RESEARCH ARTICLE

Effects of Epothilone A in Combination with the Antidiabetic Drugs Metformin and Sitagliptin in HepG2 Human Hepatocellular Cancer Cells: Role of Transcriptional Factors NF-κB and p53

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Abstract

Type 2 diabetes mellitus patients are at increased risk of many forms of malignancies, especially of the pancreas, colon and hepatocellular cancer. Unfortunately, little is known of the possible interaction between antidiabetic drugs and anticancer agents. The present study investigates the influence of metformin (MET) and sitagliptin (SITA) on the *in vitro* anticancer activity of the microtubule depolymerization inhibitor agent epothilone A (EpoA). Hepatocellular liver carcinoma cell line (HepG2) viability and apoptosis were determined by the MTT test and by double staining with PO-PRO-1 and 7-aminoactinomycin D, respectively, after treatment with EpoA, metformin or sitagliptin. The levels of nuclear factor NF-κB and p53 were evaluated in the presence and absence of inhibitors. While EpoA and MET inhibited HepG2 cell proliferation, SITA did not. EpoA and SITA induced higher p53 levels than MET. All tested drugs increased the level of NF-κB. Only MET enhanced the proapoptotic effect of EpoA. The EpoA+MET combination evoked the highest cytotoxic effect on HepG2 cells and led to apoptosis independent of p53, decreasing the level of NF-κB. These findings support the link between NF-κB and p53 in the modulation of apoptotic effects in HepG2 cells treated by EpoA. Our studies indicate that the combination of EpoA and MET applied in subtoxic doses has a stronger cytotoxic effect on liver cancer cells than each of the compounds alone. The therapeutic advantages of the combination of EpoA with MET may be valuable in the treatment of patients with diabetes mellitus type 2 (T2DM) and liver cancer.

Keywords: Apoptosis - epothilone A - hepatocarcinoma - metformin - sitagliptin

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Introduction

Studies indicate that diabetics are more likely to develop cancer, especially of the pancreas or colon, or hepatocellular carcinoma. Tumor genesis is promoted by high insulin and glucose levels pancreas, in T2DM (Saito et al., 2013; Zheng et al., 2013; Wang et al., 2015). Metformin, a commonly used drug for T2DM, reduces the incidence of various types of neoplasm. However, much less is known about the influence of the novel antidiabetic drug sitagliptin (SITA) on cancer occurrence, as well as its interaction with anticancer therapy. Additionally, the mechanisms of the possible interaction between antidiabetic drugs and anticancer agents have yet to be precisely determined.

The aim of the present paper is to determine the influence of metformin (MET) and SITA on the killing of liver cancer cells mediated by epothilone A (EpoA), a microtubule inhibitor agent.

Epothilones induce tubulin aggregation, cause mitotic arrest, and prevent microtubule depolymerization (Cheng et al., 2008). They are a newer class of microtubule-targeting agents than taxanes. Numerous studies have confirmed the antitumor activity of epothilones in several tumor cell lines, even with high levels of P-glycoprotein (Ganesan et al., 2014; Zhang et al., 2014).

The proposed mechanism of action of metformin on cancer cells is believed to be associated with reductions in the level of mammalian target of rapamycin complex 1 (mTORC1). The mTOR pathway affects the metabolism, progression and proliferation of the tumor (Chiang and Abraham, 2007). A body of evidence suggests that metformin inhibits the mTOR pathway in a manner dependent or independent on the AMP-activated protein kinase (AMPK) (Dilokthornsakul et al., 2013; Patel et al., 2015). The second studied compound, sitagliptin, is a fully reversible DPP-IV inhibitor. However, SITA is not known to have an influence on cancer cells, nor interact

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with anticancer drugs (Sangle et al., 2012).

Nuclear factor kappa B (NF-κB) is responsible for the regulation of the genetic expression of specific cytokines, chemokines, growth factors, cell surface receptors, enzymes and acute phase proteins (Wardle, 2001). It is a common component of many intracellular signaling pathways and is essential for the biosynthesis of many proteins that are actively involved in the pathogenesis of defense and inflammatory processes. The NF-κB signaling pathway is associated with survival. It is suggested that the NF-κB affects the course of various pathways in cancer cells by the transcription of specific genes encoding proteins which regulate the cell cycle (cyclin D1, GADD45, p16INK4A), apoptosis (Bcl-XL, XIAP, cIAP) and oncogenesis (p53,VCAM, ICAM). There are, however, contradictory findings concerning the role of NF-κB in cancer cells, since a number of reports attribute it with both anti-apoptotic (Ueda and Richmond, 2006) pro-apoptotic properties (Aoki et al., 2001; Ismail et al., 2015). It seems that the action of NF-κB may depend on the type of cell line. It is not known yet whether the activation or inhibition of NF-κB is a necessary and sufficient condition for the mobilization of the apoptotic cascade in HepG2 cells treated with EpoA, metformin or sitagliptin, either used alone or in combination. To better understand this requirement, the present paper investigates whether two widely-used antidiabetic drugs, metformin and sitaglipitin, enhance the action of EpoA and affect NF-κB or p53 protein levels. p53 is involved in maintaining genome stability, including the suppression of the cell cycle, DNA reparation and apoptotic changes (Hayden and Ghosh, 2012). P53 also regulates metabolism, mainly glycolysis, in cancer cells. P53 is believed to regulate NF-κB signaling via the inhibition of glycolysis through the regulation of the TIGAR (glycolysis regulator) protein (Madan et al., 2011).

The present study evaluates the effect of MET and SITA on the anticancer activity of EpoA. It also attempts to identify the common relations between two transcription factors, NF-κB and p53, in EpoA-induced HepG2 cell death.

Materials and Methods

Chemicals

Drugs: EpoA, metformin and sitagliptin were obtained from Sigma Aldrich (St. Louis, USA). Pifithirin-α (PFT-α, p53 inhibitor), sodium salicylate (SSA, an NF-κB inhibitor) were also purchased from Sigma Aldrich. NF-κBp65, Membrane Permeability/Dead Cell Apoptosis Kit were purchased from Invitrogen (Camarillo, USA), Human p53 ELISA Kit was from Dialcone (Besancon Cedex, France).

Solvents and chemicals for cell culture: medium (DMEM) and fetal bovine serum (FBS) were obtained from Cambrex (Basel, Switzerland); trypsin-EDTA, penicillin and streptomycin were acquired from Sigma Aldrich (St. Louis, USA).

Cell culture

The human HepG2 cell line used in the experiments was from American Type Culture Collection (ATCC) Rockville, MD, USA. The cells were obtained, as a generous gift, from the prof. G. Bartosz, Department of Molecular Biophysics of the University of Lodz. The cells were cultured in DMEM with 10% heat-inactivated FBS, penicillin (10 U/ml) and streptomycin (50 μg /ml) and regularly checked for mycoplasma contamination. The cells were cultured under an atmosphere of 5% CO_2 and 95% air at 37°C. The cells were removed by trypsinization.

Cell viability assay

The effects of the tested compounds on the viability of HepG2 cells were measured using MTT assay (3-(4,5- dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide). MTT is reduced by mitochondrial cytosolic dehydrogenases to a colored formazan product (Carmichael et al., 1987). This reduction reaction is characteristic for metabolically viable cells.

The cultured cells were plated into 96-well plates at a density of 10^4 cells/well. After 24 hours, the compounds were added either separately or in combination (in appropriate molar ratio) and the cells were incubated for 72 hours. Following this, 50 μl of 0.5 mg/ml MTT solution was added to the wells for 4h. The formazan crystals were dissolved in 100 µl dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm with a microplate reader (Awareness Technology Inc., USA). Cell viability was shown as a percentage of the control value i.e. for cells cultured without drugs. The IC_{50} values were calculated using IC_{50} plus v1.0.

NF-κB assay

The cells were plated ($0.5x10^6$; 60 mm Petri dishes) in 5 ml of medium. After 24h, drugs or inhibitors (sodium salicylate and pifithirin-α) were added and the cells were incubated for 72h. The cells were then washed in PBS and incubated in extraction buffer. The lysates were centrifuged at 10000 g for 30 min at 4° C. The cytosolic fraction was collected and stored at -80° C. Antibodies specific for NF-κBp65 were used for the test, regardless of phosphorylation state. The amount of colored product corresponded to the concentration of NF-κBp65 present in the probes. The reaction was stopped by 2M sulfuric acid. The depth of yellow color was measured at 450 nm (PowerWave, BioTek microplate reader).

P53 measurements

The p53 level was measured using Human p53 ELISA Kit (Dialcone) according to the manufacturer's protocol. The cells were plated into 60 mm petri dishes at a concentration of $0.5x10^6$ cells in 5 ml of medium. After 24h, the test compounds were added, and the cells were incubated for 72 h. The cell lysates were stored at -80° C in a similar manner to assess p53 level. The cells were then suspended in extraction buffer and centrifuged at 10000 g for 30 min at 4° C. The absorbance value of each well was measured at 450 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific USA).

Assessment of apoptosis

The apoptotic changes of HepG2 cells were analyzed

by using Membrane Permeability/Dead Cell Apoptosis Kit. The cells were dropped ($0.5x10^6$) in 5 ml of medium into 60 mm dishes. After 24 hours, the cells were attached to the surface of the plate, and were incubated with the test drugs for 24h-72h. Following this, 2.5 μL PO-PRO-1 stock and 1 μL 7-aminoactinomycin D (7- AAD) stock were added to each 1 mL of probe. The cells were incubated for 30 minutes on ice. After staining with PO-PRO-1 and 7-AAD, apoptotic cells show violet fluorescence, dead cells show violet and red fluorescence and live cells show little or no fluorescence, as the fluorescent violet PO-PRO-1 dye can enter apoptotic cells but the fluorescent red 7-AAD dye cannot. A violet laser and the 488 nm line of an argon-ion laser were used for excitation measurement, and fluorescence emission was evaluated using 440 nm and 670 nm band pass filters (FACS Canto, Becton Dickinson).

Statistical analysis

The data was stated as a means ± S.D. An analysis of variance (ANOVA) was used with the Tukey post hoc test for multiple comparisons (StatSoft, Tulsa, OK, USA). The significance level was set at a p-value of 0.05.

Results

Inhibition of HepG2 cancer cell growth

As shown in Figure 1, treatment with EpoA (1-300 nM) caused a concentration-dependent inhibition of cell growth, with the IC_{50} value of EpoA being 24.6 ±1.3 nM. In order to specify the relationship between NF-κB and p53 in EpoA-treated HepG2 cells, sodium salicylate (SSA), an NF-κB inhibitor, and pifithrin-α (PFT-α) a p53 inhibitor, were used. The IC_{50} value was 12 ± 1.8 mM for SSA and 260 ± 27 μM for PFT-α. The concentrations of drugs and inhibitors used for study were based on IC_{50} values. We have chosen for all the experiments the concentration of 5 mM SSA and 140 μM of PTF-α, which corresponding to survival about 80% relative to control untreated cells (Sliwinska et al., 2015).

The results of treating HepG2 cells with EpoA and SSA or EpoA with PFT-α are presented in Figure 2A. Both SSA and PFT-α increased the degree of cell death induced by EpoA. Although SSA was effective at low EpoA concentrations (1 and 10 nM), PFT-α significantly increased cell death at all tested EpoA concentrations (1, 10, 25 and 50 nM).

MET and SITA were used to explore the effect of antidiabetic drugs on apoptotic HepG2 cell death. The IC_{50} value for MET was 21± 3 mM (Sliwinska et al., 2015). Sitagliptin was non-toxic to the HepG2 cells at a broad range of concentrations (0.01-200 μM). The effect of combined treatment with EpoA (1-50 nM) and 1μM of SITA or 10 mM MET on HepG2 cell viability is shown in Figure 2B. SITA was not observed to influence the cytotoxic effect of EpoA. On the contrary, metformin significantly enhanced the loss of HepG2 cell viability (about 20-40%, p<0.05) induced by EpoA.

To examine the influence of EpoA administration on NF-κB and p53, the next part of the study investigated whether their inhibitors, SSA and PFT-α, may modulate HepG2 viability in the presence of antidiabetic drugs. As presented in Figure 3, HepG2 cells treated with 10 nM EpoA and 10 mM MET or 10 nM EpoA and SITA (1 μM) demonstrated greater loss of cell viability after preincubation with SSA or PFT-α. Hence, although SITA might modulate the level of NF-κB and p53, it may not affect the cytotoxic effect of EpoA.

Morphological changes of HepG2 cells after drugs treatment

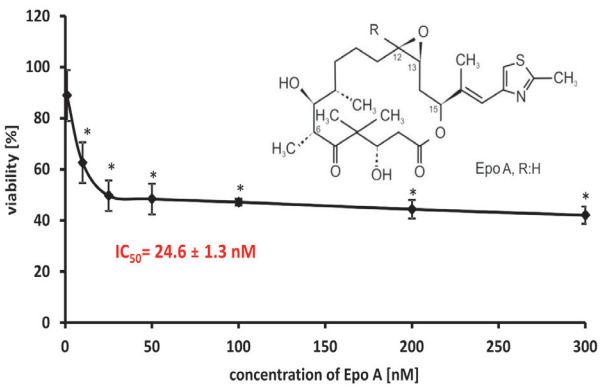

Figure 1. The Effect of EpoA on HepG2 Cell Growth. Cells were incubated with 1 – 300 nM EpoA for 72 h before the MTT assay was performed. Results are presented as means ± SD of six experiments. (*) p<0.05 statistically significant changes in comparison to untreated, control cells (taken as 100%)

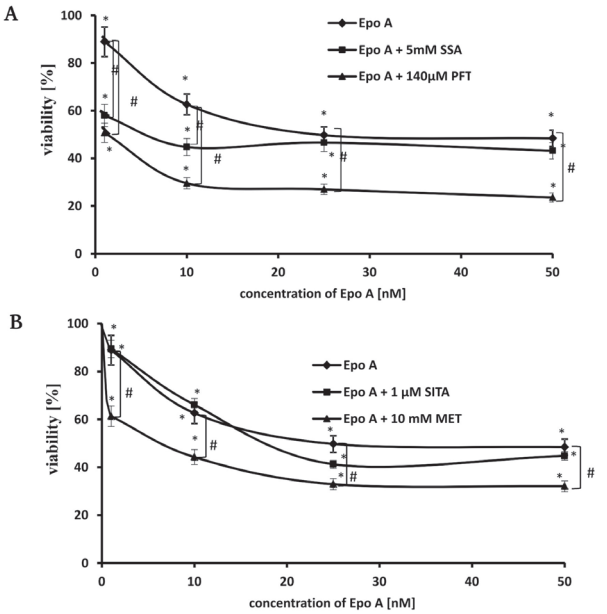

Figure 2. MTT Assay. (A) the effect of SSA (5 mM) and PFT-α (140 μM) 1h preincubation on viability of HepG2 cells treated with EpoA (1, 10, 25, 50 nM) measured by the MTT assay. (B) the impact of 10 mM metformin (MET) and 1μM sitagliptin (SITA) on the viability of HepG2 cells after EpoA treatment (1, 10, 25, 50 nM) detected by MTT assay. Results are presented as means ± SD of six experiments. (*) p<0.05 statistically significant changes in comparison to untreated, control cells (taken as 100%). (#) Statistically significant changes between effect of EpoA in comparison to (A) EpoA+ SSA or EpoA+ PFT-α, (B) EpoA+MET or EpoA+SITA, p<0.05

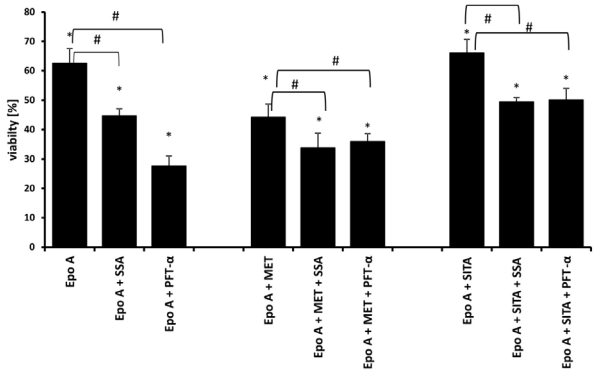

Figure 3. The Impact of 1 h Preincubation with 5mM SSA and 140 μM PFT-α on the Viability of HepG2 Cells After 10 nM EpoA, 10 nM EpoA+10 mM MET, 10 nM EpoA+1 μM SITA Treatment Determined by MTT Assay. Results are presented as means of five experiments ±SD. (*) statistically significant changes in comparison to untreated, control cells (taken as 100%), p<0.05. (#) statistically significant changes between effect of compounds added with inhibitor in comparison to the samples incubated without inhibitors, p<0.05

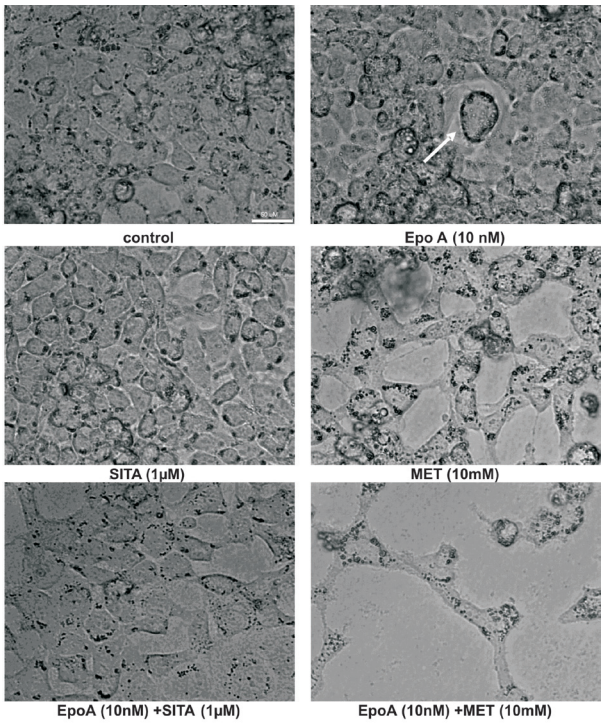

Figure 4. The Morphology of HepG2 Cells Treated for 72 h with 10 nM EpoA, 10 mM MET, 1 μM SITA Alone and in Combination. White arrow - giant cell, Olympus IX70, Japan, (scale bar = 50μm)

The morphology of cells treated for 72 h with Epo A (10 nM), MET (10 mM), SITA (1 μM), Epo A : MET (10 nM : 10 mM) or Epo A : SITA (10 nM : 1 μM) was examined and presented in Figure 4. All tested drugs induced changes in HepG2 cells in comparison to untreated cells. EpoA was found to lead to the formation of giant cells (white arrow). MET caused cell elongation. HepG2 cells incubated with SITA showed very small morphological changes. Combined treatment with EpoA and MET led to cell elongation and slightly increased the

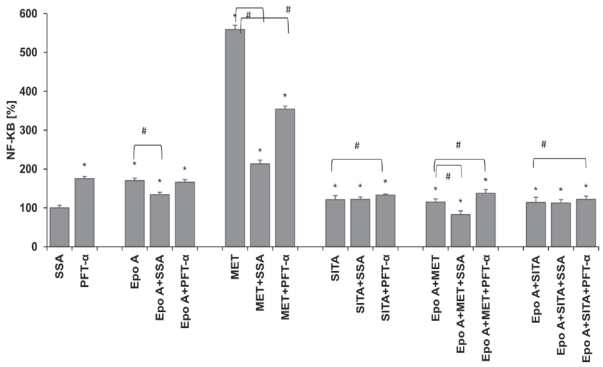

Figure 5. The Level of NF-ϰB in HepG2 Cells. The cells after 72 h treatment with EpoA, MET and SITA alone or in combination. Results are presented as means of five experiments ±SD. (*) Statistically significant changes in comparison to cells treated with 5 mM SSA (taken as 100%) $p < 0.05$. (#) Statistically significant differences observed between the probes incubated with EpoA, MET, SITA, EpoA+MET or EpoA+SITA and samples preincubated with inhibitors (SSA or PFT-α), $p < 0.05$

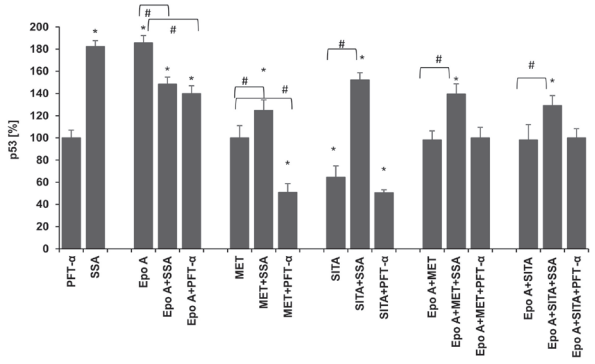

Figure 6. The Level of p53 in HepG2 Cells. The cells after 72 h treatment with EpoA, MET and SITA, alone or in combination. Results are presented as means of five experiments ±SD. (*) Statistically significant changes in comparison to PFT-α (140 μM) treated cells (taken as 100%) $p < 0.05$; (#) Statistically significant differences observed between the probes incubated with EpoA, MET, SITA, EpoA+MET or EpoA+SITA and samples preincubated with inhibitors (SSA or PFT-α), $p < 0.05$

degree of adhesion of HepG2 cells to the vessel. In turn, cells treated simultaneously with EpoA and SITA became round and had lower density.

Changes in the level of NF-ϰB protein

To determine whether MET and SITA may modulate the level of NF-ϰB after treatment of HepG2 cells with EpoA, we employed SSA and PFT-α (regardless of phosphorylation state) (Figure 5). MET was observed to evoke a significantly increased level of NF-ϰB (459%). EpoA and SITA further increased the level of NF-ϰB (EpoA 70%, SITA 21%). However, a slightly higher NF-ϰB level was noticed in HepG2 cells incubated with EpoA+ MET and EpoA+SITA. Pretreatment with SSA was found to decrease the level of NF-ϰB in HepG2 cells treated with EpoA, MET or EpoA+MET. Although PFT-α did not change the NF-ϰB level in HepG2 cells treated with EpoA, but diminished it in MET-treated cells in comparison to SITA, and enhanced it in untreated

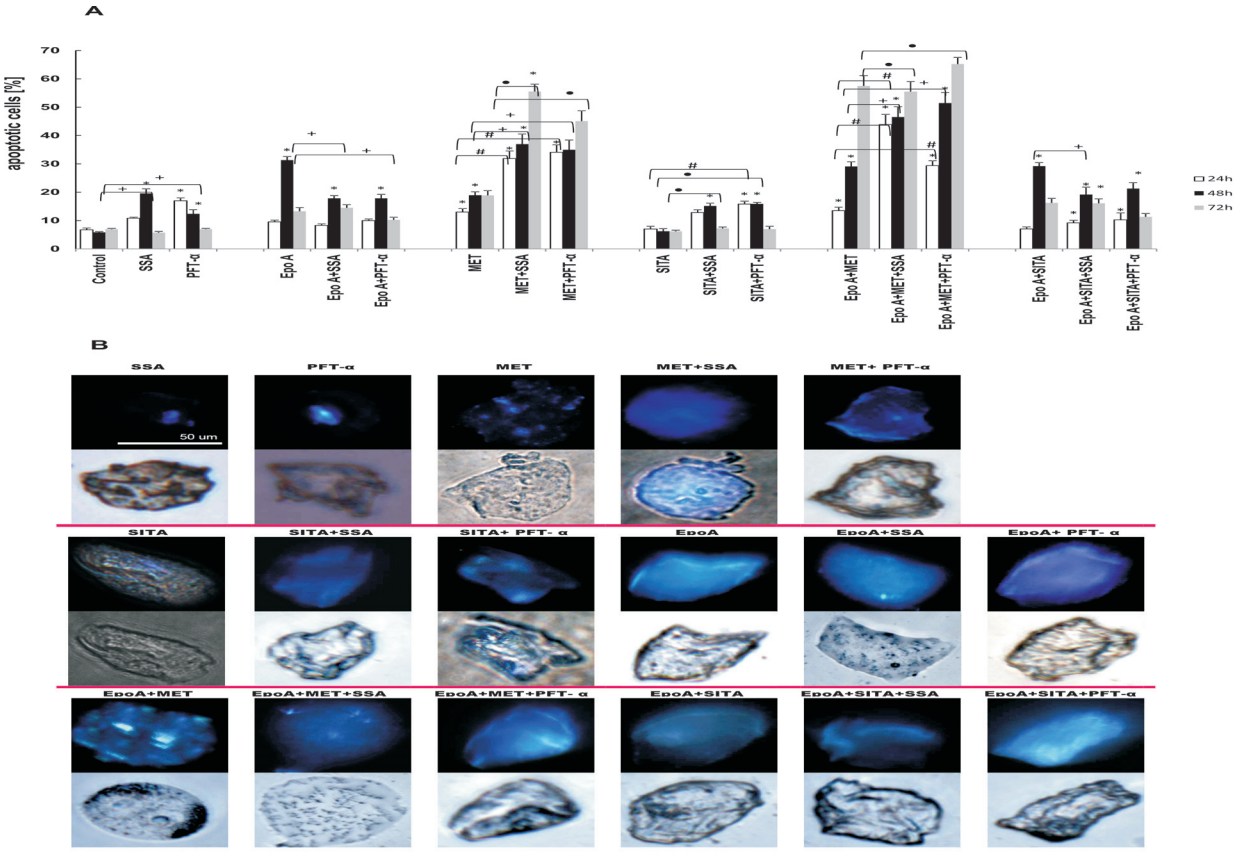

Figure 7. Flow Cytometry Analysis of Apoptosis. Cell death induced by EpoA, MET, and SITA alone and in combination at 24, 48 and 72 h in HepG2 cells measured by a Membrane Permeability/Dead Cell Apoptosis Kit with PO-PRO-1 and 7- ADD. The cells were exposed to SSA (5 mM) and PFT-α (140 μM), EpoA (10 nM), MET (10 mM), or in combination of drugs. A, quantitative results of the effect of tested drugs on the level of apoptosis. Results are presented as means ± S.D. of four experiments. (*) Statistically significant differences were found with untreated, control cells (taken as 100%) $p< 0.05$. Statistically significant differences observed between the probes incubated with EpoA, MET, SITA, EpoA+MET or EpoA+SITA in presence and absence of inhibitors (SSA or PFT-α) after 24h (#), 48h (•), 72h (+), $p< 0.05$. B, HepG2 cells stained with PO-PRO-1 and 7-ADD visualized by fluorescence microscopy (Olympus IX70, Japan; bar 50 μm). Typical observations were given after 48 h of exposure to drugs in the absence or presence of inhibitors (SSA or PFT-α). Additionally, the images are presented in contrast phase conditions

cells. The combination of EpoA+MET, as well as with EpoA+SITA, increased the level of NF-ϰB after preincubation with PFT-α.

P53 assay

The level of p53 after treatment with EpoA alone or combination of EpoA+MET or EpoA+SITA is shown in Figure 6. A significant increase of protein level was observed after EpoA treatment. MET did not influence the level of p53, but SITA decreased it. HepG2 cells treated with MET, SITA, EpoA+MET and EpoA+SITA showed significant increases of p53 level after preincubation with SSA, while those treated with EpoA demonstrated significant decreases in p53 content. This effect of SSA suggests that p53 might be involved in HepG2 cell death evoked by EpoA. As expected, PFT-α significantly reduced the level of p53 in HepG2 cells treated with EpoA, MET and SITA. Combinations of drugs did not significantly affect the level of p53 in the cells.

Confirmation of apoptosis: PO-PRO-1and 7-aminoactinomycin D assay

Both flow cytometry (Figure 7A) and fluorescence

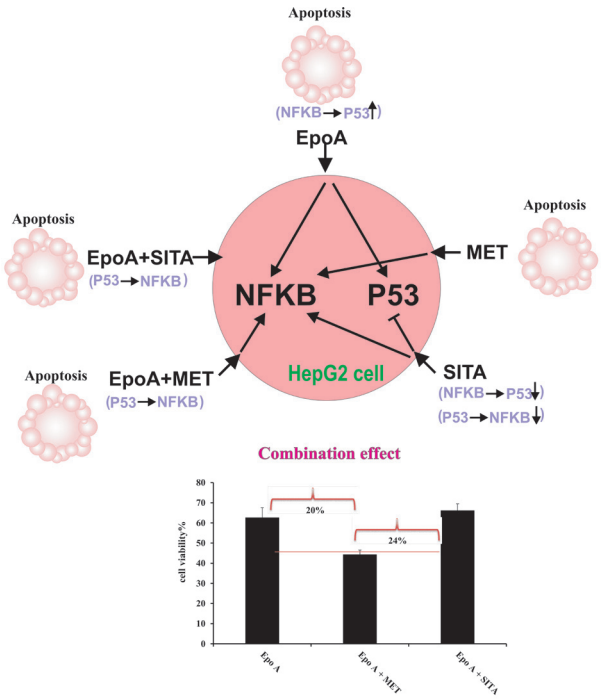

Figure 8. The Proposed Mechanism of Drugs Action

microscopy (Figure 7B) were used to evaluate the action of the drugs. Apoptotic HepG2 cells were observed to fluoresce violet after 48 h of incubation with drugs or/ and pretreatment with SSA or PFT-α (Figure 7B). A quantitative analysis of cells is presented in Figure 7A. The maximal level of apoptotic cells was detected in cells treated with EpoA (31%) or MET (19%) after 48 h of incubation. It was observed that pretreatment with SSA or PFT-α of HepG2 cells led to a decrease in the number of apoptotic cells by about 50% after 48h of incubation with EpoA. However, preincubation with SSA and PFT- α resulted in a remarkable increase of apoptosis after 48-72h of treatment with MET. SITA did not cause apoptosis of HepG2 cells. Pretreatment with SSA or PFT-α increased the level of apoptotic cells after 24 and 48 hours of cell culture, but this was not dependent on the presence of SITA.

The combination of EpoA+MET significantly increased the number of apoptotic cells. After 72h of incubation with the drugs, an increase in apoptotic cells of up to 57% was observed. Moreover, the apoptotic effect was enhanced in HepG2 cells treated for 24-48 h of EpoA+MET and preincubated with SSA or PFT-α compared to cells treated with EpoA+MET but which had not been preincubated with inhibitors. Preincubation with only SSA of EpoA+SITA-treated cells enhanced apoptotic effect at 48 h of incubation.

Discussion

Not much is known about the influence of concomitant therapies on the efficacy of anticancer drugs. This is especially true for commonly used drugs. As antidiabetics are known to have an influence on cancer cells, the present study examines the effect of metformin and sitagliptin on Epothilone A - induced apoptotic cell death in a hepatocarcinoma cell line (HepG2). The evidence suggests that metformin, a commonly-used antiglycemic drug, reduces of cancer in diabetic patients. MET is well tolerated and improves the survival rates of patients with early stage hepatocellular carcinoma (Chen et al., 2011; Harris and Smith, 2013; Miyoshi et al., 2014; Zhang and Li, 2014; Wang et al., 2015). As gene expression profiling has confirmed that microtubules are an important target in HCC treatment, epothilone, a compound belonging to a new class of microtubule depolymerization inhibitors with a strong cytotoxic effect on liver cancer cells (Chang and Wang, 2013; Zhou et al., 2013), was chosen for use in the present study. In HCC cell lines, epothilone B has previously been found to be 4- to 130-fold more potent than currently-used taxanes (Mok et al., 2006).

The present report is the first to demonstrate the ability of MET to enhance the anti-cancer effect of EpoA in the HepG2 cell line. MET and EpoA decrease cell viability to a greater degree when added in combination than when each compound is added alone. The mTOR pathway is probably involved in multidrug resistance. The simultaneous targeting of the mTORC inhibitor (by MET) and the microtubules (by Epo A) may be the reason for enhanced (Loong and Yeo, 2014; Tan et al., 2014). The combination of EpoA and SITA does not lead to statistically significant changes in cell viability.

Recent studies have revealed that the activation of NF-κB is the key factor for cell death induced by microtubule-targeting drugs (Huang and Fan, 2002; Davoudi et al., 2014). Moreover, epothilone B (Epo B) and epothilone D (Epo D) are known to cause SW620 colon cancer cell death by induction of apoptosis via regulating NF-κB/ IKK (Lee et al., 2007) (Kapahi et al., 2000). In our study both antidiabetic drugs, as well as EpoA, increased the level of NF-κB.

Hofer et al. suggest that MET amplifies the relative amount of phosphorylated versus unphosphorylated NF-κB (Hofer et al., 2014). However, our present findings contradict those of previous studies which reported that metformin inhibited NF-κB signaling through upregulation of Iκβ (Chaudhary et al., 2012). In the study performed on cancer stem cells (CSCs), metformin reduced the level of NF-κB in the nucleus and increased its level and phosphorylation of IκB in the cytoplasm. This effect was not seen in non-stem cancer cells (NSCCs). Moreover, the effect of metformin might not require AMPK/mTOR-signaling or NF-κB inhibition (Woudenberg-Vrenken et al., 2013). Previous studies suggest that inhibition of mTOR by metformin might be independent of AMPK activation, but it is associated with RAG GTPase or DNA-damage-inducible transcript 4 protein (DDIT4) (Kasznicki et al., 2014). For example, metformin enabled cell cycle arrest by the downregulation of cyclin D1 expression and/or upregulation of cyclin-dependent kinase inhibitors, such as p21Cip1, without stopping the mTOR pathway (Zhuang and Miskimins, 2008b; Cai et al., 2013).

Few studies have described the influence of SITA on NF-κB. Apart from one previous report that SITA inhibits atherosclerosis by the phosphorylation of p38 kinase in a mouse model, thus leading to the reduction of NF-κB (Aldea et al., 2014), the present study, indicating that SITA may increase the level of NF-κB in HepG2 cancer cells, appears to be one of the first.

The complicated interplay between NF-κB and p53 protein results in either synergistic or antagonistic actions in order to maintain cell homeostasis. The signaling pathway of NF-κB is an early immune response associated with p53 (Baldwin, 2012; Rengarajan et al., 2014). Our results indicate that EpoA affects the level of p53. However, sodium salicylate appears to interfere with this effect, as it was found to partially reverse an increase in p53 level evoked by EpoA. In EpoA-treated cells, both investigated proteins act proapoptically. Current studies report that apoptosis might be the reason of cell cycle arrest induced by epothilones, and that it is associated with the number of signaling trails, including p53 (Lee et al., 2007). Epothilones are also known to increase p53 expression. Paclitaxel, a well-known microtubule-stabilizing agent, significantly elevated p53 and p21 expression in both IGROV-1 and H460 cells. Although PFT - α was not found to have any effect paclitaxel-induced p53 activation, it diminished the up-regulation of p21 mediated by paclitaxel by inhibition of p53 transcription (Zuco and Zunino, 2008). Moreover, our previous result showed in OV-90 and MM14 ovarian cell lines that epothilone

B (EpoB) increases the level of p53 protein earlier than paclitaxel (PTX) and the amount of p53 return to the control value at 24h time point (Rogalska et al., 2013b). Other researchers have shown that treatment with low concentrations of EpoA or EpoB (3 nM) do not disrupt the microtubules (MT) network but suppress MT dynamics, enhance p53 nuclear accumulation, and the activation of the p53-downstream target genes leading to the apoptosis (Giannakakou et al., 2002). Epo B sensitivity in small cell ovarian carcinoma of the hypercalcemic type was associated with increased Ser15 phosphorylation of p53 (Otte et al., 2014).

Recently, we have reported the effect of epothilones on the p53 null SKOV-3 ovarian cancer cell line. We found that EpoB and EpoA induced apoptosis and mitochondrial dysfunction (Rogalska et al., 2013a; Rogalska et al., 2014). Tumors with wild-type p53 involved in cell adhesion/angiogenesis were resistant to sagopilone (SAG) therapy (Hammer et al., 2010). Treatment of A549 cells with a low (2.5 nM) concentration of SAG revealed an upregulation of direct transcriptional target genes of p53. Knockdown of p53 led to a significant increase in apoptosis induction in A549 cells (Winsel et al., 2011). These observations suggest that epothilones concentration and type of cancer cells are the main determinants of its mechanism of action (An et al., 2015) and they may affect apoptosis regardless of the level of p53.

The role of p53 in metformin action is debatable and seems to be dependent on cell type. Metformin use was associated with phosphorylation of p53 at Ser-15 (Ben Sahra et al., 2010). However, our present findings can't confirm the presence of p53 phosphorylation by MET. Similar results have been described previously (Hadad et al., 2013). It is known that metformin stops the propagation of breast cancer cells and this is not associated with p53 expression (Zhuang and Miskimins, 2008a). Results obtained using the HCC cell line suggests that the cytotoxic mechanism of metformin action is independent of p53 (Chen et al., 2013; Azlin et al., 2014). It can't be excluded that may also be the result of stimulation of poly-ADP-ribose polymerase synthesis by metformin (Chen et al., 2013). The product of poly-ADP-ribose binds to apoptosis-inducing factor on the external mitochondrial membrane. This is crucial for the activation of apoptosis-inducing factor, its translocation into the nucleus, and subsequent activation of apoptosis (Aldea et al., 2014). However, cancer cells with a functional p53 have been found to have a similar sensitivity to metformin like p53-null cells (Bost et al., 2012).

The role of p53 protein in the mechanism of action of SITA has only been described in models of diabetes and obesity, not in cancer cells. SITA was found to induce a noticeable rise in AMPK phosphorylation, TSC2 (tuberous sclerosis 2) and p53 expression (Lenski et al., 2011). In the present study, SITA increased the levels of NF-ϰB while decreasing the levels of p53. Moreover, our findings indicate that an elevated p53 level was not required for the effect of the combination therapy (EpoA+MET or EpoA+SITA). These drug combinations led to decreased level of NF-ϰB by using p53. This observation may at least partially explain the high cytotoxicity of combined EpoA+MET administration. Additionally, the significant effect on HepG2 cell viability observed after administration of EpoA+MET may result from a strong blockage of cells in the G2 – M phase. The cell cycle arrest is the main molecular mechanism of action of epothilone derivatives (Alberti, 2013). Administration of both MET and EpoA initiated apoptotic cell death, with quantitative changes and changes in cell morphology being observed (Figure 4 and Figure 7). MET at concentration of 10 mM, was found to be sufficient to promote apoptosis. Low doses of metformin in the HCC cell line induced hepatoma cell senescence (Yi et al., 2013).

In conclusion, our results indicate that inhibition of HepG2 cell growth by EpoA is significantly increased by MET (Figure 8). Combined treatment of EpoA and MET in subtoxic doses decreases the level of NF-ϰB proteins to a greater degree than each drug used alone. The combination of these compounds enhances the degree of apoptosis and cytotoxicity, especially during prolonged incubation. The possible therapeutic advantages of the combination of MET with an antimicrotubule agent may be potentially used in the treatment of patients with T2DM and liver cancer.

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