antibodies (# 2922, 1:1000, Cell signalling), and p-AKT (# 4060, 1:1000, Cell signalling) were treated with the membranes for two hours at room temperature, followed by secondary antibody GAPDH (# 8884, 1:1000, Cell signalling) for 1 h. Visualization of the immunoreactivity was assessed using horseradish peroxidase (HRP)-conjugated secondary antibodies.

2.7.Statistics

Results from the data were extracted, revised, coded and entered into the statistical software programme SPSS version 22 (IBM, Chicago, IL). All statistical analysis was conducted using two-tailed tests. Descriptive analysis based on frequency and percent distribution was performed for all categorical variables, while means with standard deviations and medians were used to display numerical variables. Statistical differences were assessed by one-way ANOVA with Tukey's post hoc multiple comparison test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****) were taken as significant.

3. Results

3.1.Cytotoxicity and selectivity Studies

We examined the effect of AdipoRon on the proliferation of MCF7, HT29 and HCT116 cell lines we used MTT assay. As shown in (Table 1) the cytotoxic activity of AdipoRon was significantly observed on the cells as the rate of inhibition was obvious in HT29, HCT116 and MCF7 in comparison

Table 1. Cytotoxic activity of AdipoRon and doxorubicin against three cell lines and one normal fibroblast (MTT 24 h, IC_{50}±SD μM) (n=6 repeated experiments).

Compounds	HT29	HCT116	MCF7	MRC5
AdipoRon	11.09±1.04	7.67±1.66	12.94±0.64	38.26±8.60
Doxorubicin	0.24±0.04	0.68±0.09	5.21±1.13	0.69±0.11

Table 2. Selectivity of AdipoRon and doxorubicin for the cancer cells compared to normal MRC5 cells (n=6 repeated experiments).

Compounds	Selectivity index [a]		
	HT29	HCT116	MCF7
AdipoRon	3.45	4.98	2.95
Doxorubicin	2.87	1.01	0.13

[a] Selectively index (SI) = IC_{50} value against the normal MRC5 cells divided by the IC_{50} value against the corresponding cancer cell line. The experiment was repeated six times.

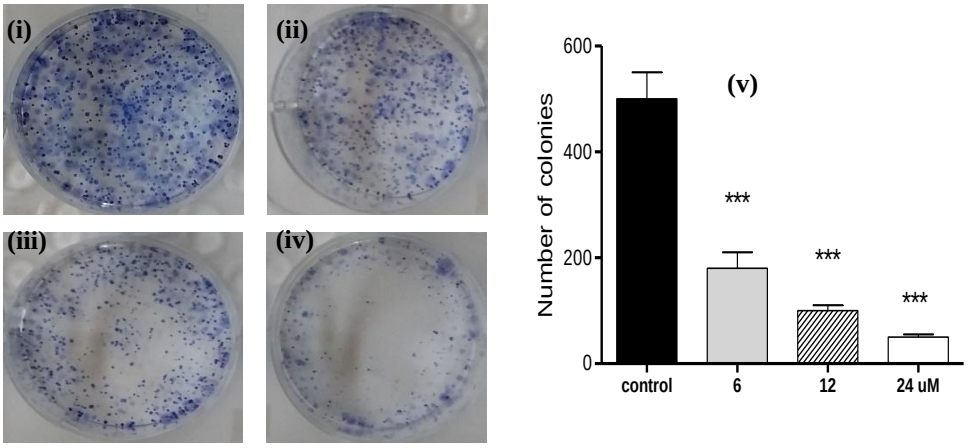

Figure 1. Clonogenicity of HCT116 cells following 24 h treatment in 6 well plate with (i): Vehicle control, (ii) AdipoRon (6 μM), (iii) (12 μM), and (iv) (24 μM). (v): bar chart showing the four treatments (x-axis), against the number of HCT116 colonies (y-axis). Experiments were repeated six times. Statistical differences compared to untreated control cells were assessed by one-way Anova with the Tukey's post-hoc multiple comparison test ($p < 0.001$ (***) was taken as significant).

in (Table 2) AdipoRon showed a SI of 3.45 which was near to 2.87 of the control doxorubicin explaining that AdipRon has a selective cytotoxic effect towards theses cell lines.

3.2. Clonogenicity of AdipoRon

The clonogenicity of Adiporon was tested using HCT116 cells after 24 hours of treatment. As showed in (Figure1). We used three concentrations of AdipoRon, 6 μM, 12 μM, and 24 μM. All these concentrations demonstrated significant decrease in the number of HCT116 colonies in comparison with the control.

3.3.Cell migration assay of Adiporon

We investigated the Anti-migration activity of AdipoRon against HCT116 cells for 0, 12, 24 h after treatment and with the three concentrations 6, 12 and 24 μM. (Figure 2) demonstrated a significant decrease in cell migration especially after 24 hours after treatment and more with 24 μM concentration of AdipoRon.

3.4.Immunofluorescence Staining

In order to investigate the antiprolofretaive effect of AdipoRon towards the colonc cancer cell line we used immunofluoresnce staining. As shwon in (Figure 3) there was an increase of apoptotic caspase 3 and decrease of anti-apoptotic Bcl-2 in HCT11 cells incubated with AdipoRon (6 μM) for 24 h.

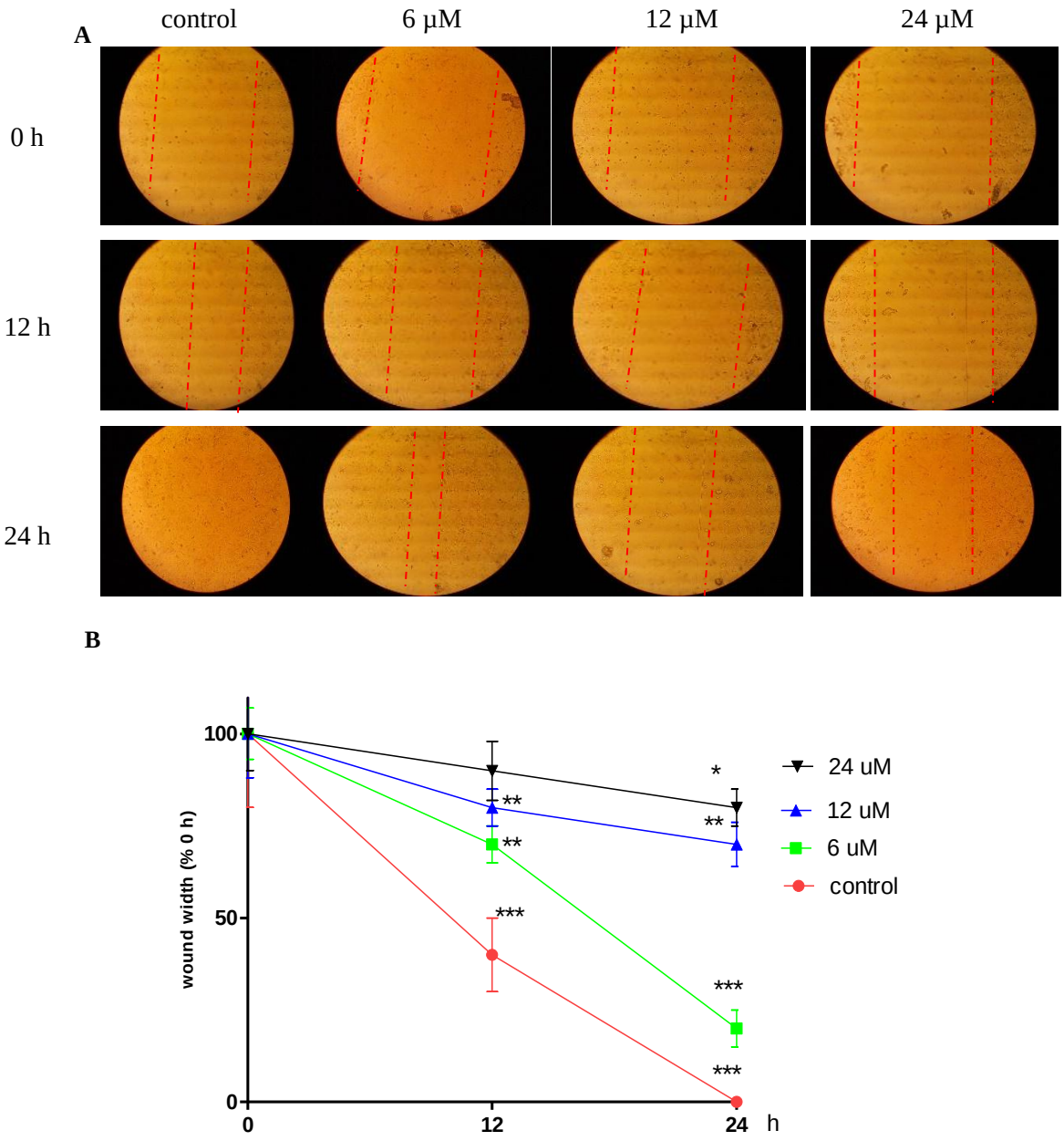

Figure 2. Anti-migration activity of AdipoRon against HCT116 cells. A: rows (0, 12, 24 h), columns (6, 12 and 24 µM), dashed lines show wound widths. B: curve showing the effect of different doses of Adiporon at different times against HCT116, x axis: h, y axis: wound width (% 0 h). Data shown is mean % inhibition ± SD (n=6). Experiment was repeated 6x. Level of significance compared to control (post hoc using Tukey): *p<0.100, **p < 0.010, ***p < 0.001.

3.5.Western blotting

To determine the effect of AdipoRon on celular signialling. We examined the level change of Cyclin D1, p27, AKT and p-AKT in HCT116 cells following their treatment with AdipoRon (6 µM) and doxorubicin (0.5 µM) for 24 hrs. Figure 4 demonstrated a decrease in cell number with AdipoRon treatment, cyclin D1 was also supressed, in addition treatment with AdipoRon increased the phosphorylation of P27 as well as p AKT.

4. Discussion

Over the last few years, several studies have shown that AdipoRon exhibits multiple beneficial effects such as anti-diabetic, anti-obesity, anti-inflammatory, anti-hypertrophic, anti-depressant and anti-ischemic [11,19-21].

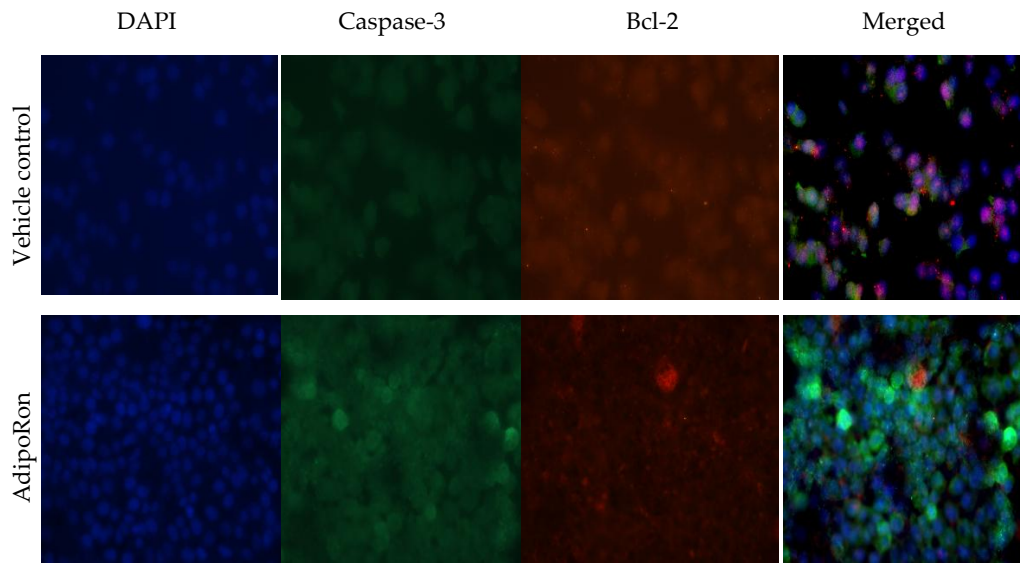

Figure 3. Detection of the immunofluorescence in HCT116 (Bcl-2: green, caspase-3: red, stained with DAPI, 15 μm; 40× objective). I: vehicle control, II: cells incubated with AdipoRon (6 μM) for 24 h.

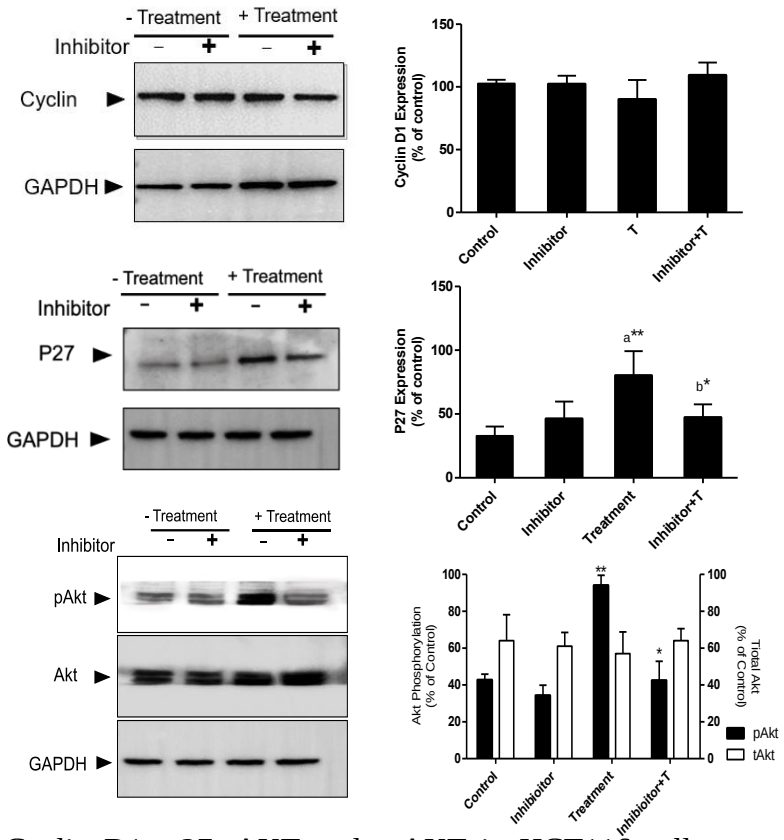

Figure 4. Expression of Cyclin D1, p27, AKT and p-AKT in HCT116 cells treated with with AdipoRon (Treatment: 6 μM) for 24 hrs. Inhibitor: Doxorubicin (0.5 μM). A: Data is represented as mean ±SD (n=6, six independent experiments). Statistical differences, compared to untreated control cells, were assessed by one-way Anova with the Tukey's post-hoc multiple comparison test. P<0.100 (*), P<0.010 (**), and P<0.001 (***) were taken as significant. B: immunoblots of Cyclin D1, p27, AKT and p-AKT and GAPDH. Image J software was used for densitometry.

Additionally, it improves post-traumatic stress disorder, anxiety and systemic sclerosis [22-24]. Interestingly, different studies have demonstrated the ability of AdipoRon as an anticancer molecule using different cancer models such as pancreatic ductal adenocarcinoma (PDAC), myeloma, breast and ovarian cancer via different mechanisms including apoptosis induction, G0/G1 blockage or autophagy [25-28]. In the cell cycle, a G0/G1 phase delay or block has been reported to play an essential mechanism by which AdipoRon causes growth arrest in different cancer models [26-31]. Thus, we conducted in vitro studies using HCT116 colon cancer cells to show the antiproliferative effect of 24 h treatment with AdipoRon (6, 12 and 24 µM). This was confirmed with Cyclin D1 as well as p27. In addition, a reduction in cell migration was observed upon AdipoRon treatment. These findings confirm that AdipoRon reduces cell proliferation rate in colorectal cancer as reported with Adiponectin (Acrp30) [31]. Two extensively expressed receptors, AdipoR1 and AdipoR2, which share structural similarities in both sequence (66.7% homology) and binding site (86%), are used by Acrp30 to perform its pleiotropic functions [32-35]. Recent AdipoR1 and AdipoR2 crystallography has shown notable structural differences, defining a distinct class of receptor structure that was previously categorized as G-protein-couplet receptors (GPCRs) [36]. Essentially, the seven-transmembrane helices create a central cavity where three different histidine residues join a zinc ion to produce a structure that may be crucial for both UCP2 overexpression and AMPK activation triggered by adiponectin. Furthermore, the extracellular layer continues to be the most plausible candidate for ligand–receptor interaction even if neither co-crystallization nor the localization and/or characterization of binding sites have been carried out as of yet [37]. In addition to metabolic-related illnesses, the involvement of Acrp30 in cancer has been extensively studied in a significant number of publications [38]. Accordingly, it has been documented that dysregulation of Acrp30 levels is adversely correlated with the incidence of breast cancer in both premenopausal and postmenopausal women [38-39]. Low serum concentrations of Acrp30 have been associated with tumor-stage and poor prognosis in colorectal cancer in addition to being associated with an increased risk of developing this particular tumor type [39-40]. Similar to this, numerous other malignancies, such as gastric, endometrial, pancreatic, and renal cell carcinoma, have shown a strong inverse connection [41].

Instead, conflicting findings already coexist in lung cancer. Having said that, although some research shows no connection at all between Acrp30 levels and lung cancer, others provide compelling evidence that hypoadiponectinemia is a clinical indicator of lung cancer progression [40- 42]. In this regard, Nigro and colleagues recently revealed that non-small-cell lung cancer (NSCLC) patients had significantly lower levels of total adiponectin than healthy people, which had a significant impact on the high molecular weight [43]. Additionally, they found that those patients had lower levels of T-cadherin and increased expression of AdipoR1. Similarly, Abooshahab et al. discovered no changes in Acrp30 levels between thyroid cancer patients and cancer-free controls, despite multiple epidemiologic studies reporting that obesity and low circulating Acrp30 levels are positively related with thyroid cancer occurrence [43].

On the cell signaling level, activation of PI3K/AKT signaling leads to reduced apoptosis, stimulates cell growth and increases proliferation. Overstimulation of the PI3K/AKT pathway may promote malignant growth. In our study we used doxorubicin as a control inhibition to compare its results to the AdipoRon effect on cancer cells. Here, we found that AdipoRon diminishes AKT phosphorylation after 24 h treatment with different concentrations of AdipoRon (6, 12 and 24 24 µM). Examination of apoptotic markers, caspase 3 and anti-apoptotic marker, Bcl-2 was reduced upon exposure to the lowest AdipoRon concentration (6 µM). These findings coincide with a previous study showing that CRC cell lines treated with adiponectin showed diminished PI3K/AKT phosphorylation [44]. Another study reported that Adiponectin inhibited colorectal adenoma growth [45]. Several studies have reported that adiponectin inhibits colorectal cancer cell growth via activation of AMPK through AdipoR1 signalling and thereby 5' AMP-activated protein kinase (AMPK), and suppression of the mammalian target of rapamycin (mTOR) pathway. AdipoRon has been identified as the first orally active AdipoR1 agonist thanks to its ability to turn on AMPK and bind to AdipoR1 and AdipoR2 in C2C12 murine myeloblast cells [14]. Multiple AMPK-dependent and independent pathways have also been reported to be impacted by

AdipoRon.

AdipoR1 and AdipoR2 have been reported to be expressed in several malignancies including human colorectal cancer and their expression are associated with progression of T-stage and differentiation of cancer cells. However, lower levels of AdipoR1 and AdipoR2 expression in poorly differentiated adenocarcinomas were observed when compared with well or moderately differentiated adenocarcinomas [46]. Whether the expression of receptors R1 and R2 is associated with the survival of patients is still controversial. One study [47] found it's a prognostic factor of colorectal cancer while another study did not [46]. Thus, further study to examine the detailed mechanism of AdipoRon and its receptor in cancer cells is needed. The impact of adipoRon on the growth of colon cancer is controversial. According to certain research, a high-fat diet does not decrease colon carcinogenesis, and an increase in serum adiponectin does not lower the risk of colon cancer [48,49]. However, other studies have shown a negative relationship between the incidence and/or progression of colon cancer and the amount of circulating adiponectin [50-52]. Additionally, under the conditions of a high-fat diet, adiponectin insufficiency stimulates colonic epithelial cell proliferation and encourages the formation of colon polyps [53]. Furthermore, there is a strong correlation between the risk of colon cancer and genetic variations of adiponectin and AdipoR1 [54]. Nonetheless, our observations that AdipoRon inhibits colon cancer cell growth suggest that AdipoRon has the potential to serve as an agent in the treatment of human colon cancer. For our findings to be beneficial clinically, further studies aimed to explore its clinical action and efficacy are yet to be carried out.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization, A.F. and Y.A.; methodology A.A, A.S.A, M.E, R.A.; formal analysis A.A; investigation, M.E, A.S.A,.; resources, A.F and Y.A data curation, A.A.; writing—original draft preparation, A.F, A.S.A A.A and Y.A.; supervision, Y.A; Writing—Review and Editing, Y.A.. All authors have read and agreed to the published version of the manuscript.

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Ethical Approval: by the Biomedical ethics committee of Umm Alqura University, Faculty of medicine, Approval number :(HAPO-02-K-012-2020-10-457).

Abbreviation list:

WHO	World Health Organization
CRC	colorectal cancer
AdipoRon	adiponectin receptor agonist
PDGF	platelet-derived growth factor
VSMC	vascular smooth muscle cell
HT29	human colorectal adenocarcinoma
HCT116	human colorectal carcinoma
MCF7	human breast adenocarcinoma
MRC5	Normal human foetal lung fibroblast
ATCC	American Type Culture Collection
SI	Selectivity index
PDAC	pancreatic ductal adenocarcinoma
AMPK	5' AMP-activated protein kinase

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Data Availability Statement

De-identified data available upon request to the corresponding author.