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Different susceptibility of colon cancer DLD-1 and LOVO cell lines to apoptosis induced by DMU-212, a synthetic resveratrol analogue

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ABSTRACT

The cytotoxic activity of DMU-212 has been shown to vary in cell lines derived from the same type of cancer, i.e. ovarian, breast and colorectal ones. However, the molecular mechanism of DMU-212 cytotoxicity has not been clarified in colon cancer cells. This study aims to elucidate the mechanism of antitumor effects of DMU-212 in two human colon cancer cell lines, DLD-1 and LOVO. We showed the stronger cytotoxic activity in DLD-1 cells in which DMU-212 evoked a greater pro-apoptotic effect as compared to that of LOVO cells. The analysis of the expression pattern of 84 apoptosis-related genes indicated transcripts specific to the mitochondria-mediated apoptosis pathway in both colon cancer cell lines used. We found that DMU-212 caused up-regulation of pro-apoptotic Bak1, Bok, Bik, Noxa, Bad, Bax, p53 and Apaf1 transcripts level in DLD-1 cell line, whereas anti-apoptotic Bcl-2, Bcl-xL and Bag1 mRNA expression was decreased. Changes in apoptosis-related genes expression were less pronounced in LOVO cells which did not express CYP1B1 protein and showed lower expression of CYP1A1 protein level than that in DLD-1 cells. Our results suggest that anticancer activity of DMU-212 is closely related to its biotransformation catalysed by these cytochrome P450 isoenzymes.

1. Introduction

Colorectal cancer is one of the most commonly diagnosed cancers and the third leading cause of cancer-related deaths worldwide. Although several screening techniques and chemopreventive strategies have been developed, over 1.2 million new colorectal cancer cases and 608,700 deaths were estimated to occur in 2008 (Jemal et al., 2010, 2011). The impaired clinical responses to therapies have been found to be associated with: (i) an incorrect patient compliance with screening recommendations and (ii) a resistance to chemotherapeutics. Dysfunctions in the signalling or execution of apoptosis are implicated in carcinogenesis and drug resistance (Hassen et al., 2012). Therefore, development of novel proapoptotic agents is currently of high significance. Apoptosis is mediated by two principal signalling pathways, the extrinsic and intrinsic ones (Herr and Debatin, 2001). The majority of cytotoxic drugs have been shown to initiate cell death by triggering intrinsic pathway through mitochondria (Debatin, 2004; Herr and Debatin, 2001; Kaufmann and Earnshaw, 2000).

A number of approaches have been considered in cancer drug development. Metabolic activation of chemotherapeutic agents by cytochrome P450 enzymes family has been found to be an important strategy in anticancer research (Bruno and Njar, 2007; McFadyen et al., 2004).

Among the multitude of antitumor compounds tested, resveratrol (3,4′,5-*trans*-trihydroxystilbene) and its hydroxylated and methylated analogues have displayed a wide spectrum of biological activity (Saiko et al., 2008). As a potential anticancer agent, resveratrol has been found to inhibit the carcinogenesis process in all three stages: initiation, promotion and progression (Delmas et al., 2006). However, the structure–activity studies have revealed that introduction of additional methoxy groups resulted in increased cytotoxic and pro-apoptotic properties of resveratrol analogues. Additionally, the methoxy groups at positions 3,5- and 3,4,5- of the trihydroxystilbene scaffold have been identified as crucial for pro-apoptotic activity of the compound (Roberti et al., 2003).

To date only a few reports have demonstrated the antiproliferative effect of DMU-212 (3,4,4′,5-tetramethoxystilbene) in

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transformed fibroblasts, prostate, cervical, ovarian, breast, hepatoma and colon cancer cells (Gosslau et al., 2005; Ma et al., 2008; Piotrowska et al., 2012; Sale et al., 2005, 2004). The cytotoxic activity of DMU-212 has been shown to vary in cell lines derived from the same type of cancer, ovarian (Piotrowska et al., 2012), breast (Ma et al., 2008) and colorectal (Sale et al., 2004) ones. However, the molecular mechanism of DMU-212 cytotoxicity has not been determined in colon cancer cells.

Therefore, the aim of this study was to elucidate the mechanism of antitumor effects of DMU-212 in two human colon cancer cell lines, DLD-1 and LOVO. We evaluated the cytotoxic activity of DMU-212 and the induction of apoptosis. Since isoenzymes of CYP1 family have been suggested to contribute to the metabolism of DMU-212 (Androutsopoulos et al., 2011; Sale et al., 2004), we assessed CYP1B1 and CYP1A1 mRNA and protein expression in both colon cell lines tested.

2. Materials and methods

2.1. Chemicals and reagents

DMU-212 (3,4,4′,5-*trans*-tetramethoxystilbene) was synthesised as described elsewhere (Androutsopoulos et al., 2011; Murias et al., 2004). The identity and purity of the compound was confirmed by NMR and LCMS. Cell Death Detection ELISA^{PLUS} kit and RealTime ready Human Apoptosis Panel 96 were purchased from Roche Diagnostics (Germany). Caspase-Glo® 3/7 Assay Kit, Caspase-8 and Caspase-9 colorimetric assay kit were obtained from Promega Co. (USA) and BioVision Inc. (USA), respectively. Rabbit polyclonal anti-CYP1A1 antibody (Ab) (sc-20772), rabbit polyclonal anti-CYP1B1 Ab (sc-32882), rabbit polyclonal anti-BAX Ab (sc-6236), rabbit polyclonal anti-BCL-2 Ab (sc-492), mouse polyclonal anti-cleaved PARP-1 Ab (sc-56196), goat anti-rabbit horseradish peroxidase (HRP)- conjugated Ab (sc-2004), goat anti-mouse HRP- conjugated Ab (sc-2005), and goat polyclonal anti-actin (ACTB) HRP-conjugated Ab (sc-1616) were provided by Santa Cruz Biotechnology (SantaCruz, CA).

All other materials were from Sigma–Aldrich St. Louis, MO unless otherwise stated.

2.2. Cell culture and cell viability assays

DLD-1 and LOVO colon cancer cell lines were purchased from the European Type Culture Collection. Both cell lines were maintained in phenol red-free DMEM supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Cells were cultivated under standard conditions at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. To investigate the effects of DMU-212 on cell viability, confluent stock cultures were detached using trypsin, and seeded in 96-well plates at a density of 2×10^4 cells/ well in 100 μl of growth medium. They were allowed to attach overnight and growing amounts of DMU-212 were then added from the stock solution prepared in DMSO at concentration of 100 mM/ml. The final concentration of DMSO in cell treatment solutions was less than 0.1%. Control cells were cultured under the same conditions with 0.1% DMSO. Cell viability was measured spectrophotometrically using MTT as a substrate, as described elsewhere (An et al., 2011). The concentration of DMU-212 which is required for 50% cell growth inhibition (IC_{50}) was determined from a plot of percent cell viability *versus* logarithm of concentration.

2.3. Assessment of apoptosis and necrosis induction

Cell Death Detection ELISA^{PLUS} kit was used to detect apoptosis and necrosis in DLD-1 and LOVO cells treated with DMU-212 according to the manufacturer's instruction. Camptothecin at concentration 5 μM was used as a positive control for apoptosis. Triton X-100 (9% solution) was used as a positive control for the induction of necrosis. Briefly, cells were seeded in a 96-well plate at a density of 2×10^4 cells per well, and after 24 h treated with DMU-212 at concentrations 5 μM and 10 μM. Twenty four hours later the supernatant and lysate were placed in a streptavidin-coated microtiter plate and incubated with a mixture of anti-histone-biotin, anti-DNA-peroxidase and incubation buffer. After 2 h of incubation, unbound antibodies were discarded by a washing step. Quantification of the nucleosomes was performed by measuring the optical absorbance at 405 nm against substrate solution as blank (reference wavelength 492 nm).

2.4. Determination of caspase-8, -9 and caspase-3/7 activity

DLD-1 and LOVO cells were seeded in a 96-well plate at a density of 2×10^4 cells per well, and after 24 h incubated with a vehicle (0.1% DMSO) or DMU-212 at 5 μM and 10 μM concentrations. After 24 h the medium was removed and the caspase-3/7 activity was determined using a luminescent Caspase-Glo® 3/7 assay kit (Promega, USA) according to manufacturer's protocol. The luminescence was measured using IVIS® Spectrum Imaging System (Xenogen, USA). The activity of caspase-8 and -9 was determined by Caspase-8 and Caspase-9 Colorimetric Assay Kit (BioVision, USA), respectively. Spectrophotometric detection was performed at 405 nm using microtiter plate reader.

2.5. PCR-array and real-time quantitative PCR (RTq-PCR) analysis

After 24 h of DMU-212 (10 μM) treatment, the cells were harvested and the total RNA was isolated (Chomczynski and Sacchi, 1987). The RNA integrity was confirmed by denaturing agarose gel electrophoresis and its concentration was quantified by measuring the optical density at 260 nm. RNA samples were treated with DNase I and reverse-transcribed into cDNA using oligo-dT primers.

PCR-array and RTq-PCR analysis were conducted in a LightCycler® Instrument 480 MultiwellPlate 96 (Roche, Mannheim, Germany) using a LightCycler® 480 Probes Master kit. Target cDNA was quantified using the relative quantification method. In the case of negative control, cDNA was not added.

RealTime ready Human Apoptosis Panel 96 (Roche, Mannheim, Germany) was used to conduct PCR-array analysis. For amplification, 20 μl cDNA solution was added to 980 μl of LightCycler® 480 Probes Master kit from Roche and then transferred in 10 μl volume to each primer- and probe- based well of the 96-well format real-time PCR. The quantity of 84 pro- and anti- apoptotic genes was standardized by seven housekeeping genes.

For RTq-PCR analysis of CYP1A1 and CYP1B1 transcript levels, 1 μl of total (20 μl) cDNA solution was added to the mixture of LightCycler® 480 Probes Master kit (Roche, Mannheim, Germany), primers and probes for CYP1A1 and CYP1B1. The quantity of CYP1A1 and CYP1B1 in each sample was standardized by ACTB and GAPDH transcript levels. Primer-sites and probe-sites were selected with the aid of the Universal Probe Library Assay Design Center of Roche Applied Science (Table 1).

2.6. SDS–PAGE and Western blot analysis

Cells were treated with RIPA lysis buffer. Next, 30 μg of protein were resuspended in sample buffer and separated on 10%

Table 1
Oligonucleotide sequences and probe number used for RTq-PCR analysis.

Transcript	Sequence (5′–3′ direction)	Probe number	Gene accession number	Product size (bp)
CYP1A1	5′ ggggcgttgtgtctttgtaa 3′ 5′ tgggttgacccatagcttct 3′	59	NM_000499.3	64
CYP1B1	5′ ggcattagagtcaactacacaaagc 3′ 5′ gaatggcaagtgccaaaaa 3′	61	NM_000104.3	67
ACTB	5′ aagtcccttgccatcctaaaa 3′ 5′ atgctatcacctcccctgtg 3′	55	NM_001101.3	91
GAPDH	5′ ctctgctcctcctgttcgac 3′ 5′ acgaccaaatccgttgactc 3′	60	NM_002046.3	112

Tris–glycine gel using SDS–PAGE. Gel proteins were transferred to nitrocellulose, which was blocked with 5% milk in Tris buffered saline/Tween. Immunodetection was performed with rabbit polyclonal anti-CYP1A1 Ab (sc-20772), rabbit polyclonal anti-CYP1B1 Ab (sc-32882), rabbit polyclonal anti-BAX Ab (sc-6236), rabbit polyclonal anti-BCL-2 Ab (sc-492) and mouse polyclonal anti-cleaved PARP-1 Ab (sc-56196) followed by incubation with goat anti-rabbit HRP- conjugated Ab (sc-2004) and goat anti-mouse HRP- conjugated Ab (sc-2005). The membranes were also incubated with anti-actin (ACTB) HRP conjugated Ab (sc-1616) to ensure equal protein loading of the lanes. Bands were revealed using SuperSignal West Femto maximum sensitivity substrate Pierce Biotechnology Inc. (Rockford, IL).

2.7. High performance liquid chromatography

The determination of DMU-212 in cells and culture medium was performed using a Shimadzu System (Shimadzu, Kyoto, Japan) equipped with a L-C-10 AD VP pump, a SCL-10 A VP interface, and a UV–Vis SPD-M10 A VP detector (set at a wavelength 326 nm). Chromatographic separation of DMU-212 was performed on a LUNA C18 column (4, 60, 250 mm; 5 μm; Phenomenex, Torrence, USA) at a flow rate of 1 ml/min. Two mobile phases were used: 30% acetonitrile, 0.1% trifluoroacetic acid (mobile phase A), and 100% acetonitrile (mobile phase B). The linear gradient system ranged from 0% to 100% of mobile phase B over 50 min of analysis. Subsequently at the end of analysis 100% phase A was used to equilibrate the column for 8 min before application of the next sample. The sample injection volumes were 100 μl. Calibration was accomplished using the external standard method at a concentration range of 0.1–75 μg/ml.

2.8. Statistical analysis

Data were expressed as means ± SD for three independent experiments. Statistical analysis was performed with one way analysis of variance ANOVA followed by Student–Newman–Keuls test. For this purpose GraphPad Prism 5.0 version for Windows was employed. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of DMU-212 on colon cancer cells viability

The inhibitory effect of compound tested was investigated in colon DLD-1 and LOVO cancer cells by MTT assay. As shown in Fig. 1, DMU-212 displayed much more potent cytotoxic effect on DLD-1 cells relative to LOVO cell line. DMU-212 inhibited DLD-1 cells by 50% at a concentration of ~1 μM (IC_{50} = 0.93 ± 0.17 μM), whereas LOVO cells were inhibited by 50% at ten-fold higher concentration (IC_{50} = 10.48 ± 1.59 μM).

3.2. Effect of DMU-212 on apoptosis

The induction of apoptosis was assayed by Cell Death Detection ELISA^{PLUS} test. The pro-apoptotic activity of DMU-212 was expressed as an enrichment factor (EF) (Fig. 2A and B). Exposure of either cell line to both concentrations tested, 5 μM and 10 μM, resulted in a statistically significant increase in the level of nucleosomes as compared to control. However, this up-regulation was more pronounced in DLD-1 lysate, EF = 4.47 and 5.08, than in LOVO, EF = 1.98 and 2.89, respectively (Fig. 2A and B). The number of necrotic cells in both DLD-1 and LOVO supernatants was also determined; no statistically significant differences as compared to controls were found (data not shown).

The activation of caspase-9 occurred in both cancer cell lines following DMU-212 treatment, however, to a smaller degree in LOVO cells (Fig. 2C and D). The increase in the activity of caspase-3/7 was similar in DLD-1 and LOVO cells (Fig. 2E and F). No effect of DMU-212 on caspase-8 activity was observed (data not shown).

3.3. Effect of DMU-212 on the expression of apoptosis related genes and proteins

To elucidate the mechanism by which DMU-212 induces apoptosis, the expression of pro- and anti-apoptotic genes in both cell lines was investigated. Significant changes were found in the expression profile of genes driving mitochondria-mediated apoptosis at 24 h after the treatment of DLD-1 and LOVO cells with 10 μM of DMU-212. DMU-212 induced a marked increase in the expression of Bax, Apaf-1 and Bad in DLD-1 cells by 5-, 4- and 3-fold, respectively. Twofold increase in the expression of p53, Bak1, Bik, Bok and Noxa was also noted (Fig. 3A). Concomitantly, DMU-212 suppressed the mRNA levels of anti-apoptotic genes related to intrinsic apoptosis pathway: Bag1, Bcl-2, Bcl-xL in DLD-1 cells, as compared to control (Fig. 3B). In LOVO cells exposed to DMU-212, we observed an increased expression of pro-apoptotic Apaf-1, p53, Bad, Bak1, Bik, Bok and Noxa genes (Fig. 3C), while decreased Bag1 and Bcl-2 mRNA levels were noted (Fig. 3D). No statistically significant differences in the expression of Bax and Bcl-xL in LOVO cell line were observed (Fig. 3C and D). As demonstrated in Western-blot analysis, no effect of DMU-212 on BAX protein expression in LOVO cells was found, however, BCL-2 was slightly decreased. On the contrary, in DLD-1 cell line we observed an increased BAX and decreased BCL-2 protein level after DMU-212 treatment (Fig. 3E), which was consistent with their mRNA expression (Fig. 3A and B). Additionally, 89 kDa fragment of cleaved PARP-1 protein was determined. Exposure of either cell line to DMU-212 resulted in an increased cleaved PARP-1 protein expression as compared to control. However, this up-regulation was more pronounced in DLD-1 than in LOVO cell line (Fig. 3F).

3.4. Effect of DMU-212 on CYP1A1 and CYP1B1 mRNA and protein expression

The relative abundance of CYP1A1 and CYP1B1 mRNA in untreated DLD-1 and LOVO cell lines is shown in Fig. 4A. We found that CYP1B1 transcript expression in LOVO cells was significantly lower as compared to DLD-1 cell line. No statistically significant difference in the expression of CYP1A1 in both colon cancer cell lines was observed (Fig. 4A). As demonstrated in Western-blot analysis, the levels of CYP1A1 and CYP1B1 proteins expression paralleled those in transcripts (Fig. 4B). CYP1B1 protein was not

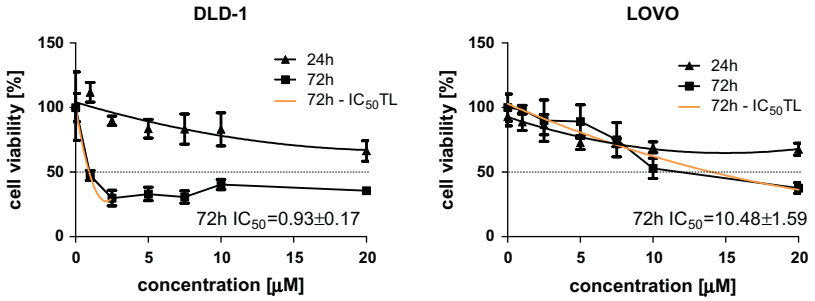

Fig. 1. Effect of DMU-212 on DLD-1 and LOVO cells viability. Cells were treated with DMU-212 at the range of concentration 0–20 μM for 24 and 72 h. IC_{50} values were determined by MTT assay for 72 h incubation. Results of three independent replicates are presented as mean ± SEM.

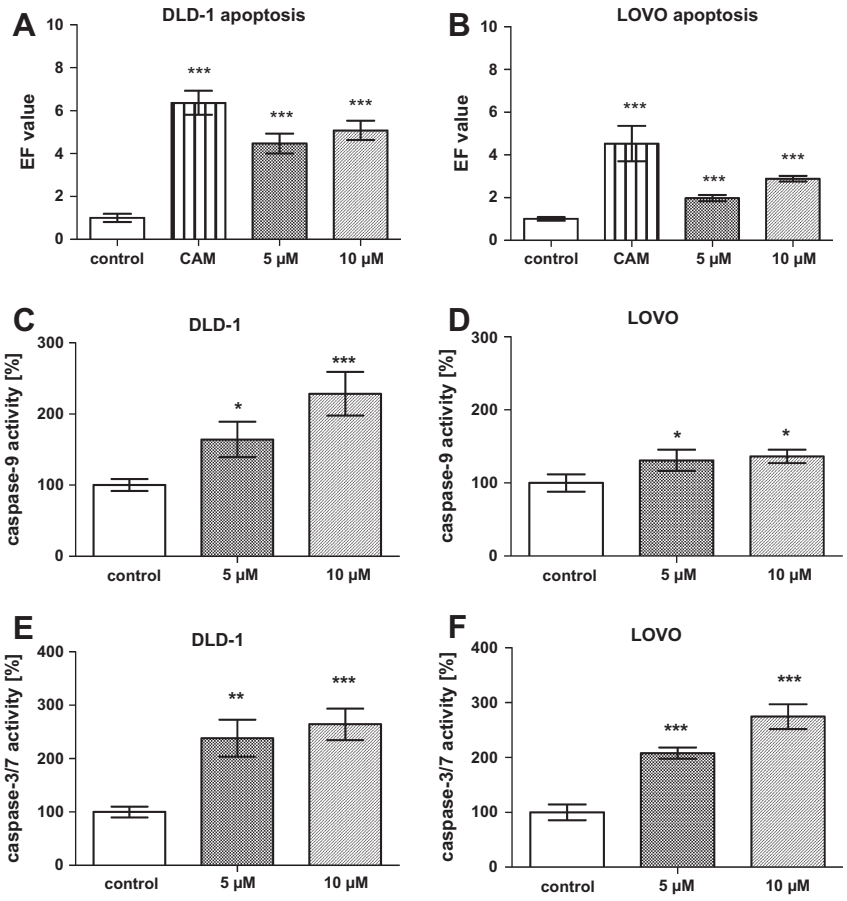

Fig. 2. Effect of DMU-212 on apoptosis induction in DLD-1 and LOVO cells. DLD-1 and LOVO cells were treated for 24 h with vehicle or with 5 μM and 10 μM of DMU-212. Apoptosis induction was assayed by ELISA test and expressed as enrichment factor (EF) in DLD-1 (A) and LOVO (B) cells. Camptothecin (CAM) was used as a positive control for apoptosis. The casp-9 activity was measured using Caspase-9 colorimetric assay kit in DLD-1 (C) and LOVO (D) cells. The casp-3/7 activity was determined by Caspase-Glo® 3/7 luminescent assay in DLD-1(E) and LOVO (F) cells. Results of three independent replicates are presented as mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate a significant difference from the control.

detected in LOVO cell line and the CYP1A1 protein level was slightly lower as compared to DLD-1 cells (Fig. 4B).

Exposure of DLD-1 cells to DMU-212 resulted in down-regulation of CYP1A1 transcript and protein expression as compared to control. Furthermore, CYP1B1 mRNA and protein were not detected in these cells after DMU-212 treatment (Fig. 5A and B). In LOVO cell line we did not observe the effect of the compound tested on CYP1A1 and CYP1B1 mRNA expression (Fig. 5A). Similarly, CYP1A1 protein level was not changed as compared to control (Fig. 5B).

3.5. Comparison of uptake and decrease of DMU-212 in DLD-1 and LOVO cells

Uptake and decline of DMU-212 in both cell lines used was investigated by HPLC. The compound tested reached the highest concentration in LOVO cells after 70 h incubation (Fig. 6). Concomitantly, in DLD-1 cells the concentration of DMU-212 was growing within the first 24 h, and then a gradual decline was observed. In the medium we noted a time-dependent decrease in DMU-212 concentration in both cell lines used (Fig. 6).

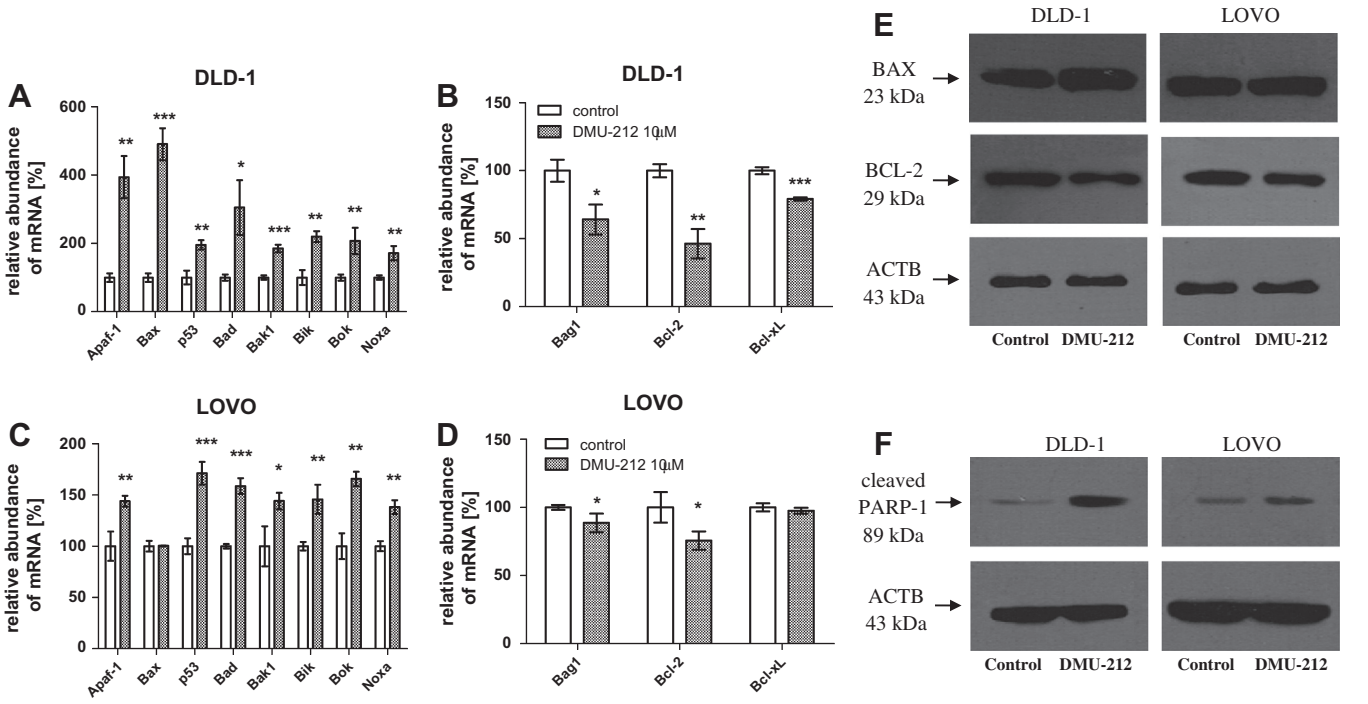

Fig. 3. Effect of DMU-212 on the expression of apoptosis-related genes and proteins in DLD-1 and LOVO cell lines. The expression of pro-apoptotic Apaf1, Bax, p53, Bad, Bak1, Bik, Bok, Noxa genes in DLD-1 (A) and LOVO (C) cells. The expression of anti-apoptotic Bag1, Bcl-2, Bcl-xL genes in DLD-1 (B) and LOVO (D) cells. The PCR-array was used to analyze the level of indicated genes in DLD-1 (A, B) and LOVO (C, D) cells treated for 24 h with vehicle or DMU-212 (10 μM). Results of three independent replicates are presented as mean ± SEM. $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control. Western blot was performed to analyse BAX and BCL-2 (E) and cleaved PARP-1 (F) protein expression in both cancer cell lines treated for 24 h with vehicle or 10 μM DMU-212.

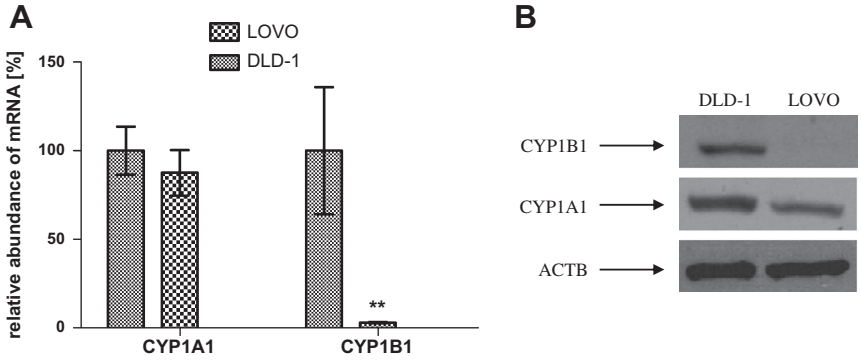

Fig. 4. Comparison of CYP1A1 and CYP1B1 mRNA and protein expression in DLD-1 and LOVO cell lines. (A) The RT-qPCR analysis of CYP1A1 and CYP1B1 mRNA relative content in untreated cells. Results of three independent replicates are expressed as mean ± SEM. $^{**}P < 0.01$ as compared to CYP1B1 mRNA relative abundance in DLD-1 cells. (B) Western blot analysis of CYP1A1 and CYP1B1 proteins in both cell lines used.

4. Discussion

In the present study we evaluated the anticancer activity of 3,4,4′,5-tetramethoxystilbene (DMU-212) in two colon cancer cell lines, DLD-1 and LOVO. The MTT assay revealed that the compound tested was more potent in DLD-1 than in LOVO cells. Our observation confirmed the previous findings evidencing varied sensitivity of colon cancer cell lines to DMU-212 (Sale et al., 2004). However, the mechanism of its cytotoxic activity has not been clarified in these cells.

Our previously published results and other authors' findings have revealed that decreased viability of ovarian, lung and breast cancer cells treated with DMU-212 was related to its ability to induce apoptosis (Gosslau et al., 2005; Ma et al., 2008; Piotrowska et al., 2012). We confirmed this mechanism in colon cancer cell lines since the stronger cytotoxic activity of DMU-212 was observed in DLD-1 cells in which the compound tested triggered the greater pro-apoptotic effect, as compared to LOVO cell line.

Although an apoptotic response upon cancer therapy often proceeds through activation of the Fas receptor/ligand system, several studies have suggested that the release of cytochrom c from mitochondria is a crucial mechanism by which cytotoxic drugs kill tumor cells (Eichhorst et al., 2000; Fulda et al., 2000; Hakem et al., 1998; Yoshida et al., 1998). DMU-212 has been shown to evoke a mitochondrial apoptotic pathway in transformed fibroblasts and breast cancer cell lines (Gosslau et al., 2005; Ma et al., 2008). However, our previously published results indicated the ability of DMU-212 to induce apoptosis in both mitochondria- and

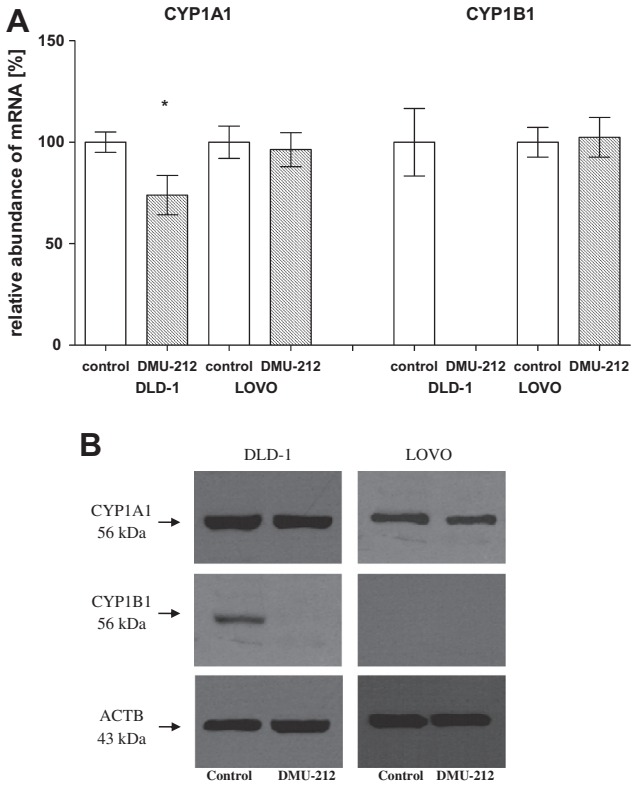

Fig. 5. Effect of DMU-212 on CYP1A1 and CYP1B1 transcript and protein levels in DLD-1 and LOVO cells. (A) The RT-qPCR analysis of CYP1A1 and CYP1B1 mRNA relative abundance in cells treated for 24 h with vehicle or 10 μM DMU-212. Results are presented as percent of change compared to controls. Mean ± SEM of three independent replicates are presented. $^{*}P < 0.05$ indicates a significant difference from the control. (B) Western blot analysis of CYP1A1 and CYP1B1 protein levels in cells treated with vehicle or 10 μM DMU-212.

receptor-mediated manner in ovarian cancer cells (Piotrowska et al., 2012). Therefore, the analysis of the expression pattern of genes driving intrinsic and extrinsic apoptosis pathways may contribute to the elucidation of the mechanism of DMU-212 anticancer action. On the basis of the data from our studies (Piotrowska et al., 2012) and those of others (Gosslau et al., 2005; Ma et al., 2008), in the current experiment we investigated the expression level of 84 pro- and anti-apoptotic genes to identify transcripts unique to DMU-212-induced apoptosis pathway. Conversely to our previous study (Piotrowska et al., 2012), we found that the expression profile of apoptosis-related genes was specific to the intracellular mechanism of apoptosis in both colon cancer cell lines used. The intrinsic apoptosis pathway is regulated by the members of BCL-2 family. They play a crucial role in inducing mitochondrial membrane changes and include both anti-apoptotic molecules, e.g. Bcl-2, Bcl-xL, and Bcl2l2-as well as pro-apoptotic members such as Bax, Bak1, Bok, and BH3 domain-only molecules (Bad, Bik, Noxa) (Antonsson and Martinou, 2000; Burlacu, 2003; Kroemer and Reed, 2000). We found that DMU-212 caused up-regulation of pro-apoptotic Bak1, Bok, Bik, Noxa, Bad and Bax transcripts level in DLD-1 cell line. It is known that Bax transcription is regulated by the tumor suppressor protein p53 (Vousden and Lu, 2002) which is one of the key factors activating Apaf-1, an important component of an oligomeric apoptosome (Adrain and Martin, 2001). On the other hand, p53 may inhibit the expression of Bcl-2, a survival gene that promotes malignant progression (Wu et al., 2001). In DLD-1 cells exposed to DMU-212, we showed a significant increase in pro-apoptotic p53 and Apaf-1 transcripts while the anti-apoptotic Bcl-2, Bcl-xL and Bag1 mRNA expression was down-regulated. Our results are consistent with the previously reported role of DMU-212 in mitochondria-mediated apoptosis (Gosslau et al., 2005; Lu et al., 2001). DMU-212 has been found to cause the induction of p53, the increase in Bax and Bax/Bcl-2 ratio and the activation of intrinsic pathway of apoptosis in transformed human fibroblasts (Gosslau et al., 2005; Lu et al., 2001).

In LOVO cell line, less sensitive to DMU-212, the expression pattern of pro-apoptotic and anti-apoptotic genes was similar to DLD-1 transcripts profile, however, changes in apoptosis-related genes expression were less pronounced. The expression of BAX and BCL-2 proteins, essential for the intrinsic pathway of apoptosis, was consistent with their mRNA level in both cell lines.

To confirm the pathway of apoptosis suggested by the discussed above expression pattern of apoptosis–related genes we have assessed the activity of caspases. Only the level of caspase-9 activation was consistent with the data related to apoptosis in both cell lines exposed to DMU-212. The higher level of apoptosis in DLD-1 cells as compared with LOVO cell line was associated with the higher activity of caspase-9, which is an initiator of caspase cascade in the mitochondrial pathway of apoptosis. No activation of caspase-8 in cells exposed to the compound tested was observed which might suggest that extrinsic pathway of apoptosis was not involved. However, there is some inconsistency between similar activation of caspases-3/7 in both cell lines and greater susceptibility of DLD-1 cells to apoptosis induction. Thus, it might be suggested that another mode of cell death could be additionally involved. To clarify this issue we have assayed the cleavage of

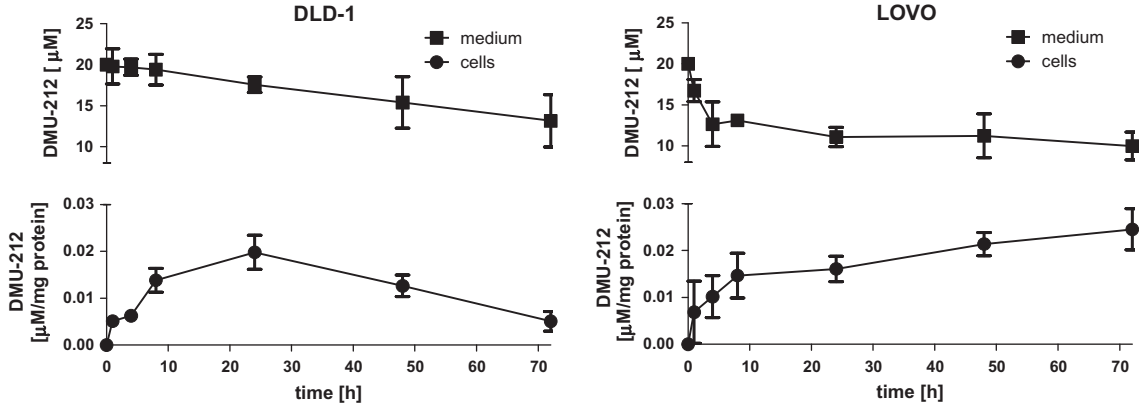

Fig. 6. Uptake of DMU-212 into cytoplasm of DLD-1 and LOVO cells (●) and decrease in DMU-212 levels in incubation medium (■). Cells were treated with DMU-212 for various time intervals, scrapped, centrifuged, lysed by freeze–thaw stress and centrifuged again. The supernatant and medium from the cells were analyzed using HPLC.

poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 is a substrate not only for caspases-3/7 but also for other proteases such as calpains, cathepsins and granzymes and their action result in several specific proteolytic cleavage fragments with different molecular weights (Zhu et al., 2009; Chaitanya et al., 2010). It has been revealed that caspases-3/7, granzyme B as well as cathepsins B and D cleave PARP-1 to a 89 kDa fragment (Zhu et al., 2009; Chaitanya et al., 2010). We have shown that the protein level of 89 kDa fragment was distinctly higher in DLD-1 cells treated with DMU-212 as compared to that in LOVO cells. These findings can suggest the involvement of additional caspases-independent mode of cell death in DLD-1 cells triggered by, for example, cathepsins or granzyme B. Since granzyme B is secreted exclusively by cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells, the contribution of cathepsins appears to be very likely. Cathepsins are a large family of lysosomal enzymes which can also initiate apoptotic cell death independent of caspases (Chaitanya et al., 2010).

Suggested additional pathway of cell death remains, however, speculative and some un-identified factors might be involved in this process.

CYP1A1 and CYP1B1 are known to catalyze the conversion of estradiol to 4-hydroxyestradiol (Hayes et al., 1996). Therefore, the compounds such as resveratrol and its derivatives, which are structurally similar to the endogenous oestrogen estradiol, may also undergo metabolism by these enzymes (Potter et al., 2002b). It has been hypothesized that DMU-212 is biotransformed to the toxic methoxy and hydroxy metabolites by CYP1B1 (Androutsopoulos et al., 2011; Potter et al., 2002a). To have an insight into CYP1 enzymes' role in DMU-212 anticancer activity we assessed the expression of CYP1A1 and CYP1B1 mRNA and protein in both untreated colon cancer cell lines. It is known that CYP1B1 mRNA is expressed in a wide range of normal tissues such as brain, breast, lung, ovary and colon, however, the protein was not detected. On the contrary, in the corresponding malignant tissues, the overexpression of CYP1B1 mRNA and protein has been revealed (McFadyen et al., 2004). Surprisingly, we found that LOVO cell line did not express CYP1B1 protein which was consistent with markedly decreased (by 90%) CYPIB1 mRNA level. It has been also shown that CYP1A1 protein level was slightly lower in LOVO than in DLD-1 cells. DMU-212 did not affect markedly CYP1A1 transcript level and protein expression in cell lines used. However, both mRNA and protein expression of CYP1B1 were completely inhibited in DLD-1 cells. Very low native level of CYP1B1 mRNA in LOVO cells was not affected by DMU-212 treatment, whereas the protein expression was not detected consistently with the lack of CYP1B1 protein in untreated cells.

In the light of our finding that LOVO cells were less susceptible to DMU-212 cytotoxicity as compared to DLD-1 cells, it could be suggested that anticancer activity of DMU-212 is closely related to its biotransformation catalysed by these cytochrome P450 isoenzymes.

To verify this hypothesis we investigated the concentration of unchanged DMU-212 in both colon cancer cell lines used. In DLD-1 cells, which express CYP1A1 and CYP1B1 enzymes, the concentration of DMU-212 was growing within the first 24 h, and then a gradual decline was observed. On the contrary, in LOVO cell line we showed a continued growing tendency in intracellular DMU-212 concentration, which was accompanied with a lower cytotoxicity, as compared to that to DLD-1 cells. Thus, it could be hypothesized that DMU-212 metabolites might be equally or even more active than the parent compound.

In summary, our study shows that DMU-212, a resveratrol analogue, displayed much more potent cytotoxic effect on colon cancer cell line DLD-1 as compared to that exerted on the cell line LOVO derived from the same type of cancer. DMU-212 inhibited the growth of both colon cancer cell lines predominantly via activation of mitochondria-mediated apoptosis pathway. Eichhorst et al. (2000) suggested that different CYP1B1 and CYP1A1 expression pattern in various tumour cells may affect their sensitivity to the potential chemotherapeutics. It cannot be excluded that the lack of the CYP1B1 protein expression in LOVO cells might contribute to the lower susceptibility of these cells towards DMU-212. Further studies aiming at elucidation of CYP1B1 and CYP1A1 role in DMU-212 cytotoxicity have been undertaken in our laboratory.

Conflict of interest

The authors declare that they have no conflict of interest.

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