

Glyceryl trinitrate-induced cytotoxicity of docetaxel-resistant prostatic cancer cells is associated with differential regulation of clusterin

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Abstract. Metastatic castration resistant prostate cancer (mCRPC) relapse due to acquired resistance to chemotherapy, such as docetaxel, remains a major threat to patient survival. Resistance of mCRPC to docetaxel can be associated with elevated levels of soluble clusterin (sCLU) and growth differentiation factor-15 (GDF-15). Any strategies aiming to modulate sCLU and/or GDF-15 in docetaxel-resistant prostate cancer cells present a therapeutic interest. The present study reports the cytotoxic effect of a nitric oxide donor, glyceryl trinitrate (GTN), on docetaxel-resistant mCRPC human cell lines and demonstrates that GTN displays greater inhibition of cell viability toward docetaxel-resistant mCRPC cells than on mCRPC cells. It is also demonstrated that GTN modulates the level of expression of clusterin (CLU) which is dependent of GDF-15, two markers associated with docetaxel resistance in prostate cancer. The results indicate that GTN represses the level of expression of the cytoprotective isoform of CLU (sCLU) and can increase the level of expression of the cytotoxic isoform (nuclear CLU) in docetaxel resistant cells. Furthermore, it was observed that GTN differentially regulates the level of the precursor form of GDF-15 between resistant and parental cells, and that recombinant GDF-15 can modulate the expression of CLU isoforms and counteract GTN-induced cytotoxicity in resistant cells. A link was established between GDF-15 and the expression of CLU isoforms. The present

study thus revealed GTN as a potential therapeutic strategy to overcome docetaxel-resistant mCRPC.

Introduction

Prostate cancer is the second most commonly diagnosed cancer in men worldwide and the fifth leading cause of cancer mortality (1). Currently, radical prostatectomy and radiotherapy represent 'salvage therapies' for individuals with localized disease. Unfortunately, treatment options for men with metastatic prostate cancer are not curative. While hormone therapy i.e. androgen deprivation therapy reduces tumor progression, relapse frequently occurs following surgical or chemical castration. Over a decade, docetaxel is the first-line systemic chemotherapy used for metastatic castration-resistant prostate cancer (mCRPC) (2).

Docetaxel is a taxane chemotherapeutic agent that sensitizes cancer cells to apoptosis by binding to β -tubulin and prevents its depolarization blocking cells in the G2/M phase of the cell cycle (3). By stabilizing the microtubules, docetaxel can inhibit a key driver of mCRPC, androgen receptors signaling (4,5). Although docetaxel demonstrated overall survival benefit, the majority of mCRPC patients eventually become refractory to this chemotherapy and carries a poor prognosis (6).

A number of previous studies have shed light into the underlying mechanisms that mediate acquired resistance to docetaxel (6-10). Notably, clusterin (CLU), an important stress-induced chaperone (when overexpressed), is one characterized way that confers docetaxel resistance in mCRPC (11). CLU, also known as Apolipoprotein J, is an ATP-independent glycoprotein present in all human tissues and fluids (12). It exists in at least two forms with different subcellular localization and antagonistic functions: The soluble clusterin (sCLU) and the nuclear clusterin (nCLU) form, reported to serve distinct roles in cancer, protumoral and antitumoral, respectively (13). The sCLU is the most predominant form. It is a heterodimeric protein comprising two subunits (α and β) of ~40 kDa each. This form bears endoplasmic reticulum signal peptide sequence that directs the protein to the endoplasmic reticulum and then to the Golgi apparatus where it undergoes

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various post translational modifications during maturation. The mature protein (80 kDa) is then cleaved by a furin-like convertase to produce the two subunits (14). Elevated levels of sCLU support a cytoprotective role through the inhibition of pro-apoptotic signaling pathways, in cells challenged with different therapeutic agents allowing them to mediate resistance to treatment induced-apoptosis (15,16). Several preclinical studies have been performed investigating the inhibition of the level of CLU and have exhibited enhanced chemosensitivity of human prostate cancer cells to treatment-induced apoptosis and delay tumor progression (17,18). This has led to the clinical development of OGX-011 (custirsén), a second-generation antisense oligonucleotide inhibitor of CLU (19,20). The results of two phase II studies of custirsén in combination with docetaxel or second-line chemotherapy in men with mCRPC reported reduced CLU expression within tumor cells, as well as lowered serum CLU levels which are correlated with improved survival (21-23).

An alternative splicing of CLU mRNA generates nCLU (55 kDa) that lacks the endoplasmic reticulum signal peptide sequence and localizes in the nucleus. In contrast, the cytotoxic activity of nCLU in prostate cancer cells is acknowledged. Evidence for an antitumoral role of nCLU is demonstrated through the promotion of cell cycle arrest and antimetastatic activity in prostate cancer cells by decreasing their motility and progression (24,25). Besides sCLU and nCLU, other intracellular non-secreted CLU isoforms are expressed within stressed cells but at a very low level (26).

Cytokines, including growth differentiation factor-15 (GDF-15), a member of the transforming growth factor (TGF) superfamily, have been reported to be substantially induced in prostate cancer cells exposed to docetaxel and mitoxanthrone chemotherapy and to contribute to tumor cell therapy resistance (27,28). Increased serum GDF-15 levels following one cycle of docetaxel regimen was associated with a shorter overall survival suggesting that GDF-15 could predict for early docetaxel resistance (28). Notably, GDF-15 has been reported to exert also a proapoptotic function in prostate cancer cells (29).

There are now novel therapeutic agents approved in the treatment of mCRPC following docetaxel failure, including cabazitaxel, a second-generation of taxane (30). Nevertheless, there is a growing interest in developing novel therapeutic approaches to overcome resistance to docetaxel and to provide better disease control in mCRPC. Previous preclinical studies have demonstrated the efficacy of nitric oxide (NO) donor therapy for the treatment of prostate cancer (31,32). Many NO donors have been demonstrated to be potent chemosensitizer and/or radiosensitizer against a wide variety of human tumor cells (33-36). It is now well documented that NO exerts dual activities in cancer: Protumoral or antitumoral. NO donors-induced anti-tumoral activities in prostate cancer cells are due to their ability to simultaneously inhibit cell survival, cell growth pathways and sensitize tumor cells to apoptosis. In prostate cancer cells, apoptosis can be positively regulated by NO through the S-nitrosylation and inhibition of nuclear factor-kappa B (NF- κ B) and subsequent regulated resistant factors such as Yin Yang 1 (YY1) and B cell lymphoma-2 (BCL2)/BCL-extra large (BCL-XL) (32). In addition, a direct role for NO with the S-nitrosylation of YY1 has

been evidenced in reversing tumor necrosis factor-related apoptosis-inducing ligand-resistant prostate cancer cells (37). Furthermore, the therapeutic efficacy of NO donors in the inhibition of epithelial-to-mesenchymal transition phenotype and metastasis has been demonstrated in metastatic human prostatic cancer cells (38,39).

In this present study the sensitivity of docetaxel-resistant human prostate cancer cells to the NO donor glyceryl trinitrate (GTN) and the regulation of resistant markers were examined to explore novel therapeutic strategies for targeting mCRPC.

Materials and methods

Cell culture. The human mCRPC cell line DU145 was obtained from the American Type Culture Collection (Manassas, VA, USA). The PC3 AG and docetaxel-resistant derivative PC3-D12 cell lines were kindly provided by Professor B. Watson (University College Dublin, Dublin, Ireland) (40). The docetaxel-resistant DU145 (DU145-DR) cell line was established within the team. The DU145 parental cells were seeded in T25 flasks (2×10^6 cells) and treated twice a week with increasing doses (0.01, 0.1, 0.5, 0.75, 1, 5 and 10 nM) of docetaxel (Sanofi S.A., Paris, France). Doses were increased at intervals of 2-3 weeks, dependent on the rate of cell proliferation. Following each step, when the cells stopped proliferating and exhibited a modified morphology, as observed via microscopy, docetaxel treatment was ceased immediately and the cells were placed in complete medium. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-4.5 g/l glucose supplemented with 10% fetal calf serum (FCS) (both from Dominique Dutscher SAS, Brumath, France) at 37°C in a humid atmosphere of 5% CO₂. All cell lines were mycoplasma free.

Viability test. Cells were seeded in 96-well plates (2,000 cells/well) in complete DMEM medium (100 μ l/well). Following 24 h, the DMEM medium was replaced with Opti-MEM™ medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for DU145 lines and Opti-MEM™ supplemented with 1% FCS for PC3 cell lines. The cells were treated with docetaxel (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, 102.4, 204.8, 409.6 or 819.2 nM) or GTN (25, 50, 100, 200 or 400 μ M; Merck KGaA, Darmstadt, Germany) supplemented or not with 5 ng/ml of human recombinant GDF-15 (R&D Systems, Inc., Minneapolis, MN, USA). Following 72 h of treatment, cell viability was evaluated by adding MTS (Promega Corporation, Madison, WI, USA). Absorbance (abs) was read following 4 h at 490 nm. The following formula was used to obtain the percentage of cell viability: % viable cells = [(abs_{sample} - abs_{blank}) / (abs_{control} - abs_{blank})] x 100. Half maximal inhibitory concentration (IC₅₀) calculations were determined using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA).

Cell transfection. Cells were seeded in 6-well plates (DU145 and DU145-DR at 3×10^5 cells/well; PC3 AG and PC3-D12 at 2×10^5 cells/well) in complete medium and incubated at 37°C the day prior to transfection. DU145 and DU145-DR cells were transfected with 50 nM small interfering RNA (siRNA) control and SmartPool anti-human CLU-siRNA (cat. no. L-019513-00-0005; GE Healthcare Dharmacon, Inc.,

Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as a transfection reagent. The transfection of PC3 AG and PC3-D12 cell lines was carried out with 5 nM siRNA using Dharmafect 2 (GE Healthcare Dharmacon, Inc.) as a transfection reagent. At 5 h following transfection, the medium was replaced and GTN treatments at 100 μ M were performed. Cells were incubated for 72 h.

To establish the DU145-DR fluorescent cell line, DU145-DR cells were stably transfected with 2.5 μ g/ μ l pCMV-DsRed plasmid (Clontech Laboratories, Inc., Mountainview, CA, USA), supplemented with 60 μ l Superfect (Qiagen GmbH, Hilden, Germany). The positive cells were selected using Genitocin (750 μ g/ml, determined as the minimum effective concentration to kill non-resistant cells; Gibco; Thermo Fisher Scientific, Inc.) and sorted by flow cytometry following 2 weeks of selection.

Western blotting. Western blotting was performed to examine the expression of clusterin and pro-GDF-15. Cells were treated with the indicated concentrations of GTN in presence or absence of human recombinant GDF-15. Cells were lysed in boiling buffer (150 mM NaCl, 150 mM TrisHCl pH 7.4, 1% SDS and 1 mM sodium orthovanadate) supplemented with protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The viscosity of the samples was reduced by several passages through a 26-gauge needle. Proteins concentrations were measured using the DC™ protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The proteins (from whole-cell extracts or cell supernatants) were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad Laboratories, Inc.). After blocking non-specific binding sites overnight at 4°C by 5% non-fat milk in PBS with Tween-20 0.1%, the membranes were incubated with the appropriate primary antibody: Monoclonal mouse anti-human clusterin (MAB29372; 1:1,000) polyclonal goat anti-human GDF-15 (BAF940; 1:1,000) (both from R&D Systems, Inc.), monoclonal mouse anti-human β -actin (SC-47778; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) or monoclonal mouse anti-human heat shock cognate 70 (HSC70; SC-7298; 1:1,000; Santa Cruz Biotechnology, Inc.) overnight at 4°C, then with the secondary antibody peroxidase AffiniPure goat anti-mouse IgG (H+L; 115-035-003; 1:5,000; Jackson ImmunoResearch Europe, Ltd., Newmarket, UK) or streptavidin protein Dylight 800 (1:10,000; Thermo Fischer Scientific, Inc.) for 1 h at room temperature. The level of protein expression was analyzed using the Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA) or the Molecular Imager Chemi Doc™ XRS+ (Bio-Rad Laboratories, Inc.). β -actin or HSC70 were used as loading control for cell extracts, and Ponceau red (Sigma-Aldrich; Merck KGaA) staining was used as the loading control for cell supernatants. Densitometric analyses of protein levels were performed using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA).

Zebrafish model. All zebrafish experiments were conducted according to the French and European Union guidelines concerning laboratory animal handling. The animal procedures described were reviewed and approved by the local Ethics Committee (C2EA 'Comité d'Ethique en Expérimentation Animale', Grand Campus Dijon, registered

no. 105 by the national Ethics Committee CNREEA 'Comité National de Réflexion Ethique sur l'Expérimentation Animale'). In the present study a transgenic zebrafish line *flila*: Enhanced Green Fluorescent Protein (EGFP) expressing EGFP in endothelial cells under the *flila* promoter was used (Zebrafish International Resource Center, University of Oregon, Eugene, OR, USA). This model allows the following of eventual neovascularization at the yolk sac level following a microinjection of tumor cells (41). Adult zebrafish (15 males and 15 females) were maintained in a recirculating aquaculture system (Müller & Pfleger GmbH & Co. KG, Rockenhausen, Germany) with a temperature range of 26-28°C, and a 14/10-h light/dark cycle as previously described (41). They were fed twice a day with dried flake food. The mean ranges for conductivity and pH in the system were 600-700 μ S and 6.0-8.0, respectively. At 2 days prior to the xenotransplantation, males and females were placed in the same tanks and mating was triggered by light stimuli the following day. Eggs were collected in sourcing water a few hours following the fertilization, counted, sorted and up to 50 eggs placed at 28°C per Petri dish.

Xenotransplantation and treatment procedure. Zebrafish larvae were dechorionized by pronase (1 mg/ml; Roche Diagnostics) for 20 min prior to micro-injection and arrayed on a Petri dish. The injection of DsRed expressing DU145-DR cells was carried out under a fluorescence magnifying glass (Leica MZFLII) using micro-injectors (Eppendorf Femtojet) (20-100 tumor cells/injection) as described previously (41,42). The injected larvae were incubated at 32°C in saturated humid conditions. At 1 day following the injection, the larvae were anesthetized with 0.17 mg/ml tricaine (Sigma-Aldrich; Merck KGaA) and sorted according to their red fluorescence using a Leica MZFLIII fluorescence stereomicroscope bearing appropriated filters with 12.5:1 zoom leading to a maximum magnification of x800 with 10x micro objectives (Leica Microsystems, Inc., Buffalo Grove, IL, USA). The positive larvae were then treated with 10 μ M GTN for 5 days. Non-positive larvae were euthanized with tricaine (0.3 mg/ml).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from zebrafish larvae using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and 1 μ g RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase with random hexamers (both from Promega Corporation) according to manufacturer's protocol. cDNA was quantified by qPCR on a 7500 Fast system (Life Technologies; Thermo Fisher Scientific, Inc.) using the standard SYBR-Green PCR Master mix detection protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Standard reaction volume was 20 μ l and contained 10 μ l SYBR-Green mix, 2 μ l cDNA template and 125 nM of primers. The thermocycling conditions were composed of an activation step at 50°C for 2 min, a denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 sec and primer annealing/extension at 60°C for 1 min. The final step was a 60°C incubation for 1 min. All reactions were performed in triplicate. The mRNA abundance was calculated according to the $2^{-\Delta\Delta C_q}$ method analysis (43). Expression of all genes was normalized respective to human L32 and zebrafish actin expression levels. The primers used were as

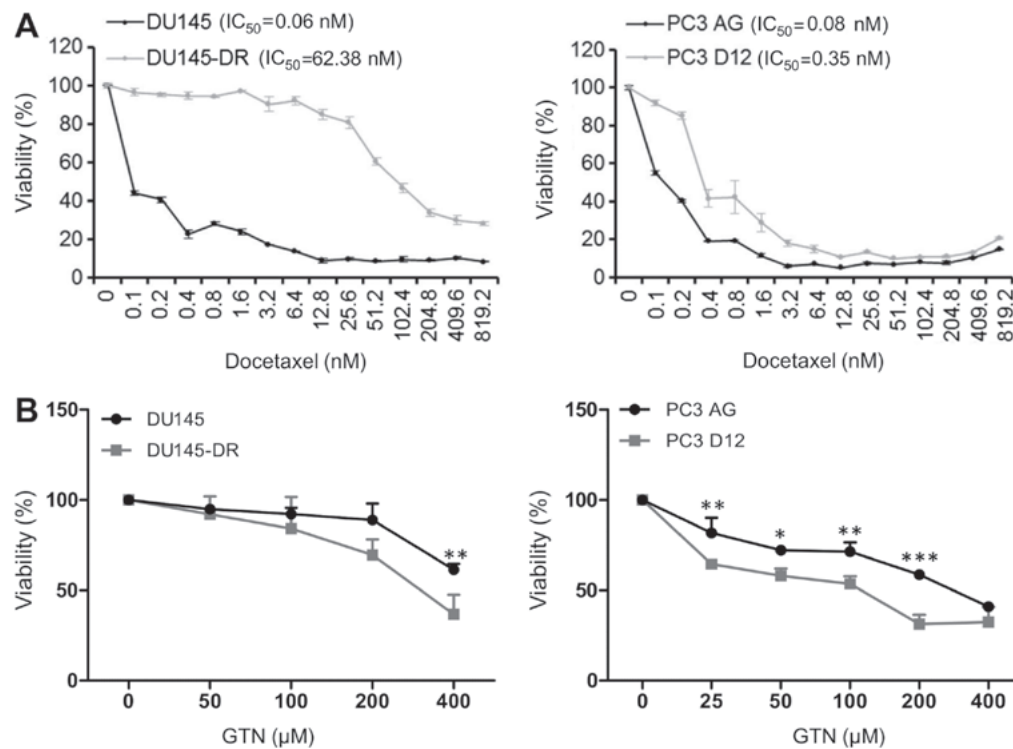


Figure 1. Docetaxel-resistant mCRPC cells are sensitive to GTN. Docetaxel-resistant (DU145-DR and PC3 D12) or parental (DU145 and PC3 AG) mCRPC cell lines were treated for 72 h with a range of (A) docetaxel or (B) GTN concentrations as indicated. Cell viability was determined and the half maximal inhibitory concentration values following docetaxel and GTN treatments were estimated from the concentration-response curve. Data are presented as the mean \pm SD of three independent experiments. Significant differences were determined by applying a two-way analysis of variance test for each pair (DU145 vs. DU145-DR and PC3 vs. PC3 D12). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. DU145-DR or PC3 D12 as appropriate. mCRPC, metastatic castration resistant prostate cancer; GTN, glyceryl trinitrate; ANOVA, analysis of variance.

follows: Human clusterin, forward, 5'-CCG CAA AAA GCA CCG GGA GGA-3' and reverse, 5'-GGG CTG CAG CTC ATC TTG GGG-3'; zebrafish clusterin, forward, 5'-AAG AGG AAG AAT CAA AGC AGG TGT-3' and reverse, 5'-GTA GAG GAG AAA CAG CCC CAG-3'; human cyclin D1, forward, 5'-CCC TGA CAG TCC CTC CTC T-3' and reverse, 5'-GAA GGG GGA AAG AGC AAA G-3'; human L32, forward, 5'-TGT CCT GAA TGT GGT CAC CTG-3' and reverse, 5'-CTG CAG TCT CCT TGC ACA CCT-3'; and zebrafish actin, forward, 5'-CCC AGA CAT CAG GGA GTG AT-3' and reverse, 5'-CAC AAT ACC GTG CTC AAT GG-3'.

Cytokine array analysis. Cell culture supernatants from DU145, DU145-DR, PC3 AG and PC3-D12 were analyzed using RayBio® human cytokine array C3 and C9 according to the manufacturer's recommendation (RayBiotech, Inc., Norcross, GA, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation of the indicated number of experiments. Significant differences were evaluated using Student's t-test, one-way analysis of variance with a post-hoc Tukey's test or two-way analysis of variance with a post-hoc Bonferroni's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Docetaxel-resistant human prostatic cancer cells are sensitive to GTN. In the current study, two human prostatic cancer cell

lines were utilized, both chemosensitive (DU145 and PC3AG) and chemoresistant (DU145-DR and PC3-D12) to docetaxel. In order to generate DU145-DR cells, DU145 cells were treated with increasing concentration of docetaxel. The cell viability in the chemosensitive and chemoresistant cells to docetaxel was first assayed by MTS assay in the presence of a range of docetaxel concentrations. In response to increasing concentrations of docetaxel, an increased survival rate was observed in prostatic resistant cancer cell lines DU145-DR and PC3-D12 in comparison with the parental cells, which confirmed the docetaxel resistant phenotype of DU145-DR and PC3-D12. DU145-DR and PC3 D12 cells demonstrated a greater resistance to docetaxel compared with their parental counterparts (DU145 DR IC_{50} =62.38 nM vs. DU145 IC_{50} =0.06 nM; PC3D12 IC_{50} =0.35 nM vs. PC3-AG=0.08 nM; Fig. 1A).

Notably, when compared with the parental cell lines, DU145-DR and PC3-D12 cell lines chemoresistant to docetaxel exhibited increased sensitivity to the antiproliferative effect of GTN used at various concentrations (ranging from 25-400 μ M) over a time course of 72 h (Fig. 1B).

GTN-induced cytotoxicity is attributable to differential regulation of CLU. Having demonstrated that docetaxel-resistant cell lines were more prone to GTN cytotoxicity, it was attempted to identify the molecular mechanisms underlying this effect. The level of clusterin, a key docetaxel resistant marker in human prostatic cancer, was investigated. The basal levels of the secretory form of clusterin (sCLU; anti-apoptotic function) in the supernatant from docetaxel-resistant and parental mCRPC

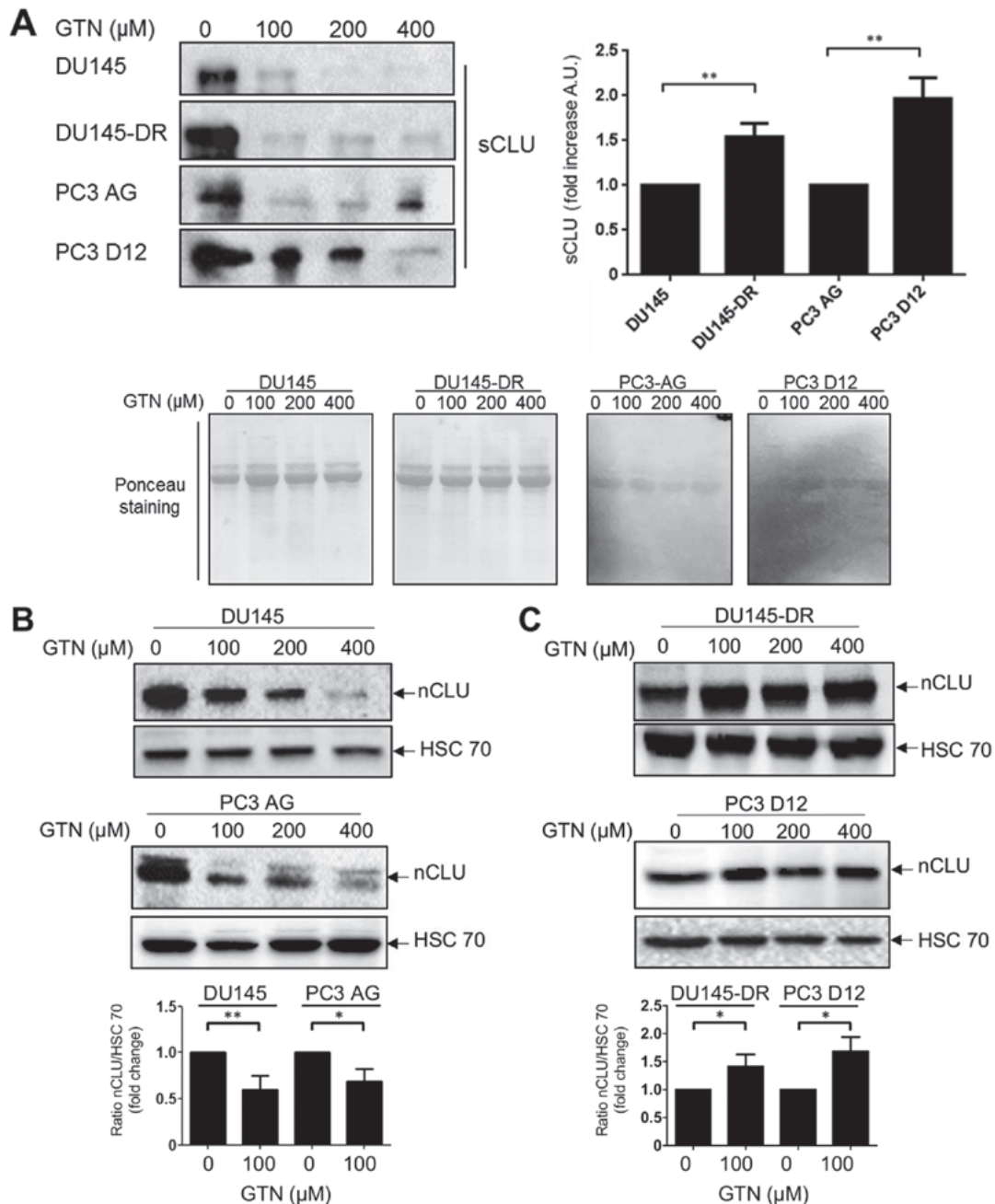


Figure 2. Differential regulation of clusterin isoforms by GTN. Western blot analysis of sCLU and nCLU levels in (A) culture supernatants and (B and C) whole-cell extracts from mCRPC cells. The blots presented are representative of three independent experiments. The histograms present quantification of sCLU and nCLU using densitometric analyses of three independent experiments. (A) The data are presented as mean-fold increase (\pm standard deviation) over basal level in DU145 and PC3 AG cells. The blots were stained with Ponceau solution as loading control of cell culture supernatant samples. (B and C) Docetaxel-sensitive/resistant mCRPC cell lines were treated for 72 h with GTN concentrations ranging from 0-400 μM . The constitutive HSC70 was used as loading control. The immunoblotting analysis of sCLU and nCLU in both (B) docetaxel-sensitive and (C) -resistant cells (PC-3 or DU145) was performed in parallel. Data represent the mean ratio nCLU/HSC70 \pm standard deviation in mCRPC cells treated with GTN (100 μM) over basal values (cells left untreated) from three independent experiments. Statistically significant differences were determined using a Student's t-test. * $P < 0.05$; ** $P < 0.01$. GTN, glyceryl trinitrate; sCLU, soluble clusterin; nCLU, nuclear clusterin; mCRPC, metastatic castration resistant prostate cancer; HSC70, heat shock cognate 70.

cell lines were evaluated. It was observed that the level of sCLU was significantly higher in docetaxel-resistant cells compared with parental cells (Fig. 2A). Notably, a reduction in sCLU protein expression was observed in all cell lines upon GTN treatment at 100 μM for 72 h, except for the PC3-D12 cells, in which a reduction of the level of expression was observed only with higher concentrations of GTN (400 μM). The nCLU protein expression level was then evaluated. It was observed that GTN induced a significant reduction in

the levels of nCLU compared with control in the parental cells (Fig. 2B) and an increase in the levels of nCLU compared with control in the resistant cell lines (Fig. 2C), in accordance with their sensitivity to GTN-induced cytotoxicity (Fig. 1B). These results may suggest that GTN regulates the balance of proapoptotic and antiapoptotic levels of expression of CLU, thereby favoring death over survival.

The biological involvement of the modulation of CLU expression in GTN-induced cytotoxicity for

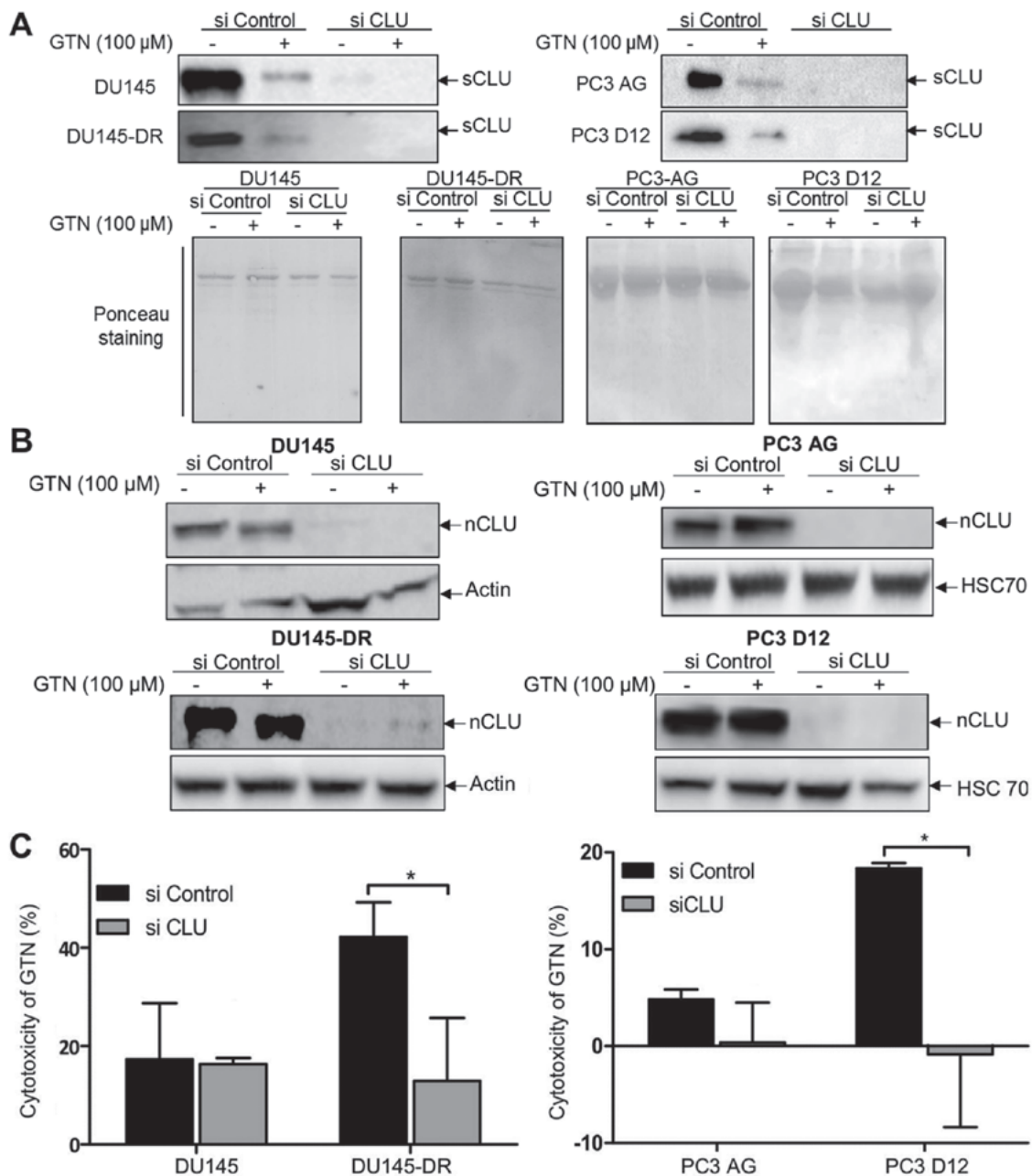


Figure 3. GTN-induced cytotoxicity is dependent on levels of clusterin isoforms. Analysis of (A) sCLU and (B) nCLU levels by western blotting with extracts obtained from mCRPC cell lines transiently transfected with control siRNA (si-Control) or clusterin siRNA (si-CLU) and treated or not with 100 μ M GTN for 72 h. sCLU level was evaluated in the culture supernatants and nCLU in total lysate extracts. Ponceau staining is shown as loading control for analysis of sCLU in cell culture supernatants samples. β -actin and HSC70 (for analysis of nCLU in whole-cell extracts) were used as loading controls. GTN-induced cytotoxicity on siRNA transfected docetaxel-sensitive/resistant human prostate cancer cells was measured. (C) Cytotoxicity was indirectly determined by subtracting the viability values from the total values (100%) in non-treated cells. Transfected mCRPC cells were treated with 100 μ M GTN for 72 h. Data represent the mean \pm standard deviation of 3 independent experiments. Significant differences were determined by using a two-way analysis of variance test. * $P < 0.05$. GTN, glyceryl trinitrate; sCLU, soluble clusterin; nCLU, nuclear clusterin; siRNA, small interfering ribonucleic acid; HSC70, heat shock cognate 70; mCRPC, metastatic castration resistant prostate cancer.

docetaxel-resistant cell lines was then examined. Thus, whether CLU silencing (sCLU and nCLU) could affect the cytotoxic effects of GTN in the four human prostate cancer cell lines was explored. As presented in Fig. 3A and B, western blotting indicated that sCLU and nCLU levels of expression were dramatically decreased with CLU siRNA compared with controls, both in the absence or presence of GTN (even though the transfection reagent increases the amount of CLU). Furthermore, sensitive cells (DU145 and PC3-AG cells) transfected with CLU siRNA and then

treated with GTN did not exhibit any marked changes in cytotoxicity compared with the control (cells transfected with control siRNA). In accordance with our previous findings (Fig. 1B), docetaxel-resistant control cells (control siRNA) were more sensitive to GTN-induced cytotoxicity than the parental control cells (control siRNA). However, clusterin silencing significantly reduced the cytotoxic effect of GTN in both DU145-DR and PC3-D12 docetaxel-resistant cells compared with the control cells to reach a similar level of cytotoxicity observed in sensitive cells (Fig. 3C).

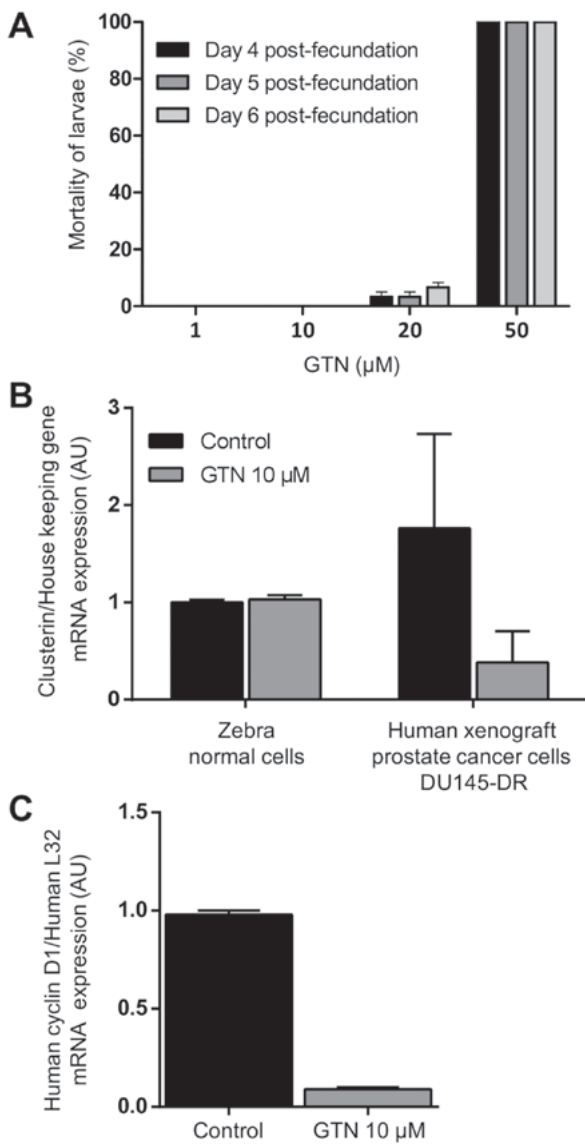


Figure 4. GTN downregulates the expression of human clusterin and cyclin D1 at a transcriptional level. (A) Non-xenotransplanted larvae were treated with increasing concentrations of GTN (1, 10, 20 and 50 μ M) for 3, 4 or 5 days to determine their sensitivity to the nitric oxide donor. A batch of 10 larvae was used for each treatment. Data represent the mean \pm standard error of the mean. Larvae were xenotransplanted with DU145-DR docetaxel-resistant human metastatic castration resistant prostate cancer cells then treated with 10 μ M of GTN for 5 days. (B) Zebrafish and human clusterin expression and (C) human cyclin D1 expression were analyzed in xenotransplanted larvae by reverse transcription-quantitative polymerase chain reaction. Each condition was obtained from the mRNA extracted from the batch of 10 larvae. Data are representative of 2 independent experiences. GTN, glyceryl trinitrate.

Taken together, these findings indicate that the cytotoxic effect of GTN in sensitive cells is not dependent of clusterin expression. On the contrary, the cytotoxic effect of GTN in docetaxel-resistant cells is dependent of clusterin expression. Although the present study cannot distinguish the role of sCLU and nCLU (siRNA CLU indifferently targets sCLU and nCLU), these results suggest that GTN cytotoxicity may be most likely dependent of the nuclear form of the clusterin, upregulated by GTN treatment in docetaxel-resistant cells.

GTN modulates the level of human clusterin in vivo. The role of GTN in modulating the level of clusterin expression

in human prostatic cancer cells *in vivo* was then investigated by experimental approaches in zebrafish. The tolerance of zebrafish larvae (4-6 days post-fertilization) to GTN was tested and mortality rate was recorded. All larvae treated with 1 and 10 μ M GTN during the 4, 5 and 6 days post-fertilization exposure sets survived. For the larvae exposed to 20 μ M GTN, the mortality rates were 3.3% in the 4 and 5 days post-fertilization and 6.6% in the 6 days post-fertilization group. All zebrafish larvae exposed to 50 μ M died in each stage of larval development tested (Fig. 4A). The maximum tolerated dose for GTN selected for further studies was 10 μ M, corresponding to 100% zebrafish larvae survival. A DU145-DR xenograft model in zebrafish was then generated, and the effects of GTN on the level of clusterin transcription were assessed *in vivo*. Following GTN treatment, it was observed that the level of zebrafish clusterin gene expression remains unchanged compared with controls, whereas the level of human clusterin gene expression in human tumor cells was reduced (Fig. 4B). The level of expression of human cyclin D1 upon GTN treatment was also measured. Following 5 days post-xenotransplantation exposure of GTN, the level of expression of cyclin D1 gene (key cell cycle regulator) was decreased (Fig. 4C), which suggests a reduction in the amount of clusterin in DU145-DR cells and the inhibition of proliferation status of these tumor cells *in vivo*.

GTN and GDF-15 modulate the level of CLU. As cytokines such as TGF- β can regulate the level of expression of CLU (44), the presence of cytokines in the docetaxel-resistant and sensitive cellular model (using a cytokine array, data not shown) was investigated to better delineate the role of CLU in cancer cell sensitivity to GTN. A signal was detectable for GDF-15, a member of TGF- β family, that lead us to evaluate the impact of GTN on GDF-15 regulation. Following GTN exposure, the intracellular form of GDF-15 (pro-GDF-15, a precursor form of GDF-15) is differentially regulated between sensitive and docetaxel-resistant prostate cancer cells. It is significantly increased in docetaxel-resistant cells with the lower concentration of GTN (100 μ M) compared with control, but not in parental cells (Fig. 5A). DU145 and DU145-DR cells were not used here due to an undetectable level of the intracellular form of GDF-15 (data not shown).

A GDF-15 recombinant protein was used to elucidate whether it could affect the level of expression of sCLU and nCLU in PC3 AG and PC3 D12 cell lines. Cells were treated with recombinant GDF-15 or GTN or the combination of both. Notably, GDF-15 induces sCLU and nCLU levels of expression mainly in PC3 D12 docetaxel-resistant cells. However, when GTN is added to GDF-15 treatment, the level of sCLU is completely reduced and the level of nCLU is increased compared with GDF-15 treatment alone (Fig. 5B). Meanwhile, the effect of GTN, GDF-15 and GTN/GDF-15 was tested on the viability of PC3 AG and PC3 D12 cells. Although no significant effect was observed on PC3 AG viability under these conditions, recombinant GDF-15 along with GTN in docetaxel-resistant PC3 D12 cells significantly abrogates the GTN-induced decrease in viability in these cells (Fig. 5C). This effect is in accordance with a lower amount of nCLU in comparison with GTN treatment alone (Fig. 5B). Conversely, although recombinant GDF-15 treatment resulted in an increase in nCLU (pro-apoptotic) in PC3 cell lines, GDF-15

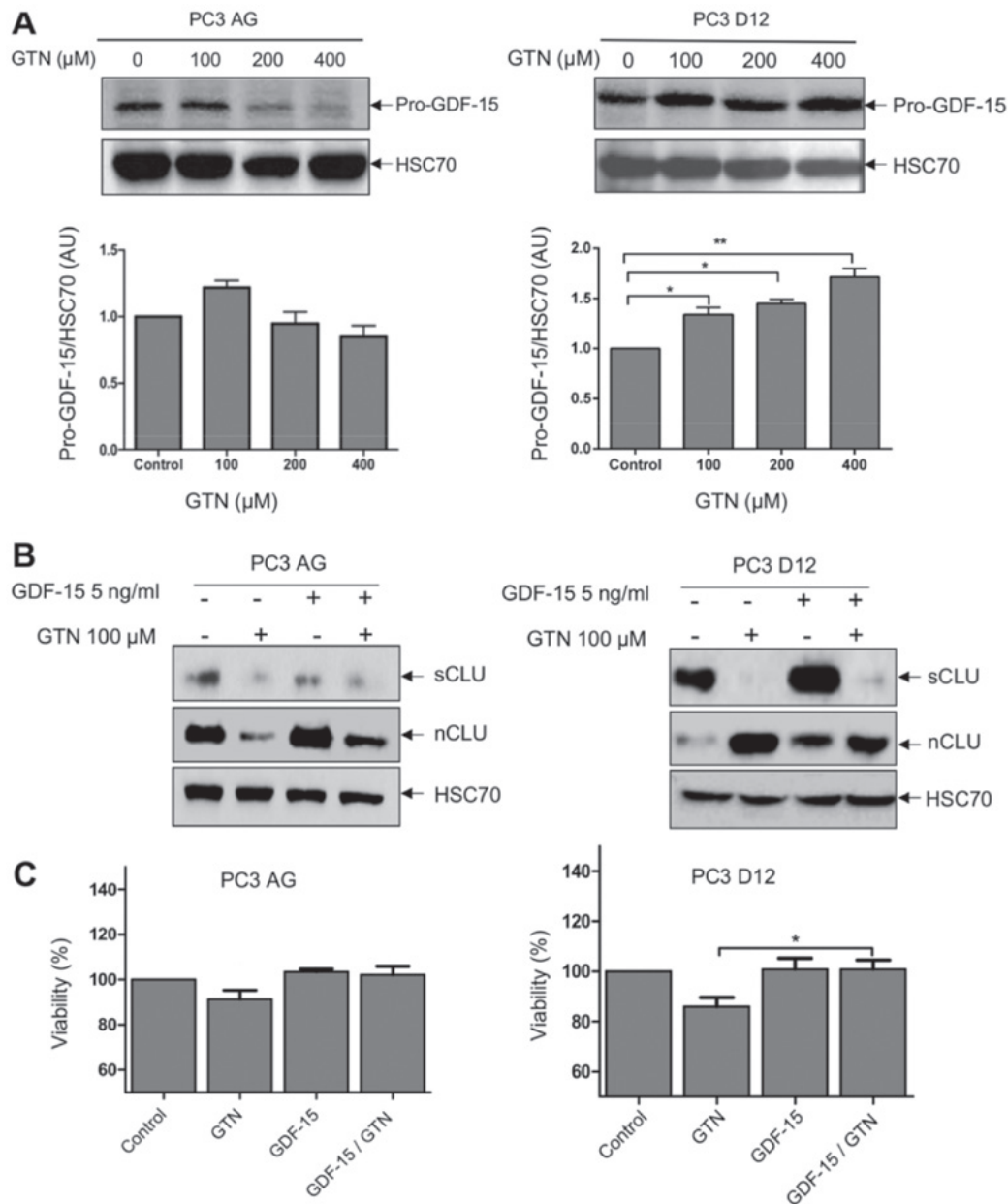


Figure 5. The regulatory effect of GTN on clusterin levels involves GDF-15. (A) PC3-AG and PC3-D12 mCRPC cell lines were treated for 72 h with GTN concentrations ranging from 0–400 μ M and the pro-GDF-15 levels were evaluated by western blotting. The histograms present ratio expression of pro-GDF-15 over HSC70 using densitometric analyses. (B) sCLU and nCLU levels in PC3-AG and PC3-D12 docetaxel sensitive/resistant mCRPC cells were analyzed by western blotting. Cells were treated or not with 100 μ M GTN in the presence or absence of recombinant GDF-15 (5 ng/ml). These results are representative of three independent experiments. HSC70 protein was used as protein loading control. (C) Cells were treated or not with 100 μ M of GTN, and with or without 5 ng/ml recombinant GDF-15. The viability of the cells was measured. The error bars represent mean \pm SD of three independent experiments. Significant differences were determined using a one-way analysis of variance test. * $P < 0.05$; ** $P < 0.01$. GTN, glyceryl trinitrate; GDF-15, growth and differentiation factor-15; mCRPC, metastatic castration resistant prostate cancer; HSC70, heat shock cognate 70; sCLU, soluble clusterin; nCLU, nuclear clusterin.

treatment did not result in an increase in cytotoxicity. Due to its various biological functions and role in other signaling pathways, its cytotoxicity could have been counteracted.

Together, these results demonstrate that GTN promotes a differential regulation of the level of sCLU and nCLU in favor of a cytotoxic effect in docetaxel-resistant PC3 D12 cells which appears to be associated with the level of GDF-15.

Discussion

In an attempt to overcome resistance to docetaxel-based therapy in mCRPC, the effect of the NO donor GTN was studied in

docetaxel-resistant human prostatic cancer cells (PC3-D12 and DU145-DR) in comparison with the docetaxel-sensitive parental counterpart. GTN was revealed to be more effective in inhibiting cell viability in docetaxel-resistant cells than in docetaxel-sensitive cells. It was observed that GTN regulates the level of expression of two known markers of resistance in mCRPC, CLU and GDF-15. More precisely, a differential regulation of soluble and nuclear CLU isoforms in docetaxel sensitive and resistant prostate cancer cells by GTN was observed. Also, GTN modulated CLU isoforms in favor of a cytotoxic effect associated with increased nCLU pro-death isoform and decreased sCLU cytoprotective

isoform in docetaxel resistant cells. Notably, targeting CLU expression by siRNA abrogated the ability of GTN to sensitize docetaxel-resistant cells. Altogether an association between the levels of nCLU expression and GTN-induced cytotoxicity was demonstrated.

These findings support the hypothesis that the response of docetaxel-resistant mCRPC cells to GTN is governed by the balance between the pro-death and cytoprotective isoforms of the CLU protein. These findings suggest that docetaxel resistance is mediated to a certain extent by sCLU and that GTN can overcome docetaxel resistance. CLU is a key contributor in mediating survival in chemoresistant cancer cells. A number of studies have demonstrated the cytoprotective role of sCLU against docetaxel in prostate cancers and multiple chemotherapeutic agents in a wide range of late-stage tumors (45,46). Mechanistically, a previous report highlighted the importance of protein kinase B (Akt) pathway activation, responsible for sCLU induction in docetaxel resistance in prostate cancer cells (15). Notably, an inhibitory effect of NO donors S-Nitroso-N-Acetyl-D,L-Penicillamine and S-nitrosoglutathione on Akt pathway activation through S-nitrosylation of the kinase at cysteine 224 has been described (47). Notably, the present study demonstrated *in vivo*, in a xenograft zebrafish model, the ability of GTN to specifically regulate the level of human clusterin expression in cancer cells and most likely the growth *in vivo* of xenografted human DU145-DR cells. The use of xenograft human prostate cancer cell models have already been revealed to be a useful tool to develop functional cancer models using PC3 cells (42) or to evaluate docetaxel treatment in DU145-xenografted zebrafish (48).

Altogether these findings suggest that GTN could act as a negative transcriptional regulator of sCLU, but the exact mechanism remains unclear. Given that the Akt pathway is commonly activated in several human cancers it is reasonable to speculate that NO donors may be exploited for counteracting sCLU-dependent docetaxel resistance. Furthermore, Zhou *et al* (16) demonstrated that acquired resistance to docetaxel in prostate cancer cells was linked to sCLU induction triggered by High Mobility Group Box 1 (HMGB1) produced from dying cells in a HMGB1/Toll Like Receptor 4 - Receptor for Advanced Glycation End-products/sCLU pathway. This pathway involves the activation of the transcription factor NF- κ B to promote sCLU gene expression. Notably, it has been demonstrated that NF- κ B activity can be inhibited by S-nitrosylation of critical thiol in both p65 and p50 subunits (49,50).

The inhibition of sCLU in prostate cancer and its impact on the cytotoxic effect of various chemotherapeutic agents has been extensively investigated in preclinical studies and provided rationale for the use of CLU antisense inhibitor OGX011 (or custirsene) as a therapeutic target (51-55). Following encouraging results of phase II clinical studies, two phase III clinical trials for custirsene in combination with docetaxel or cabazitaxel and prednisone for patients with mCRPC but no survival benefit was reported in patients treated with the combination compared with patients treated with chemotherapeutic agents alone (56,57). The disappointing result from phase III clinical trials may suggest that a therapeutic strategy directed against sCLU alone is not sufficient and that a therapeutic strategy that

would modulate the two isoforms (sCLU and nCLU) in favor of an antitumoral response may present an interesting strategy.

The nuclear sub-localization of CLU (nCLU) was demonstrated to promote pro-apoptotic signaling in many cells. Kim *et al* (58) demonstrated that nCLU mediates apoptosis by sequestering BCL-XL, thereby releasing Bax which triggers mitochondria cytochrome *c* release and activation of caspase-3. The current study demonstrates that GTN treatment enhances the level of nCLU which is correlated with GTN-induced cytotoxicity. The present study is, to the best of our knowledge, the first report on GDF-15-mediated regulation of CLU, and the findings suggest an implication of GDF-15 in the regulatory mechanism of nCLU by GTN. The regulation of CLU by other cytokines, including TGF- β 1 and interleukin 24, has also been reported, but the mechanism of action remains largely unknown (59,60).

A number of preclinical and clinical studies have demonstrated the role of GTN as an anti-cancer agent for prostate cancer. A phase II study of GTN in patients with prostate cancer has revealed an inhibitory effect on prostate-specific antigen progression following primary treatment failure (surgery or radiotherapy) (61). Notably, it was demonstrated that GTN attenuates hypoxia-induced hypoxic cells and enhances the *in vitro* (hypoxic PC3 cells) and *in vivo* antitumor effect of doxorubicin in mice bearing PC3 prostate tumor xenograft model (31). Furthermore, the NO donor DETANONOate was demonstrated to sensitize prostate cancer cells to cisplatin both *in vitro* and *in vivo* (32). Therefore, novel approaches for combination therapy strategies (chemotherapy/NO donor) are required for the effective treatment of prostate cancer.

Recently, transcriptomic signatures associated with docetaxel-resistant mCRPC cells were analyzed with the goal of identifying putative new therapeutic target to overcome docetaxel resistance. RNA sequencing analysis revealed upregulation of genes associated with cancer stem cells-like characteristics (62). Further studies would be required to determine whether NO could directly or indirectly affect these targets and molecular pathways to overcome docetaxel resistance.

Taken together, the present results demonstrate that NO donors, such as GTN, may be an interesting therapeutic agent to disrupt the resistant pathways mediated by docetaxel. NO can interfere with multiple signaling pathways involved in resistance to drugs and many NO donors have been demonstrated to sensitize cancer cells to various chemotherapeutic agents (63). In accordance with the literature, the present data support a key role of CLU in cancer chemoresistance with sCLU being overexpressed following intensive exposure of docetaxel. Thus, as GTN differentially modulates the two CLU isoforms (sCLU and nCLU), it makes GTN a promising therapeutic agent to combine with other chemotherapies.

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Availability of data and materials

The dataset generated from cytokine arrays are not publicly available to fully exploit data for future research project and publication. All other data generated or analyzed during this study are included in this article.

Authors' contributions

SB and LM carried out the experiments and contributed to the interpretation of the results. LD carried out experiments with support from LM. SB and LM designed the figures. SR was involved in the establishment of docetaxel-resistant cells. SB wrote the material and methods section. VL conceived, designed, supervised the study and analysed data. JFJ contributed to the elaboration of the project with NI underlining clinical challenges. SP partially co-supervised the work and took the lead in writing the manuscript with support from VL. AB contributed to the interpretation of the results, proposed experiments and reviewed the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Ethics approval and consent to participate

The animal procedures described in the present study were reviewed and approved by the local Ethics Committee of the University of Burgundy.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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