https://doi.org/10.31925/farmacia.2023.1.7 **ORIGINAL ARTICLE**

IN VITRO ASSESSMENT OF THE POTENTIAL CYTOTOXIC EFFECT OF METFORMIN ON COLORECTAL CANCER CELLS

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Abstract

Metformin (Met), a biguanide molecule, is successfully used in the treatment of type 2 diabetes as the first line of therapy for many years now. Beside its antidiabetic effect exerted through the inhibition of gluconeogenesis in liver and increase of glucose uptake in peripheral tissues, it seems to possess an anticancer potential, demonstrated in breast, lung, hepatic and colorectal cancers. Colorectal cancer is responsible for 950 000 deaths just in 2020 and is considered to be one of the most prevalent types of cancer. The aim of the current study was to evaluate through a series of *in vitro* tests the cytotoxicity of Met in HT-29 and HCT- 116, two colorectal cancer cell lines and in a healthy colon cell line, CCD- 841 CoN. All three lines were stimulated with five concentrations of Met (5, 10, 25, 50, 75 mM) for 72 hours. Cellular viability was determined, followed by microscopical morphology analysis and immunofluorescence staining. Met has shown no cytotoxic effect towards CCD 841 CoN cells, whereas in both cancer cell lines showed a concentration-dependent decrease of viability. The morphology of the cancer cells was also changed directly proportional with the concentrations used, rounded, floating cells and decrease in confluency being observed. Moreover, Dapi staining was performed in order to spot changes at a nuclear level. After fixation and permeabilization of the cells, Dapi was added and nuclei shrinkage, intense fluorescence, condensation and fragmentation were seen. Therefore, Met exerts a selective cytotoxic effect in colon cancer cells through inducing apoptotic-like alterations.

Rezumat

Metformin (Met), moleculă din clasa biguanidelor, este utilizat cu succes de mulți ani ca terapie de primă linie în tratamentul diabetului de tip 2. Pe lângă efectul său antidiabetic exercitat prin inhibarea gluconeogenezei în ficat și creșterea captării glucozei în țesuturile periferice, pare să posede un potențial anticancerigen, demonstrat în cancerele de sân, pulmonar, hepatic și colorectal. Cancerul colorectal este considerat responsabil pentru 950 000 de decese doar în 2020 și este considerat a fi unul dintre cele mai răspândite tipuri de cancer. Scopul studiului actual a fost de a evalua printr-o serie de teste *in vitro* citotoxicitatea Met în HT-29 și HCT 116, două linii celulare de cancer colorectal și într-o linie celulară de colon sănătoasă, CCD 841 CoN. Toate cele trei linii au fost stimulate cu cinci concentrații de Met (5, 10, 25, 50, 75 mM) timp de 72 de ore. Viabilitatea celulară a fost determinată, urmată de analiza morfologică microscopică și colorarea prin imunofluorescență. Met nu a arătat niciun efect citotoxic asupra celulelor CCD 841 CoN, în timp ce în ambele linii de celule canceroase a arătat o scădere a viabilității dependentă de concentrație. Morfologia celulelor canceroase a fost, de asemenea, modificată direct proporțional cu concentrațiile utilizate, observându-se celule rotunjite, plutitoare și scăderea confluenței. Mai mult, colorația Dapi a fost realizată pentru a identifica modificările la nivel nuclear. După fixarea și permeabilizarea celulelor, s-a adăugat Dapi și s-a putut observa contracția nucleilor, fluorescența intensă, condensarea și fragmentarea nucleilor. Prin urmare, Met exercită un efect citotoxic selectiv în celulele canceroase de colon prin inducerea unor modificări de tip apoptotic.

Keywords: metformin, colorectal cancer, viability, morphology

Introduction

Metformin (Met), part of the biguanide class, is the first line of treatment for type 2 diabetes (T2D), its first use being recorded in the late 1950s. Today, 150 million people use this molecule for controlling their glucose levels, due to its affordability and long-term safety, Met being on the "essential medicine" list of World Health Organization [14, 23]. Met exerts its anti-diabetic activity through the activation of adenosine monophosphate activated protein kinase (AMPK), this stimulation being followed by synthesis of fatty acids, inhibition of gluconeogenesis in the liver, and by an increased skeletal muscle glucose uptake [18, 19]. Met was shown to possess more properties than just anti-diabetic ones. Studies have shown the beneficial effect Met has in case of patients with obesity, liver, cardiovascular, age-linked diseases and kidney fibrosis [15]. In addition, metformin appears to have an anti-tumour effect, which attracted even greater attention. In this regard, Met proved effective in preventing different types of cancer, including liver, lung, and colorectal cancers [26]. Also, Met reduces the risk of cancer apparition in diabetics patients, several risk factors being common in both diseases: obesity, age, sex, smoking [19]. Although the exact mechanism of the anticancer effect is not clearly elucidated, a number of mechanisms were considered. It is known that cancer cells need high quantities of glucose in order to proliferate, their metabolism being altered from normal cells. Met may inhibit the tumour growth through its glucose level lowering effect. Also, over-expression of insulin growth factors is a common feature in cancer. Met acts on hyperinsulinemia, inhibiting thus the progression of cancer [4]. Regulating epigenetic modifications, modulating NF-κB signalling pathway, inducing autophagy and suppressing angiogenesis are also thought to be ways through which Met exerts its antitumor effect [26]. *In vitro* and *in vivo* studies have uncovered the potential anti-tumour effects of metformin, and the drug is now being tested in a variety of clinical trials [4].

Colorectal cancer (CRC) has one of the highest incidences among cancer, occupying the third place worldwide. With 950,000 deaths estimated in 2020 by the GLOBOCAN study, CRC ranks second regarding cancer mortality [22]. Between the risk factors for the apparition of CRC are included not only genetic factors, but diet, obesity, alcohol and smoking [10]. The conventional treatment methods include surgery, chemotherapy and radiotherapy, while several molecules are considered potential chemopreventive agents [9, 13]. Met is considered to be a candidate in CRC chemoprevention, inhibiting though its complex mechanism of action CRC's development and progression [15]. There is evidence suggesting that T2D patients treated with Met have a decreased risk of developing CRC, while the T2D patients who have undergone surgery for CRC have an increased overall survival rate in comparison with diabetic patients who have not been treated with Met [6].

In light of these premises, the current study aimed to evaluate the cytotoxic potential of Metformin. Consequently, two different types of colon cancer cells as well as a healthy colon cell line were evaluated for cell viability. To further understand Met's biological effects, both cell morphology and its impact on nuclei were examined.

Materials and Methods

Reagents

Analytically pure metformin hydrochloride, trypsin-EDTA solution, phosphate saline buffer (PBS), di-methyl sulfoxide (DMSO), foetal calf serum (FCS), penicillin/streptomycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] were acquired from Sigma Aldrich, Merck KgaA (Darmstadt, Germany). Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003™) and McCoy's 5a Medium Modified (ATCC® 30-2007™) were purchased from ATCC (American Type Culture Collection).

Cell culture

The normal colon cell line CCD 841 CoN (CRL-1790™) and the cancer cell lines HCT 116 (CCL-247™) and HT-29 (HTB-38™) were acquired from ATCC. CCD 841 CoN cells were cultured in EMEM supplemented with 10% FCS and 1% penicillin (100 U/mL) - streptomycin (100 μg/mL) mixture, while HCT 116 and HT-29 cells were cultured in McCoy's 5a Medium Modified supplemented with 10% FCS and 1% penicillin (100 U/mL)-streptomycin (100 μg/mL) mixture. All cell lines were maintained in standard conditions in an incubator, at 37°C and 5% CO_2.

Cellular Viability Evaluation

In order to determine CCD 841 CoN, HCT 116 and HT-29 cellular viability, MTT assay was conducted at 72 h following Met treatment, according to the following protocol [2, 3]. In brief, 96-well plates were seeded with 1×10^4/200 μL cells/well and after reaching 90% confluence, Met test solutions were added. A stock solution of Met was prepared by dissolving it in water for injection, followed by the preparation of five different concentrations in culture media: 5, 10, 25, 50, 75 mM. After 72 h, test solutions were replaced with 100 μL fresh culture medium, step followed by the addition of 10 μL MTT reagent. The plates were left in an incubator at 37°C for 3 h. After the incubation time, 100 μL Solubilization Solution was added and the plates were left at room temperature, protected from light. Absorbance was read at 570 and 630 nm with the Cytation 5 instrument (BioTek Instruments Inc., Winooski, VT, United States). The second wavelength was used in case of background signals. Data obtained

was expressed as percentage (%) of viable cells normalized to control cells.

Cellular Morphology

Microscopic analysis was performed in order to spot the impact Met had on the normal colon cell line CCD 841 CoN and on the colorectal cancer cell lines, HCT 116 and HT-29. After the same time interval, the cells were examined under bright field illumination with the Cytation 1 (BioTek Instruments Inc., Winooski, VT, United States). Gen5™ Microplate Data Collection and Analysis Software (BioTek Instruments Inc., Winooski, VT, United States) was used for processing the images taken.

Immunofluorescence staining

Cells were subjected to immunostaining in order to gain new information about the mechanism through which Met exerts its effect. For this matter, Dapi was used to visualize the cell nuclei [1]. The analysis was conducted in a similar manner as described before [17]. CCD 841 CoN, HCT 116 and HT-29 cells were seeded at a density of 1×10^5 cells/well in 12-well plates and after reaching 90% confluence, they were treated with 5 and 75 mM Met for 72 h. After the desired treatment time, the cells were washed with cold PBS and fixed with paraformaldehyde 4%. After being left 30 min at 4°C, they were washed again with PBS. The permeabilization was realized with a solution of TritonX 2%, the plate was left 30 min at room temperature, after which TritonX 0.01% was used for washing the cells before adding the blocking solution. With the blocking solution, cells were left at 4°C for 30 minutes and afterwards they were washed again with TritonX 0.01%. The final steps consisted of adding 300 µL/well of Dapi stain, keeping the plate for 15 min at 4°C. After washing the cells with PBS, images were captured with Olympus IX73 inverted microscope (Olympus, Tokyo, Japan) and analysed with cellSens Dimensions v.17 Software (Olympus, Tokyo, Japan).

Statistical analysis

Results are expressed as means ± SD (standard deviation), the one-way ANOVA test, followed by Dunett`s multiple comparison post-test being applied. The software used the statistical analysis was Graph-Pad Prism version 9.4.0 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The statistically significant differences between data are marked with * (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

Results and Discussion

Met is an antidiabetic molecule used successfully in the treatment of T2D, its glucose lowering effect being due to inhibiting the glucose production in the liver and due to increasing peripheral glucose uptake [26]. Being in therapy for over 50 years, it is considered a safe molecule which has many other properties beside the antihyperglycemic one [5]. Particularly, the effect Met has on different cancers has drawn attention. Cancer cells, through what is known as the Warburg effect, need high quantities of glucose for the growth and proliferation of the cells. Met has the ability to reduce the available glucose, depleting cancerous cells of something otherwise vital [20]. Also, there seems to be a link between patients with diabetes and cancer development, observational studies showed a decrease in the survival rate of patients diagnosed with cancer and diabetes [16]. One of the most prevalent types of cancer is colorectal cancer, its incidence being continuously growing [10]. CRC is a complex disease, as well as diabetes, several risk factors being in common in both cases. It is known that high levels of insulin and insulin resistance enhance tumorigenesis. Through its mechanism of action, Met stimulates glucose uptake in peripheral tissues and reduces plasma insulin, improving insulin resistance [11].

With these premises in mind, the current study evaluated Met`s cytotoxic activity in two colorectal cancer cell lines, HCT 116 and HT-29. Also, a healthy colon cell line was used to test Met`s effects, namely CCD 841 CoN. For determining cellular viability in all three cell lines used, MTT assay was performed. After a careful review of the literature, the following concentrations of Met were chosen for testing: 5, 10, 25, 50, 75 mM [21, 25, 27]. In the healthy colon cell line, 72 h treatment with Met showed no cytotoxic effect. The 5mM concentration increased cellular viability to 101%, while the concentrations raging from 10 mM to 75 mM decreased cellular viability in a concentration-dependent manner, but neither of them below 79% (Figure 1).

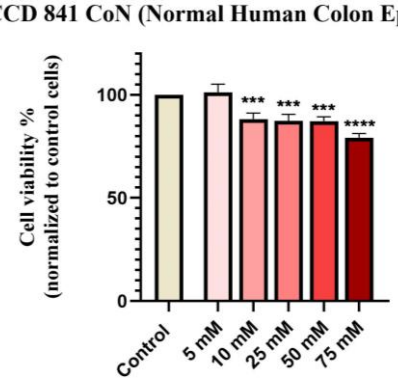

Figure 1.

In vitro assessment of Met's (5, 10, 25, 50 and 75 mM) impact on CCD 841 CoN cells' viability at 72 h post-stimulation by MTT assay

The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. The statistical differences between data are marked with "*" (*** p < 0.001)

The ISO Standard 10993-5:2009 states that a compound exerts a cyto-toxic effect if it causes a decrease in cell viability by more than 30% [8]. Therefore, Met does not appear to exert cytotoxicity towards normal human colon cells.

The colorectal adenocarcinoma cell line, HT-29, showed sensibility to treatment with Met. All test solutions decreased cellular viability in manner directly proportional with the concentrations used, but a cytotoxic effect was seen starting from 25 mM Met, where a viability of 69% was obtained (Figure 2). 75 mM affected cells the most, 56% of cells being left viable after 72 h stimulation.

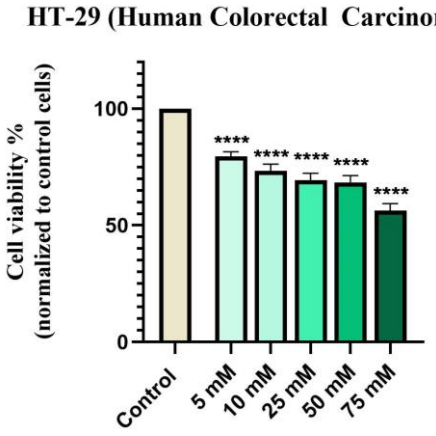

Figure 2.

In vitro assessment of Met's (5, 10, 25, 50 and 75 mM) impact on HT-29 cells' viability at 72 h post-stimulation by MTT assay

The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. The statistical differences between data are marked with "*" (****p < 0.0001)

In case of HCT 116, the colorectal carcinoma cell line, the therapeutic response was stronger than in HT-29 cells. Again, a concentration-dependent loss of viability was observed, Met causing a cytotoxic effect starting from 10 mM (Figure 3). At 75 mM, the viability of the cells dropped to 39%.

For the current study, MTT assay, a colorimetric test which allows the determination of cellular viability by determining the mitochondrial function of the cells, was chosen. The dehydrogenase enzyme from viable cells converts 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide into formazan crystals, which after solubilization, can be quantitatively measured by spectrophotometry at 570 nm [12]. Similar results as those presented in the current study were reported in the literature. In a study conducted by *Zheng* and colleagues, through the MTT assay, 20 mM Met was shown to decrease cellular viability of two colon cancer cells in a time-dependent manner [27]. The same results were obtained by *Yip* and collaborators who tested various concentrations of Met on HT-29, SW480 and SW620 colon cancer cells for 24, 48 and 72 h. Met showed a time and concentration-dependent inhibition of cell proliferation [25].

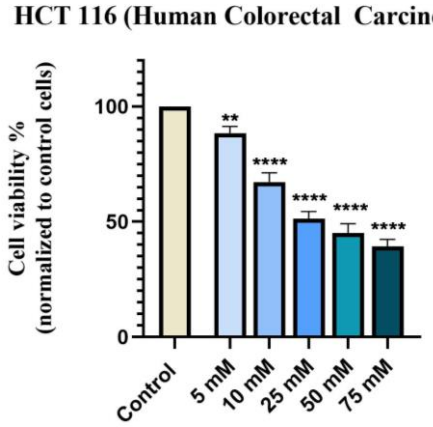

Figure 3.

In vitro assessment of Met's (5, 10, 25, 50 and 75 mM) impact on HCT 116 cells' viability at 72 h post-stimulation by MTT assay

The results are expressed as cell viability percentage (%) normalized to control (unstimulated) cells. The data represent the mean values ± SD of three independent experiments performed in triplicate. The statistical differences between data are marked with "*" (**p < 0.01 and ****p < 0.0001)

Beside cellular viability assessment, the morphology of the cells was evaluated under bright field illumination. CCD CoN cells, after 72 h treatment with various concentrations of Met, showed no alteration to their epithelial-like morphology, as seen in Figure 4.

In line with the viability results, the morphological analysis revealed concentration-dependent changes in HT-29 cells. Confluency reduction, detachment from the plate and rounded cells were seen at each concentration, but they were most visible following treatment with 25, 50 and 75 mM (Figure 5).

In case of HCT 116 cells, the microscopy analysis revealed shrunken and rounded cells. As a sign of severe plate detachment, floating cells were seen, the Met`s effect being manifested in a concentration-dependent manner (Figure 6). Higher concentrations affected confluency of the cells the most.

Similar to our results, *Sena* and colleagues observed plasma membrane detachment in a concentration-dependent manner when visualizing the morphology of HT-29 cells after 48 h treatment with 10, 25 and 50 mM Met [21].

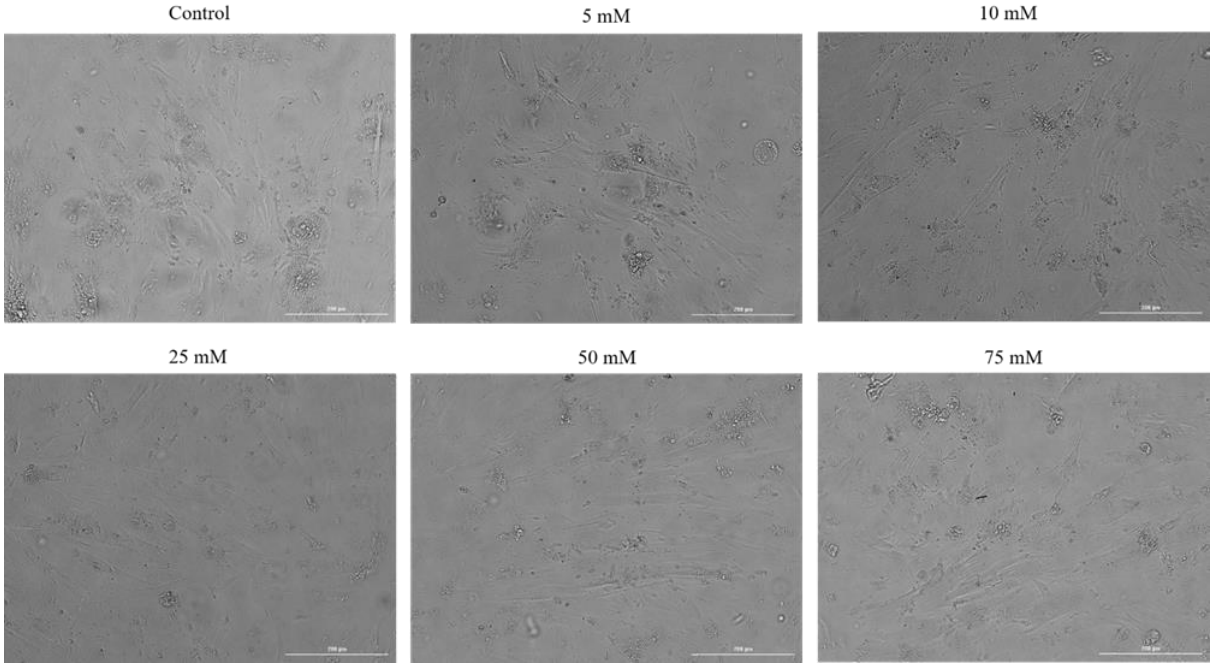

Figure 4.
Representative images of the morphological features of CCD 841 CoN cells after treatment for 72 h with Met (5, 10, 25, 50 and 75 mM). The scale bar was 200 μm

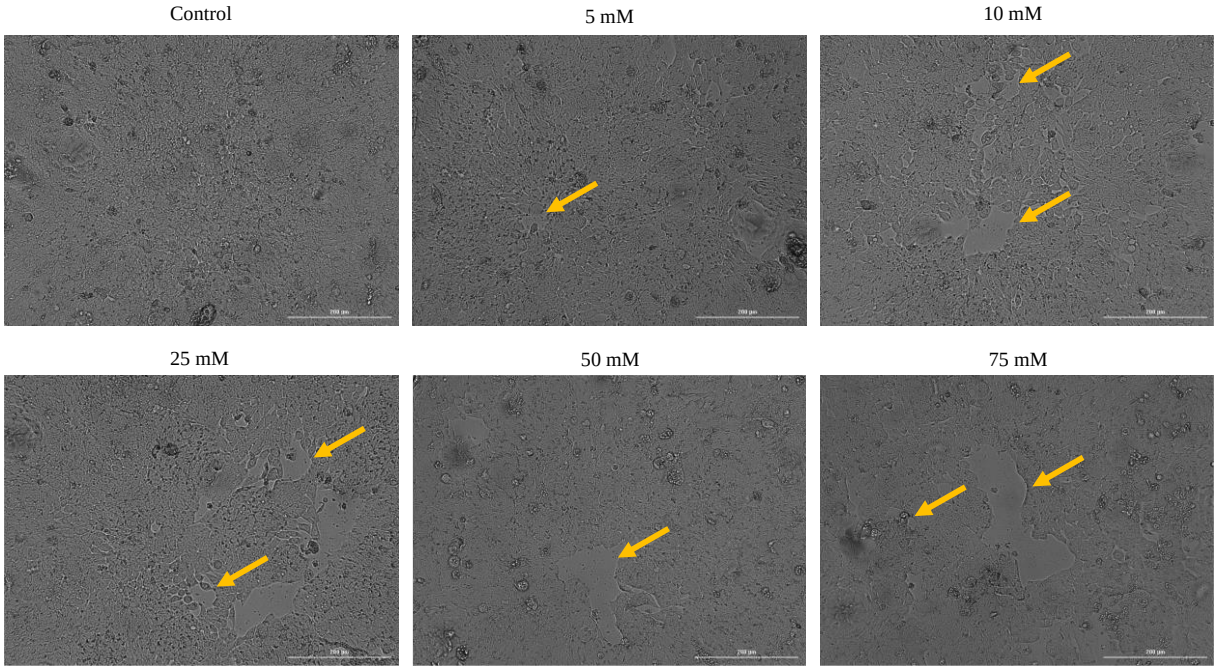

Figure 5.
Representative images of the morphological features of HT-29 cells after treatment for 72 h with Met (5, 10, 25, 50 and 75 mM). Morphological changes are highlighted with yellow arrows. The scale bar was 200 μm

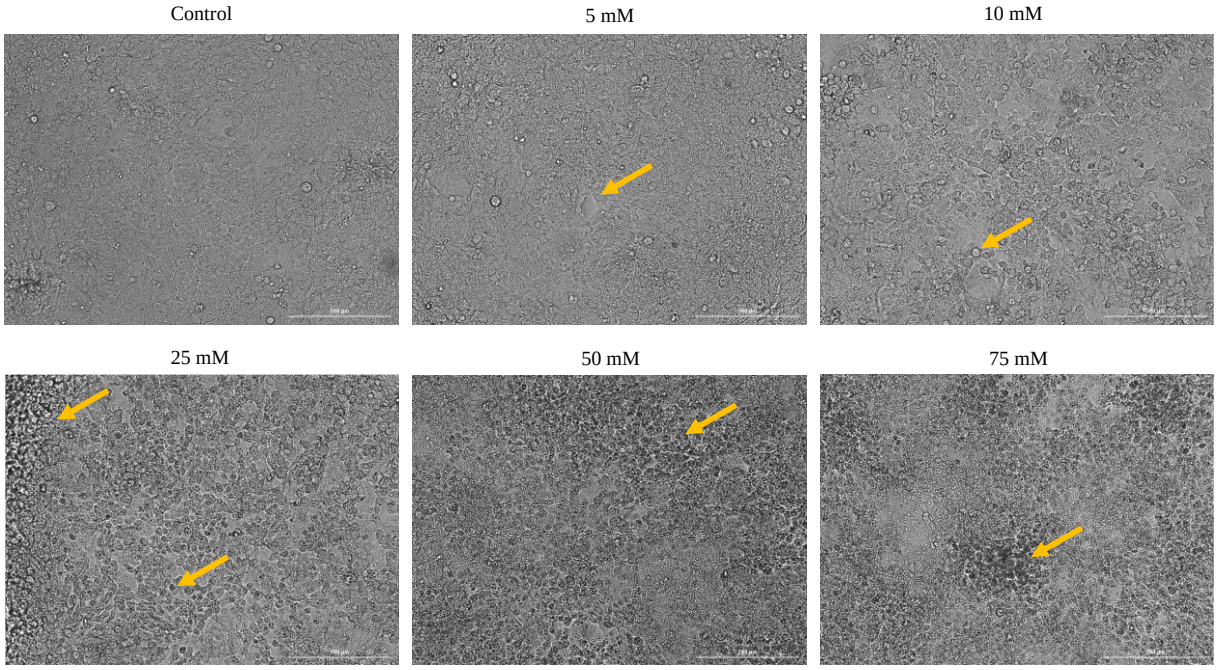

Figure 6.
Representative images of the morphological features of HCT-116 cells after treatment for 72 h with Met (5, 10, 25, 50 and 75 mM). Morphological changes are highlighted with yellow arrows. The scale bar was 200 µm

Immunofluorescence staining was performed in order to visualize the changes that occurred in the morphology of cell nuclei after 72 h treatment with 5 and 75 mM Met. The lowest and the highest concentrations were chosen in order to understand the extent of the cytotoxic effect of Met, 5 mM allowing to better visualize the starting of the changes, while 75 mM allowed the assessment of the alterations to full extent. Under fluorescence microscopy, no significant nuclear changes were noticed in CCD 841 CoN cells, beside slight nuclear condensation at the higher concentration used (Figure 7).

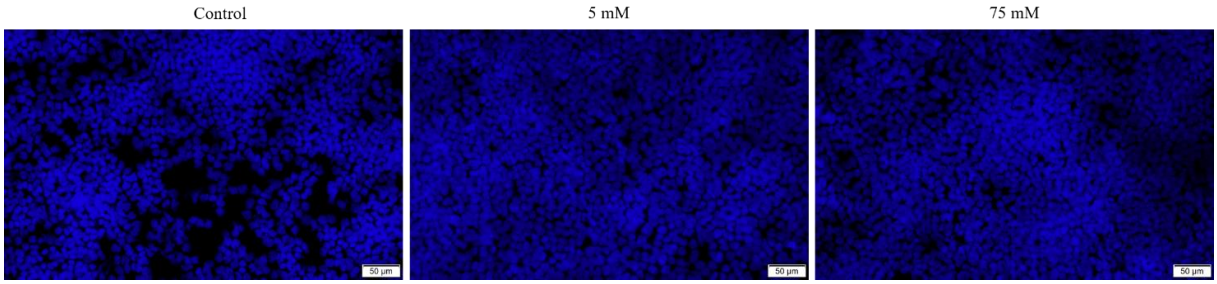

Figure 7.
Dapi staining of CCD 841 CoN cells' nuclei following the 72 h treatment with Met 5 and 75 mM. The scale bars represent 50 µm

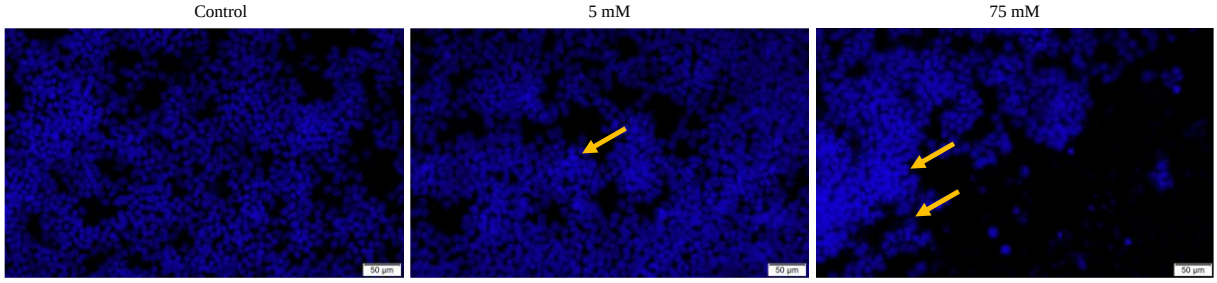

Figure 8.
Dapi staining of HT-29 cells' nuclei following the 72 h treatment with Met 5 and 75 mM. Nuclear changes are highlighted with yellow arrows. The scale bars represent 50 µm

Dapi staining revealed condensed nuclei in HT-29 cells. The 75 mM concentration impacted cells the most, a more prominent fluorescence in rounded and shrunken nuclei being seen (Figure 8).

HCT 116 cells` nuclei were affected the most by Met treatment. As well as in the case of HT-29 cells, cells` nuclei suffered rounding and shrinkage, characterized by intense fluorescence (Figure 9). Also, fragmentation of the nuclei took place after 75 mM treatment.

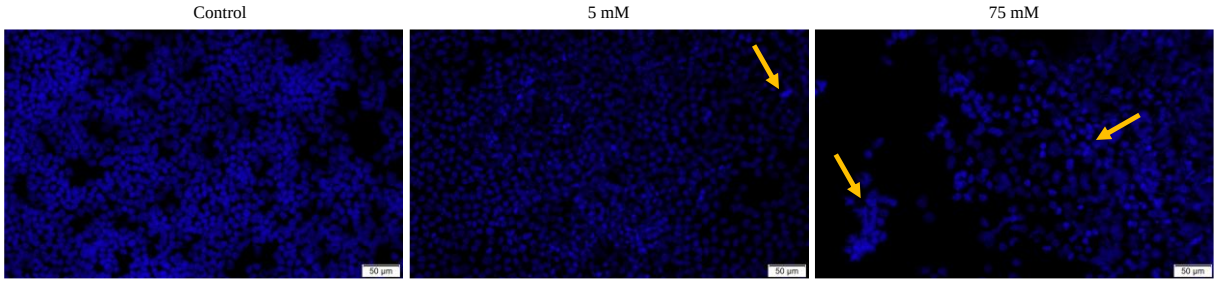

Figure 9.
Dapi staining of HCT 116 cells' nuclei following the 72 h treatment with Met 5 and 75 mM. Nuclear changes are highlighted with yellow arrows. The scale bars represent 50 µm

The analysis gave better understanding of the possible type of death that lays behind the loss of viability that Met treatment produced. Dapi, a fluorescent dye that binds to A-T rich regions of DNA, allowed the visualization of the alterations that took place in the cells` nuclei [1]. In the biomedical domain, apoptosis has been under the magnifying lamp of researchers. Signs of a cell undergoing apoptosis are shrinkage of the cell, condensation of chromatin, fragmentation of cell nuclei and formation of apoptotic bodies [7, 24]. If a molecule possesses the ability to induce voluntary death in cancer cells, it is considered to be of an important therapeutic potential [24]. The present study revealed nuclear condensation and fragmentation of colon cancer cells` nuclei, along with intense fluorescence, cell rounding and shrinkage, Met exerting thus an apoptotic-like effect.

Conclusions

The present study analysed through a series of *in vitro* evaluations the cytotoxic effect of metformin in two colon cancer cell lines. In order to see if the observed effect was cancer cell selective, a normal human colon cell line was used. The results of the study revealed that Met exerts no cytotoxicity in the healthy cell line, while it decreases the viability in both cancer cell lines in a concentration-dependent manner. Moreover, Met seems to produce morphological changes after 72 h treatment in HCT 116 and HT-29 cells. Furthermore, Met treatment produced alterations in the structure of cell nuclei, apoptotic-like signs being observed. These findings point to the possible antitumor effect Met exerts in one of the most prevalent types of cancer. Further studies are necessary in order to better understand the antitumor potential of Met and the exact mechanism that lies behind the effect.

Conflict of interest

The authors declare no conflict of interest.

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