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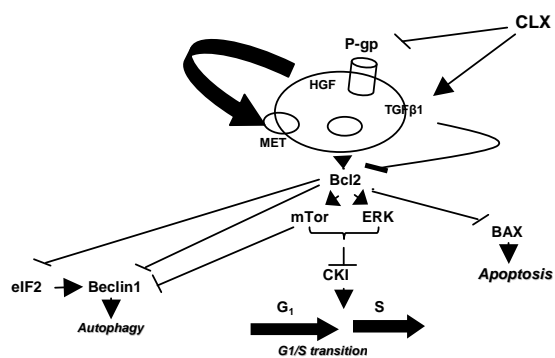
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Graphical Abstract

The *in vitro* pro-autophagic, antiproliferative and pro-apoptotic effect of celecoxib on MDR-positive cells is mediated by the action of P-gp on the HGF/MET autocrine loop.



Down-regulation of the HGF/MET autocrine loop induced by celecoxib and mediated by P-gp in MDR-positive human hepatocellular carcinoma cell line.

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Running Title: celecoxib-induced autophagy in MDR positive cells.

ABSTRACT

Many tumors are resistant to drug-induced cell cycle arrest and apoptosis. We have reported that apoptosis can be restored in human multidrug-resistant (MDR) hepatocellular carcinoma cell lines by celecoxib. Here we show that P-gp mediates cell cycle arrest and autophagy induced by celecoxib in human MDR overexpressing hepatocellular carcinoma cell line by down-regulation of the HGF/MET autocrine loop and Bcl-2 expression. Exposure of cells to a low concentration of celecoxib down-regulated the expression of mTOR and caused G1 arrest and autophagy, while higher concentration triggered apoptosis. Cell growth inhibition and autophagy were associated with up-regulation of the expression of TGF β 1, p16^{INK4b}, p21^{Cip1} and p27^{Kip1} and down-regulation of cyclin D1, cyclin E, pRb and E2F. The role of P-glycoprotein expression in resistance of MDR cell clone to cell cycle arrest, autophagy and apoptosis was shown in cells transfected with *MDR1* small interfering RNA. These findings demonstrate that the constitutive expression of P-gp is involved in the HGF/MET autocrine loop that leads to increased expression of Bcl-2 and mTor, inhibition of eIF2 α expression, resistance to autophagy/apoptosis and progression in the cell cycle. Since mTor inhibitors have been proposed in treatment of “drug resistant” cancer, these data may help explain the reversing effect of mTor inhibitors.

Keywords: MDR, celecoxib, P-gp, HGF/MET, autophagy

1. INTRODUCTION

Most anticancer therapies kill cancer cells primarily by apoptosis. Inactivation of tumor cell apoptosis thus contributes to the development of cancer spreading and resistance to therapy [1]. The development of multiple drug resistance (MDR) characterized by intrinsic P-glycoprotein (P-gp) expression, is a general phenomenon occurring in several tumors, including hepatocellular carcinoma (HCC) [2]. A large number of chemical, physical or environmental stimuli may lead to P-gp expression, which can enhance cell survival in adverse conditions [3]. The expression of P-gp must be considered a type of marker of tumor aggressivity and is often associated with expression of the angiogenic phenotype in HCC cell lines, as we have recently shown [4,5]. Since P-gp expression seems to be controlled by COX-2 activity, COX-2 inhibitors could have an inhibitory effect on the MDR phenotype. We have recently shown that celecoxib, a specific inhibitor of COX-2 activity, reverses MDR-positive HCC cells to apoptosis by a P-gp-dependent mechanism [6].

Apoptosis is not the only cell response to drugs, toxins and stress conditions. It has been suggested that another response to therapeutic interventions can involve autophagy and autophagic cell death. Autophagy is activated in response to γ -irradiation and various anticancer drugs [7,8] and can also be considered as a tumor suppressor mechanism since defects in autophagy profoundly perturb organism development. The haploinsufficient essential mammalian autophagy gene, Beclin1, promotes carcinogenesis in mice, especially as liver cancer [9]. It has been demonstrated that Yoshida AH-130 hepatoma and primary HCC cells have lower autophagic activity than normal hepatocytes [10]. Acquired drug resistance to chemotherapy may cause resistance to cell cycle arrest and autophagy [11]. Apoptosis, autophagy and cell cycle progression require several regulator proteins, suggesting that they share the same molecular pathways [12]. COX-2 inhibitors may exert their anticancer activity by slowing down cell proliferation and inducing autophagy and cell-cycle arrest [13,14]. Cell-cycle progression is promoted by a number of cyclin-dependent kinases which have to complex with regulatory proteins, namely cyclins, to drive the cell through the cycle. They

are negatively regulated by cell-cycle inhibitory proteins called cyclin-dependent kinase inhibitors, CKI. Two classes of CKI have been identified: the INK4 family, including p16^{INK4b}, p15^{INK4a}, p18^{INK4c} and p19^{INK4d}, which inhibit cyclin D associated kinases (cdk2, 4 and 6) and the Cip/Kip family, including p21^{Cip1}, p27^{Kip1} and p57, that negatively regulate cyclin E/cdk2 and cyclin A/cdk2 complexes [15]. Interestingly, p16^{INK4b}, p21^{Cip1} and p27^{Kip1}, whose main function is to induce cell cycle arrest and apoptosis, induce autophagy [16]. p27^{Kip1} also can down-regulate P-gp [17]. Thus, enhancement of CKI signaling is particularly noteworthy among the putative molecular targets other than COX-2 in CLX-mediated *in vitro* antiproliferative effects in cancer cells [13,14].

On these bases, this study was designed to address the following questions: 1) Is the CLX-induced inhibition of MDR-positive HCC cell growth due to cell-cycle arrest? 2) Is cell-cycle arrest related to induction of autophagy? 3) Does CKI activation regulate cell cycle, autophagy and apoptosis of MDR-positive HCC cells? 4) Does P-gp directly regulate the expression of cell-cycle progression and autophagy-related proteins such as HGF/MET, Bcl-2, Beclin1 and CKI?

2. MATERIALS AND METHODS

2.1. Cell lines. Experiments were performed on a human HCC line PLC/PRF/5, by using the drug-sensitive clone (P5) and the highly MDR-positive subclone P1(0.5) [18]. The P1(0.5) subclone was developed by serial prolonged exposure to increasing concentrations of doxorubicin (Pfizer Global R & D, USA) starting from parental P5 cells, and subsequently cultured in DMEM containing 0.5 µg/mL doxorubicin and 10% fetal bovine serum (FBS), as shown elsewhere [5]. The cell lines were cultured at 37°C in an atmosphere containing 5% CO₂.

2.2. Cell treatment. Doxorubicin-free, serum-free medium containing celecoxib (CLX) (2.5, 5, 10 and 20 µM) was used in all experiments.

2.3. Colony formation efficiency test. (P5) and the subclone P1(0.5) were seeded in triplicate onto 35-mm dishes containing agarose and DMEM and were treated with 2.5, 5, 10 and 20 µM celecoxib in doxorubicin-free, serum-free medium. The plates were incubated at 37°C, in 5% CO₂, for 2 weeks and photographed.

2.4. Cell cycle analysis by flow cytometry. 50000 cells were plated onto 60-mm Petri dishes and experiments were done in triplicate; cells were treated with 2.5, 5, 10 and 20 µM CLX for 24 h. Cells were fixed with 70% ethanol at 48°C. RNaseA (20 µg/mL) and PI (50 µg/mL) were then added, and cell suspensions were incubated for 30 min in the dark. Stained cells were analyzed on a FACScan flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ).

2.5. Cell viability. P5 and P1(0.5) cells were plated in 60-mm Petri dishes and grown in DMEM supplemented with 10% FBS. On the next day, cells were treated with 2.5, 5, 10 and 20 µM celecoxib in doxorubicin-free, serum-free medium. After 48 h, the dead cells were identified using the trypan blue staining test. Adherent cells were detached, resuspended in DMEM, and both adherent and suspended cells were mixed with an equal volume of 0.04% (w/v) trypan blue. The percentage of dead cells stained was determined by counting. For MTT assay, 25,000 cells were plated in each well of a 96-well plate. After 48 h, the cells were treated with celecoxib 2.5, 5, 10

and 20 μ M. An equal volume of DMSO was added to the control well; after 48 h, 10 μ L MTT (5 mg/mL) in growth medium were added per well, and the plates were incubated at 37°C for 4 h. The reduced MTT dye in each well was solubilized in 200 μ L DMSO. Absorbance was measured on an ELX-800 microplate reader at 540 nm.

2.6. Immunocytochemistry. Cells were plated onto a coverslip and grown in culture medium containing 10% FBS. After 24 h, cells were treated with 2.5, 5, 10 and 20 μ M celecoxib in doxorubicin-free, serum-free medium. For immunocytochemistry, cells, after 24 h, were fixed with paraformaldehyde (3.7% w/v), permeabilized with 0.05% (v/v) Triton X-100 for 10 min, blocked with 10% (w/v) normal goat serum, then incubated overnight with primary antibodies at an appropriate dilution (1:100) p16^{ink4} (mouse monoclonal), p21^{cip1} (rabbit polyclonal) p27^{kip1} (rabbit polyclonal), p53 (mouse monoclonal), cyclin D1 (mouse monoclonal), cyclin E (rabbit polyclonal), TGF β 1 (mouse monoclonal) and E2F (rabbit polyclonal) (Santa Cruz Biotechnology, USA). After washing, samples were incubated with Dako-Envision dual link secondary antibody at room temperature. Then the slides were colored with chromogen DAB, countercolored with hematoxylin and mounted with Eukitt. Total expression and site of expression of proteins were determined by counting 100 cells in 10 different microscope fields. For immunofluorescence studies, cells were fixed with paraformaldehyde (3.7% w/v) after 1 h, permeabilized with 0.05% (v/v) Triton X-100 for 10 min, blocked with 10% (w/v) normal goat serum, and samples were incubated overnight with primary antibodies for Beclin1 (rabbit polyclonal), Bcl-2 (rabbit polyclonal) and LC3 (rabbit polyclonal) (Santa Cruz Biotechnology, USA) and then incubated with FITC- or TRITC-conjugated secondary antibodies (Alexa Fluor, Molecular Probes, The Netherlands) at room temperature and analyzed with Nikon fluorescent microscope.

2.7. Western blot analysis. Cells were lysed in a buffer (50mM Tris/HCl pH 7.4, 150mM NaCl, 5mM MgCl₂, 1% TX-100, 1mM EGTA, 1mM DTT), containing protease inhibitors and NaF 0.1 M. Protein concentrations were determined with the Bradford assay. The lysates were centrifuged and proteins (25 μ g) were subjected to electrophoresis on Tris-glycine SDS

polyacrylamide gel. After blotting onto nitrocellulose with filter papers, non-specific binding sites were blocked by incubation and carried out for 1 h at room temperature in phosphate buffered saline (PBS) with 5% milk. The blots were then probed with primary antibodies at the appropriate dilution (1:500) overnight, p16^{ink4} (mouse monoclonal), p21^{cip1} (rabbit polyclonal), p27^{kip1} (rabbit polyclonal), cyclin D (mouse monoclonal) and E (rabbit polyclonal), pRb (goat polyclonal) and E2F (rabbit polyclonal) (Santa Cruz Biotechnology, USA), Bcl-2 (rabbit polyclonal), mTOR (rabbit polyclonal), P-mTOR (rabbit polyclonal), eIF2 α (mouse monoclonal), P-eIF2 α (rabbit monoclonal), HGF (rabbit polyclonal), MET (mouse monoclonal) (Cell Signaling, USA). After being washed, blots were incubated with secondary antibodies 1:5000 (Bio-Rad Laboratories Ltd., Hemel Hemstead, UK) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence according to the manufacturer's instructions (Pierce Biotechnology, USA).

2.8. Incorporation of monodansylcadaverine (MDC). Cells were incubated with 0.05 mM MDC in PBS at 37°C for 20 minutes. After incubation, cells were washed twice with PBS and immediately analyzed by Nikon fluorescence microscope.

2.9. Small interfering RNA transfection. Positive control GAPDH small interfering RNA (siRNA), sicontrol non-targeting siRNA and a pool of four different double-stranded RNA oligonucleotides directed against MDR1 (siGENOME SMARTpool, human ABCB1) were purchased from Dharmacon. P1(0.5) cells were seeded onto 60-mm dishes to reach 30% to 50% confluence after 24 h of incubation, and transfected with a total of 100 nmol/L siRNA using LipofectAMINE 2000 (Invitrogen Life Technologies, Inc.) in antibiotic-free medium, according to the manufacturer's instructions. 72 h after transfection proteins were extracted for Western blot analysis and cells were analyzed for immunocytochemical analysis and flowcytometric analysis.

2.10. Statistical analysis. The significance of the differences between groups was determined by ANOVA. $P < 0.05$ was considered significant.

3. RESULTS

3.1. G1 phase arrest induced by CLX in MDR-positive HCC cell line. To clarify the mechanism underlying CLX-mediated *in vitro* antitumor action, the growth-inhibitory effect of various concentrations of CLX (2.5, 5, 10 and 20 μM) was assessed in a serum-free, doxorubicin-free medium. Cell viability was measured by MTT assay and trypan blue exclusion test after cells were exposed to CLX for 48 h. Clonogenic cell survival assays were also performed with low concentrations of CLX and colonies were counted after 14 days.

Cell viability was affected, in a concentration-dependent manner, especially in MDR-positive P1(0.5), but not in P5 cells either by MTT (Fig. 1A) or trypan blue exclusion test (Fig. 1B) and quantified by an increase in doubling time (38 ± 0.9 vs 120 ± 2.3). Flow cytometric analysis of P5 and P1(0.5) cell lines was performed after 48 h without CLX and serum, and it was found that both cell lines were in the same stage of the cell-cycle (Fig.1 C). CLX treatment induced concentration-dependent inhibition of cell growth in MDR-positive P1(0.5) cells, but not in P5 (Fig 1D and data not shown). Cell number and colony size were inversely related to the CLX concentration.

Whether CLX inhibition of MDR-positive cell growth was caused by cell-cycle arrest was also determined. Both P5 and P1(0.5) cells were treated with different concentrations of CLX in serum-free, doxorubicin-free medium and the phases of cell cycle distribution were determined by flow cytometric analysis (Fig. 2A and 2B). DNA content of P1(0.5) cells revealed that CLX caused an accumulation of G1 cells in an inversely concentration-dependent manner (Fig. 2B). Exposure to the lowest concentration of CLX (2.5 μM) resulted in the most evident accumulation of cells in G1 phase. With increasing CLX concentrations, a gradual reduction in the accumulation of P1(0.5) cells in the G1 phase until the control value (from 70% to 53%) was observed (Fig. 2B). This was associated with a concentration-independent decrease in the S fraction (from 20% to 11%) and no significant effect on G2/M transition except for the decrease shown using CLX at 2.5 μM . This

suggests that the inhibitory effect of CLX on P1(0.5) cell growth leads to G1 cell cycle arrest. At higher concentrations of CLX (10 and 20 μM), caused an increase of sub-G1 phase in P1(0.5) cells, consistent with an increase of apoptotic cells, as shown previously (Fig. 2B) [6]. On the contrary, no significant changes in DNA distribution of P5 cells were observed (Fig. 2C). Altogether, these data show that the inhibitory effect of CLX on MDR-positive cell growth leads to a cell-cycle inhibition primarily at the G1/S checkpoint as well as to apoptosis, depending on the drug concentration. Both events are likely to contribute to reduced cell growth of P1(0.5) cells triggered by CLX 10 μM .

3.2. CLX-induced autophagy in MDR-positive HCC cell line during G1 phase arrest

Since G1 arrest is frequently associated with activation of the autophagic process, we first verified whether CLX at low concentrations was able to stimulate the lysosomal vacuolar formation in P1(0.5) cells by measuring the incorporation of monodansylcadaverine (MDC) into the lysosomes [19]. The vacuolar compartment was hardly detected as dispersed, faint basal MDC staining in P1(0.5) cells (Fig. 3A), while MDC-labeled vacuoles were present in P1(0.5) cells following treatment with 2.5 μM and 5 μM CLX for 3 and 6 hours (Fig. 3A).

To have a better insight into the effect of CLX on the autophagic process, we looked for the mammalian microtubule-associated protein 1 light chain, LC3-II, a specific autophagosome/autolysosome marker, known as a structural component during autophagosome formation [20]. Since LC3-II is rapidly degraded with the sequestered material by lysosomal proteases, its turnover but not its concentration, is a good mirror of autophagic activity, as recently proposed by Tanida et al. [20]. We therefore measured the lysosomal turnover of LC3-II by immunocytochemical analysis (Fig 3B). LC3 granular staining can be quantified as 0.4 LC3-II-labeled vacuoles/cell section in untreated P1(0.5) cells, value comparable with that measured in HeLa cells. Treatment of P1(0.5) cells with CLX at a low concentration induced approximately a 4-fold increase in the immunofluorescence of punctat of endogenous LC3 (Fig. 3B). Since the autophagic process can be dissected with agents acting at different phases, we measured the LC3-II

turnover also in P1(0.5) cells pre-incubated with 10 mM 3-MA, an inhibitor of the sequestration phase [21,22]. As expected, accumulation of LC3-II-labeled vacuoles was prevented by the PI3-kinase inhibitor (Fig. 3B).

Once it was shown that the lysosomal vacuolar system is activated by CLX, it was determined whether the activation of Beclin1, a novel Bcl-2 interacting protein needed for the nucleation of the autophagosome, was required for CLX-induced autophagy. P1(0.5) cells were stained immunocytochemically with anti-Beclin1 antibodies and immunoreactivity was measured. Immunoreactivity was hardly detected in cells not treated with CLX whereas it was clearly enhanced in CLX-treated cells, being already evident after 1 h (Fig. 3C). Because Bcl-2, that is known to be an anti-apoptotic and anti-autophagic protein, was down-regulated in 10 μ M CLX-treated P1(0.5) [6], its expression was estimated after exposure to 2.5 and 5 μ M CLX. Bcl-2 expression decreased when P1(0.5) cells were exposed to CLX (Fig. 3D).

To verify whether CLX-mediated activation of the autophagic process was related to an unbalance equilibrium between the expression of eIF2 α , the best known inducer of autophagy, and that of mTOR, the cell stress sensor and inhibitor of autophagy-induction [23], Western blot analysis was carried out. CLX up-regulated the phosphorylation of eIF2 α , down-regulating mTOR phosphorylation in P1(0.5) cells, consistently with what was observed on autophagy (Fig. 3E).

3.3 CLX-induced up-regulation of p16^{INK4a}, p21^{cip1} and p27^{kip1} and TGF β 1 during HCC growth arrest

To examine the molecular mechanism that may be underlying the changes in cell-cycle progression, autophagy and apoptosis, we investigated the levels of CKI expression, p16^{INK4a}, p21^{Cip1} and p27^{Kip1}. CKIs are known to prevent the transition of cells from G1 to S phase. The treatment with 5 μ M and, especially 10 μ M CLX enhanced CKI expression in P1(0.5) but not in P5 cells, as measured by Western blot analysis (Fig. 4A). Because CKIs work in the nucleus and are also present in the cytoplasm as inactive proteins, an immunocytochemical study was performed

(Fig. 4B). All three CKIs we measured increased at a nuclear level after CLX treatment. In particular, p27^{Kip1} expression increased about four times (Fig. 4C), largely confirming the Western blot results.

To date, TGFβ1 is considered to be the main growth inhibitory factor for epithelial cells acting in the late G1 phase upstream of CKI function [24]. Some HCC cells are known to exhibit an autocrine loop for TGFβ1 to regulate their growth. To assess whether this was the case, it was determined whether P1(0.5) cells produced TGFβ1 in response to CLX treatment to induce CKI expression. Quantification of the TGFβ1-expressing cells, as reported in Fig. 4D, showed a clear increase of TGFβ1 production in the CLX-treated P1(0.5) cells, but not in P5, consistently with the inhibitory effect on cell growth. We also examined p53 expression, since p53 is known to control p21 levels. No significant changes following CLX treatment (Fig. 4D) were found, indicating a p53-independent enhancement of p21 in P1(0.5) cells.

3.4. Down-regulation of cyclin D1 and cyclin E, p-Rb, E2F, MET and ERK induced by CLX during HCC growth arrest

Since the main function of CKI is the inhibition of the cyclin-dependent kinases, the downstream events linking CKI function to G1 arrest were investigated. The effects of 10 μM CLX on cyclin D1 and E expression level, known to be involved in G1 and G1/S cell cycle progression, were examined. Fig. 5 shows that CLX treatment of P1(0.5), but not of P5, induced a slight decrease in cyclin D1 expression and about a three-fold reduction in cyclin E protein levels as determined by Western blot (Fig. 5A). This result was confirmed by immunocytochemical analysis (Fig. 5B).

The product of the Rb gene prevents cells from entering into S phase. G1/S progression is mediated by cyclin D1 and cyclin E in complex with the cyclin-dependent kinases, which phosphorylate Rb, leading to inactivation of its growth-suppressive function and resulting in cell-cycle transition. We, therefore, investigated whether CLX treatment could affect phosphorylated Rb

(pRb). We found that pRb expression was decreased in P1(0.5), but again not in P5, following CLX treatment, consistently with a down-regulating expression of cyclin E (Fig. 5A).

Because pRb inhibits cell-cycle progression by interacting with transcription factors such as E2F, when Rb becomes phosphorylated, E2F is released and stimulates cell proliferation. Thus, further experiments were carried out to check whether CLX treatment might influence E2F expression. A reduction in E2F expression in P1(0.5) cells was evident by Western blotting (Fig. 5A) and quantification of the immunocytochemical analysis demonstrated a 5-fold decrease in E2F expression levels in P1(0.5) cells, while no significant changes were observed in P5, following CLX treatment (Fig. 5B).

Since some evidence indicates a critical role of the HGF/MET and downstream MEK/ERK pathway in the control of hepatocyte proliferation both *in vitro* and *in vivo* as well as of p21^{Cip1} expression [25], additional experiments were carried out to detect and quantify MET and ERK expression after CLX treatment. Western blot analysis of these experiments showed that CLX down-regulated MET and ERK expression only in P1(0.5) cells (Fig. 5A).

3.5. CLX-induced p27^{kip1} and Beclin1 up-regulation, sub G1 peak and G1 cell accumulation in P1 cells are mediated by P-gp expression

To validate P-gp as primary target responsible for the *in vitro* anti-proliferative, pro-autophagic and pro-apoptotic effect of CLX, further experiments were performed. The expression of P-gp was specifically down-regulated by transient transfection with an *MDR1*siRNA. As controls, parallel cultures were either not transfected or transfected with an inefficient duplex oligonucleotide not targeting *MDR1*mRNA (sham-transfected). Proper conditions were assessed to achieve optimal down-regulation of P-gp expression, which was monitored by immunoblotting determination of P-gp protein level (Fig. 6A). *MDR1*siRNA transfection successfully down-regulated P-gp expression in *MDR1*siRNA transfected P1(0.5) culture (Fig. 6A). In *MDR1*siRNA-transfected cells, P-gp protein level, normalized against the actin protein level, was reduced by

>90%, as measured by densitometry (data not shown). A parallel set of cultures was performed to evaluate p27^{Kip1} expression by Western blot and immunocytochemistry as well as Beclin 1 expression and DNA distribution by flowcytometric analysis. The *MDR1* siRNA induced an increase in the levels of p27^{Kip1} expression under the basal conditions, as measured by Western blot (Fig. 6A). However, immunocytochemical analysis indicated that, when P1(0.5) cells were exposed to CLX, nuclear p27^{Kip1} expression increased in a concentration-dependent manner in untransfected and sham-transfected cultures (Fig. 6B). Immunocytochemistry analysis of Beclin1 showed that its levels in CLX treated-*MDR1*siRNA-transfected P1(0.5) cells were maintained at approximately the value in *MDR1* siRNA transfected P1(0.5) culture, while no effect on *MDR1* siRNA was observed under basal conditions of untreated cells (Fig. 6C). Consequently, flowcytometric analysis of DNA distribution showed no changes in either sham-transfected or *MDR1*siRNA transfected P1(0.5) cells in comparison with untransfected cells. On the contrary, it demonstrated the disappearance of the 10 μ M CLX-induced sub-G1 peak and G1 cell accumulation in *MDR1*siRNA-transfected P1(0.5) cells compared with CLX-treated untransfected cells (Fig. 6D). Because the *MDR1*siRNA-mediated CLX effects on P1(0.5) growth might depend on the action of MDR1 on the HGF/MET autocrine loop, we also investigated whether P-gp siRNA could modulate HGF/MET expression as well as that of potential downstream mediators. It was found that *MDR1* siRNA down-regulated the levels of expression of both HGF and MET as well as that of Bcl-2, a candidate mediator of CLX effects, without significantly affecting p27^{Kip1} or Beclin1 expression (Fig. 6A and C).

4. DISCUSSION

HCC is one of the most frequent malignancies worldwide, and in addition it is extremely resistant to currently used chemotherapeutics [26]. We present here evidence demonstrating that P-gp, the protein encoded by the MDR1 gene, regulates the autocrine loop mediated by HGF/MET in P1(0.5) cells, leading to resistance to drug-mediated growth inhibition. CLX, the specific inhibitor of COX-2 activity, effectively overcomes P-gp mediated drug resistance and suppresses the anchorage-independent growth of resistant HCC cells by inducing G1 arrest, autophagy and apoptosis. In addition, CLX decreases Bcl-2 expression by down-regulation of the MET pathway and phosphorylation of mTOR in human HCC MDR-positive cancer cells.

High levels of Bcl-2 sustain the drug-resistant phenotype, while down-regulation of Bcl-2 is associated with reduced drug resistance [27,28]. In the present study the role of Bcl-2 in the negative regulation of Beclin1 and Bax expression and resistance to autophagy and apoptosis was found to correlate with phosphorylation of mTor and ERK MAPK.

Some evidence supports the key role of Bcl-2 in the CLX effect of suppressing cell drug resistance. The crosstalk between autophagy and apoptosis is partly mediated by the functional and physical interaction between Beclin1, an essential autophagy gene, or Bax, the best known proapoptotic factor, and Bcl-2, one of the pragmatic apoptosis-inhibitory proteins [29,30]. When Bcl-2 interacts with calcineurin, it allows ERK phosphorylation by Raf 1 and cell-cycle progression [31]. Thus, Bcl-2 function seems to depend on its localization, since only endoplasmic reticulum-targeted Bcl-2 inhibits autophagy and only mitochondria-targeted Bcl-2 inhibits apoptosis [29]. Cytosolic Bcl-2 might actively maintain cell survival by favoring cell-cycle progression.

Several mechanisms have been proposed to explain the antiproliferative effects of COX-2 inhibitors, besides their action as COX-2 activity inhibitors [13,14]. In this study, it is shown that CLX at low concentration induced autophagy whereas at higher concentration apoptosis as confirmed by the appearance of the subG1 peak according to flow cytometric analysis only in HCC

cells over-expressing P-gp (Fig. 2B and 2C). The lower concentrations of CLX (2.5 and 5 μ M) were unable to trigger apoptosis in P1(0.5) cells, while they were effective in activating autophagy, suggesting that autophagy could be the earliest event involved in the reversal of drug-resistance by CLX, operating upstream of apoptosis. The induction of autophagy in P1(0.5) cells by CLX at low concentration is supported by the down-regulation of mTOR associated with up-regulation of eIF2 α , leading to enhancement of Beclin1 expression within 30 minutes, which causes the accumulation of LC3-II-labeled autophagosomes and then MDC-labeled autolysosomes. The observation made in the present work that CLX at concentration lower than 10 μ M triggers autophagy whereas at higher concentration causes apoptosis in P-gp over-expressing HCC cell lines might be important to explain controversial results reported in the literature concerning the antiproliferative effect of CLX. In fact, CLX effect on cancer cells, at least HCC cell lines, would depend on the expression of P-gp and CLX concentration in the microenvironment. To obtain the “killing” effect by CLX (apoptotic death) at least two conditions should be present, P-gp overexpression and concentration of the drug of about 10 μ M or more. Without both conditions, CLX may leads to autophagy or to COX-2 activity inhibition only, slowing down the growth rate of cancer without killing cancer cells. With regards this point, autophagy can be considered a mechanism by which cells, also cancer cells, survive to stress, especially those characterized by hypoxia and/or DNA denaturation.

Celecoxib-induced accumulation of P1(0.5) in G₀/G₁ phase together autophagy induction might be seen as a sort of defence by MDR positive cells to survive to CLX induced toxicity. It is possible that in case autophagy should not evolve to autophagic death or autophagic-induced apoptosis, MDR positive cells could recover and quickly start again to proliferate. If it is the case, treatment with CLX should allowed to reach drug concentration to cause apoptosis, otherwise it could favor cancer growth. It is important to consider that targeting autophagy to control cancer growth it is probably unsafe at present, as the knowledge of the entire mechanism regulating autophagy-apoptosis is too much partial to be exploited for therapy attempts. As suggested recently, we agree

that precaution should be used before using drugs that target autophagy as the results could be quite different from what wait for [32]

Although autophagy has received attention recently, there is still debate about its role as a mechanism for cell survival or cell death under certain conditions. As a survival mechanism, autophagy is known to prevent apoptosis especially to handle cell damages caused by stress condition. However, during prolonged starvation or oxidative stress, autophagy can switch to apoptosis or autophagic cell death [33]. For these reasons autophagy might inhibit cancer growth by regulating cell mass. Recent studies have shown that autophagy has multiple roles in the regulation of cell death, differentiation and antimicrobial response in mammals [34,35]. Conditions known to induce autophagy inhibit cell growth by inducing cell-cycle arrest, indicating that cell-cycle progression largely depends on cell protein mass [22]. Consistently, here the flow cytometric analysis revealed that CLX at low concentration caused accumulation of cells in G1 phase that decreased from 2.5 to 10 μ M when the sub-G1 peak appeared, indicating a shift to apoptosis [6].

Although p53 is a well known mediator of cell-cycle arrest, apoptosis and autophagy, in the present work the CLX effect on MDR-positive cells appeared to be p53-independent, even if it was mediated by enhanced p21^{Cip1} expression. We hypothesize the involvement of the pathway of ERK1/2, because CLX reduces the phosphorylation of ERK1/2 in P1(0.5) cells. This event results in enhanced expression of p16^{INK4b}, p21^{Cip1} and, particularly, p27^{Kip1}, whose main function is to negatively regulate cell cycle progression [13,14]. The observation that CLX enhances CKIs, especially p27^{Kip1}, which inhibit cell growth by acting on G1 phase of the cell cycle (Fig.4) supports the concept that drugs acting on cell cycle could be used for cancer treatment. The hypothesis is in line with the observation that elemene, a natural antitumor drug, sensitizes chemoresistant ovarian carcinoma cells to cisplatin-induced growth suppression through up-regulation of p53, p21^{Cip1}, p27^{Kip1}, Gadd 45 and down-regulation of cdk2-cyclin B1, resulting in G2/M arrest (36) and overexpression of p27^{Kip1} induced autophagy. The hypothesis is supported by other reports [37-39].

The concept of cell-cycle mediated drug resistance and how novel CKIs are used to overcome this resistance led us to study the effects of CLX on the expression of both cyclins D1 and E. We found that CLX down-regulates cyclins D1 and E in P1(0.5), suggesting that the coxib may prove to be a valuable tool for the inhibition of cyclin D1/cdk2 and cyclin E/cdk4 complex in resistant HCCs. As a consequence, CLX also down-regulates the phosphorylation of RB, whose main function is to promote the G1/S transition. In line with data shown in the present work, Narayanan *et al.* (2003) report G1 arrest of prostate cancer cells by CLX by modulating cell cycle regulatory proteins such as cyclin D1 and cyclin E, pRB, p27^{Kip1} and p21^{Cip1}(13). On the whole, these data support the concept of sharing key molecules such as Bcl-2, CKI, TGFβ among the pathways of autophagy, cell cycle progression and apoptosis [23,30].

It has been proposed that drug resistance can be overcome by conventional therapies by activating the autophagic process [7,8,40,41]. Rapamycin, a mTOR inhibitor and a well known autophagy inducer, selectively reverses drug resistance in Akt overexpressing and PTEN deficient lymphomas [42] or it cooperates with the selective MET inhibitor PHA56672 to suppress growth and invasion of lung cancer [43].

Exposure to radiation induces autophagy, G1 arrest and p27^{Kip1} expression in radiosensitive cell lines, while the growth of resistant cells is associated with low levels of p27^{Kip1} expression [44].

It has been reported that autophagy can degrade NFκB-inducing kinase and IκB kinase, leading to NFκB inactivation, one of the mechanisms proposed for reversal of drug/immuno-mediated resistance [45]. In line with these observations, here the sensitizing effects of CLX in human MDR-positive HCC cells appear to help to switch off P-gp expression. First, the antiproliferative and pro-apoptotic effect of CLX, observed as G1 cell accumulation and appearance of sub-G1 peak in DNA distribution, appeared to be dependent on P-gp expression, because suppression of P-gp by *MDR1*siRNA makes CLX ineffective. Interestingly, silencing P-gp expression also prevents activation of autophagy and p27^{Kip1} by CLX and these findings fits with our previous data on prevention of apoptosis [6].

Data shown in the present study are schematically diagrammed in Fig. 7. Overexpression of Bcl-2 can be detrimental for response of tumor cells to drug-mediated autophagy/cell cycle arrest/apoptosis and Bcl-2 might be proposed as a main target for reversal of drug resistance.

In conclusion, to the best of our knowledge, this is the first report showing that CLX induces growth suppression by stimulating autophagy, besides apoptosis and G1 arrest, and identifies Bcl-2 as a possible common mediator of these events. These results illustrate that P-gp is responsible for the *in vitro* antiproliferative, pro-autophagic and pro-apoptotic effect of CLX. MDR1 siRNA down-regulates the levels of expression of both HGF and MET and CLX decreases Bcl-2 expression by down-regulation of the MET pathway in P1(0.5) cells. For these reasons the CLX effects on P1(0.5) might depend on the action of P-gp on the HGF/MET autocrine loop. In the future, it would be useful to extend these preclinical *in vitro* results to evaluate the effect of the above used concentrations of CLX in mouse tumor models to support potential clinical studies.

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Acknowledgments

This work is dedicated to "ad memoriam" of Prof. Luciana Tessitore. Luciana tragically died last summer in her beloved mountains while was climbing Monte Rosa together her son. She was an expert climber and an outstanding scientist, she dedicated her life to science, teaching and son. Ciao Luciana, we will not forget you.

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Figure legends

Figure 1. CLX inhibits the growth of MDR-positive cells. (A) MTT assay and (B) Trypan blue exclusion test; cells were exposed to CLX (2.5, 5, 10 and 20 μ M for 48 h); data shown are means \pm standard deviation (SD) of three independent experiments. (C) Cell cycle phase distribution of HCC cells grew without CLX and serum for 48 h (D) Colony formation of MDR-positive cells in soft agar (day 14 after CLX treatment). Colonies showing more than 25 cells/colony were counted and presented as percentage of control.

Figure 2. CLX induces accumulation of subG1 and G1 MDR-positive cells. (A) Representative DNA distribution of HCC cells treated with CLX at the indicated concentrations (0-2.5-5-10-20 μM) for 24 h. (B) Cell cycle phase distribution of P1(0.5) cells treated with various concentrations of CLX for 24 h. Each tabulated percentage represents the average of three independent experiments. (C) Cell cycle phase distribution of P5 cells treated with various concentrations of CLX for 24 h. Data shown are means \pm SD.

Figure 3. CLX induces autophagy of P1(0.5) MDR positive cells. Cells were exposed to CLX for the indicated times and analyzed by Nikon fluorescence microscopy. (A) MDC (0.05 mM) incorporation in autolysosomes. (B) Staining of LC3-II punctat. Cells were exposed to CLX in the presence and absence of 10 mM of 3MA for 1h. (C) Immunocytochemical analysis of Beclin1 expression after 1 h exposure of CLX. (D) Immunocytochemical analysis of Bcl-2 expression after 1 h exposure of CLX. Data are representative of three separate experiments and were calculated from inspection of at least 10 randomly chosen fields (40-50 cells/field). (E) Western Blot analysis of eIF2 α , P- eIF2 α , mTor and P-mTor in P5 and P1(0.5) cells at basal conditions and after treatment with CLX 5 μM for 2 hours. Three separate experiments ere done and one representative of all is shown.

Figure 4. Up-regulation of CKI during CLX-induced P1 growth arrest and up-regulation of TGF- β by 10 μM CLX for 3 h in P1(0.5) cells. Cells were cultured for 24 h in the presence of CLX. (A) Western blot analysis of p16^{ink4a}, p21^{cip1} and p27^{kip1} expression in HCC cell lines. (B) Expression of CKI by immunocytochemistry (10 μM CLX). (C) Quantification of the levels of nuclear CKI expression detected by immunocytochemistry; error bars represent SD of four independent experiments, * p < 0.05 versus control; # p < 0.05 versus P5. (D) Quantification of TGF β 1 and p53 expression by immunocytochemical analysis (10 μM CLX).

Figure 5. Down-regulation of the expression of proteins involved in the mitogenic signal transduction by 10 μ M CLX for 3 h in P1(0.5) cells. (A) Western blot analysis of cyclins D and E, pRb, E2F, ERK and MET expression. (B) Quantification of cyclin D, cyclin E and E2F expression by immunocytochemical analysis; data are means and error bars denote SD of three separate experiments, *p = 0.05.

Figure 6. Suppression of P-gp expression by MDR1siRNA prevents CLX-induced enhancement of p27^{kip1} expression and Beclin1, G1 arrest and apoptosis in P1(0.5) cells. (A) Representative Western blot of P-gp, HGF, MET, Bcl-2 and p27^{kip1}. (B) Levels of p27^{kip1} expression by immunocytochemical analysis after CLX treatment and MDR1siRNA and nuclear quantification of p27^{kip1}. (C) Immunofluorescence of Beclin1. (D) DNA distribution by flow cytometric analysis, *p<0.005.

Figure 7. Hypothetical model of how CLX induces autophagy, cell cycle arrest and apoptosis in P1(0.5) cells.

Fig. 1A

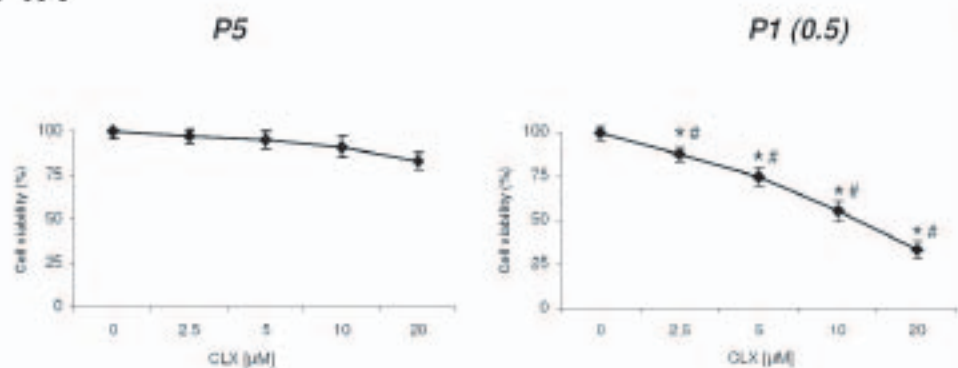


Fig. 1B

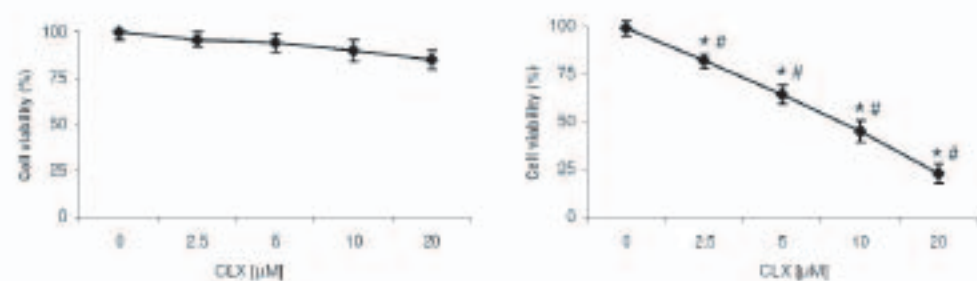
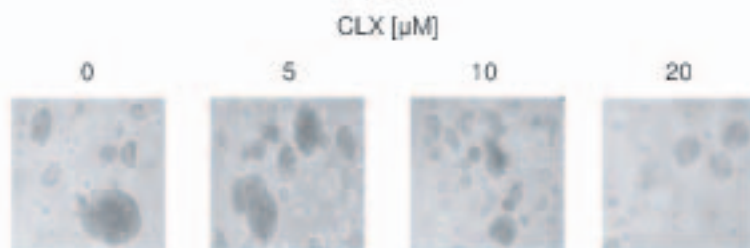


Fig. 1C

Cell Lines	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
P5	0.6 ± 0.7	52.0 ± 3.5	32.0 ± 4.2	16.1 ± 0.7
P1(0.5)	0.3 ± 0.5	54.1 ± 1.2	27.2 ± 0.3	18.6 ± 1.2

Fig. 1D



CLX (µM)	Colony number (colony count/plate)	Colony size (µ ²)
0	101 ± 3.6	60 ± 4.5
2.5	97 ± 5.1	55 ± 4.9
5	89 ± 4.7	40 ± 3.2
10	50 ± 3.2 *	20 ± 3.1 *
20	35 ± 6.1 *	18 ± 3.9 *

Fig. 2A

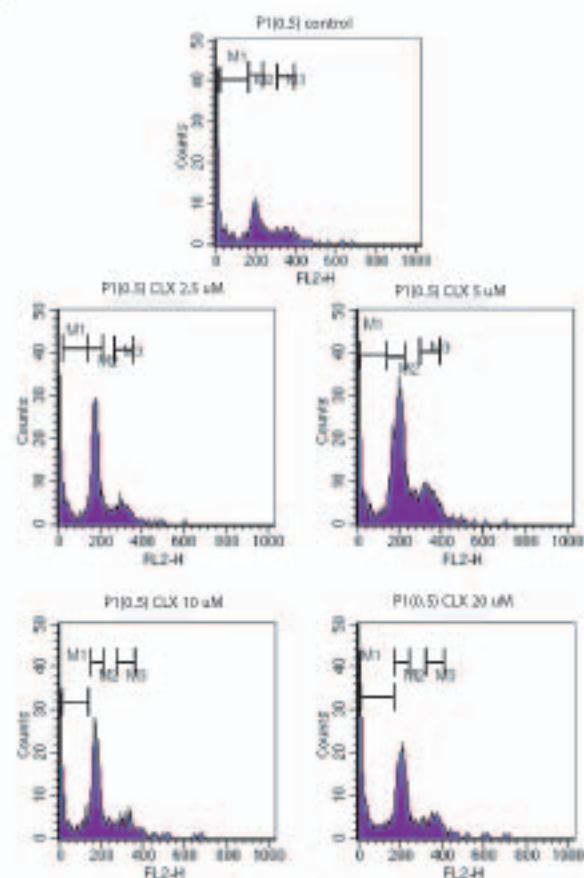


Fig. 2B

CLX (μM)	P1(0.5)			
	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
0	4.4 \pm 0.6	51 \pm 4.6	20 \pm 1.2	29 \pm 2.4
2.5	5.6 \pm 0.9	70 \pm 5.2 [*]	11 \pm 0.9	19 \pm 1.2
5	6.7 \pm 1.0	66 \pm 5.5 [*]	12 \pm 2.3	22 \pm 2.3
10	17.9 \pm 2.3 [*]	62 \pm 4.5 [*]	14 \pm 0.9	24 \pm 2.9
20	22.1 \pm 3.3 [*]	53 \pm 3.2	15 \pm 2.3	32 \pm 3.6

Fig. 2C

P5				
CLX (M)	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
0	0.1 \pm 0.1	57.7 \pm 0.2	34.1 \pm 0.4	8.2 \pm 0.6
2.5	1.1 \pm 1.1	57.8 \pm 1.0	34.3 \pm 1.3	7.9 \pm 0.8
5	0.67 \pm 0.97	56.7 \pm 0.9	35.16 \pm 0.22	8.19 \pm 0.63
10	1.4 \pm 1.0	58.0 \pm 0.97	33.3 \pm 0.8	8.68 \pm 0.35
20	0.7 \pm 0.7	57.5 \pm 1.7	34.7 \pm 1.7	7.7 \pm 0.7

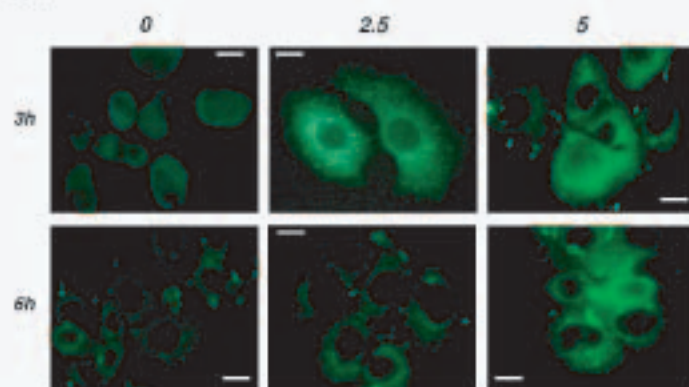
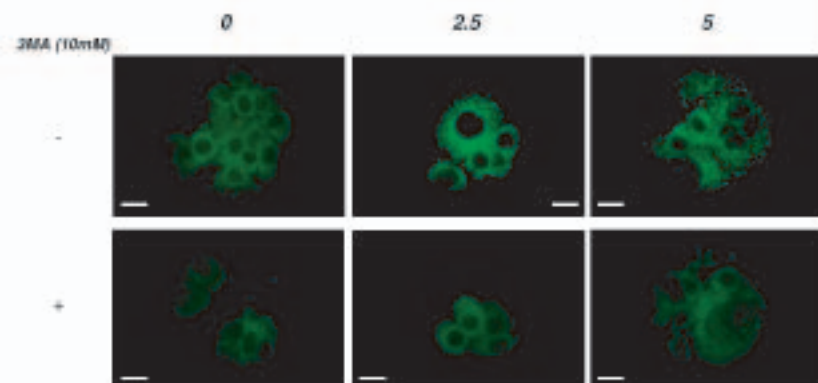
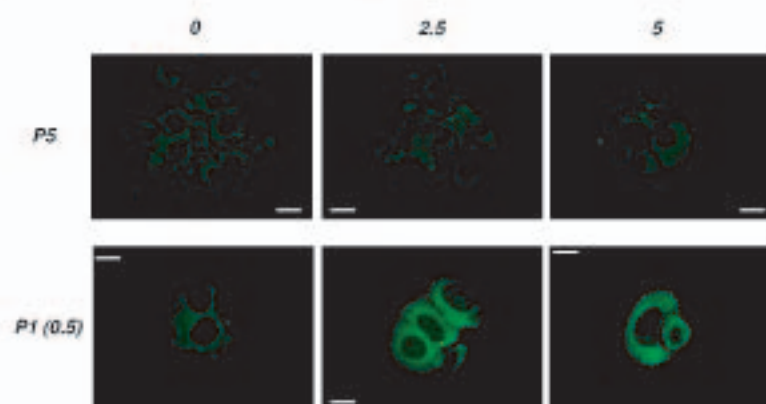
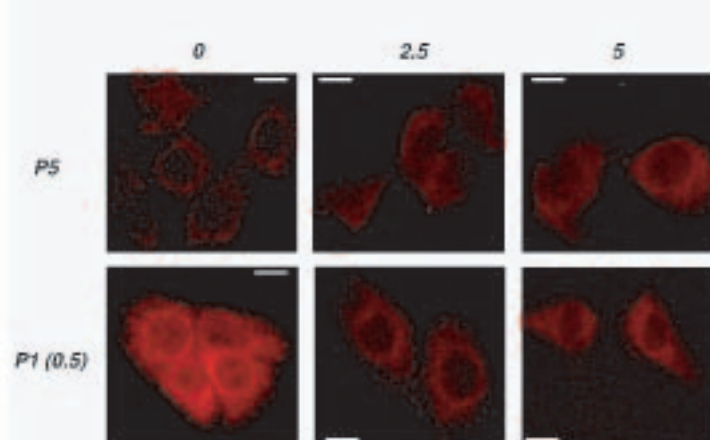
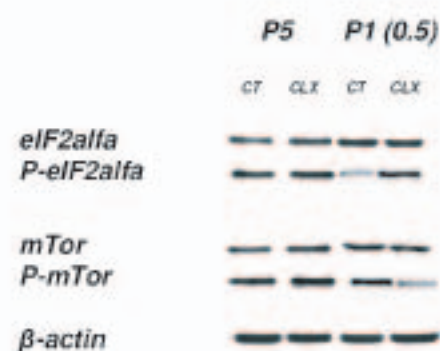
Fig. 3A*CLX* [μ M]**Fig. 3B***CLX* [μ M]**Fig. 3C***CLX* [μ M]**Fig. 3D***CLX* [μ M]**Fig. 3E**

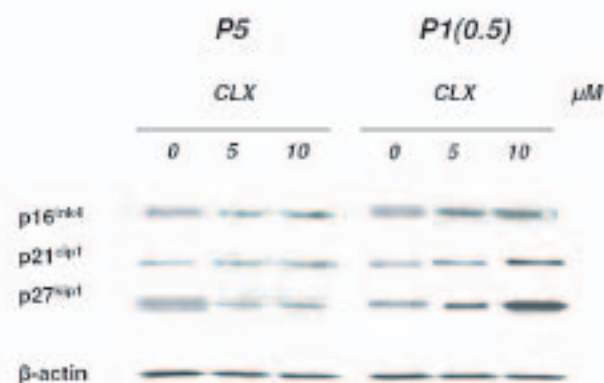
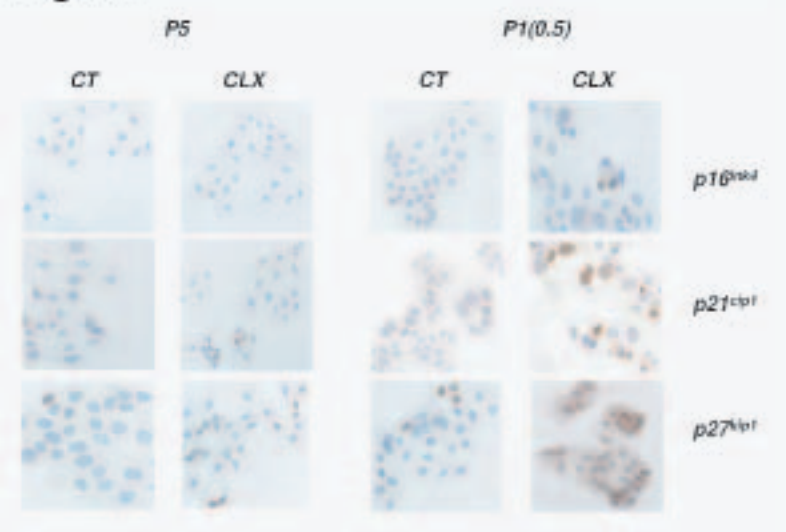
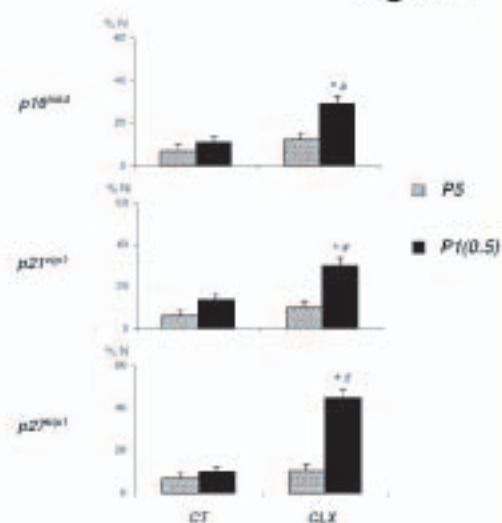
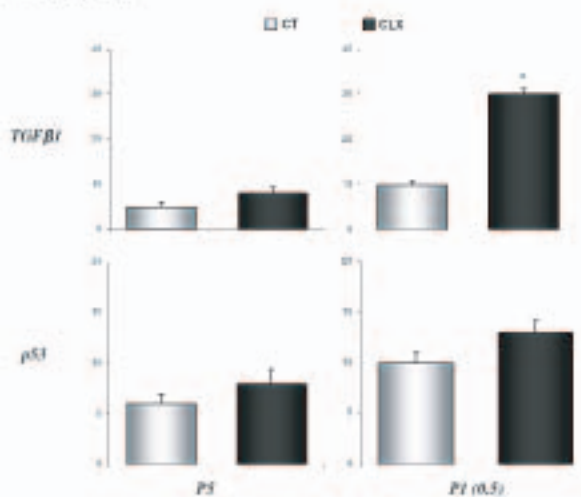
Fig. 4A**Fig. 4B****Fig. 4C****Fig. 4D**

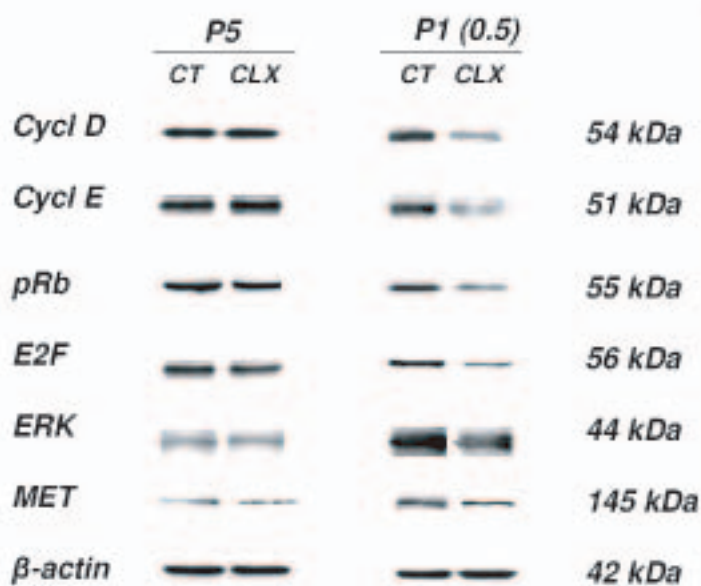
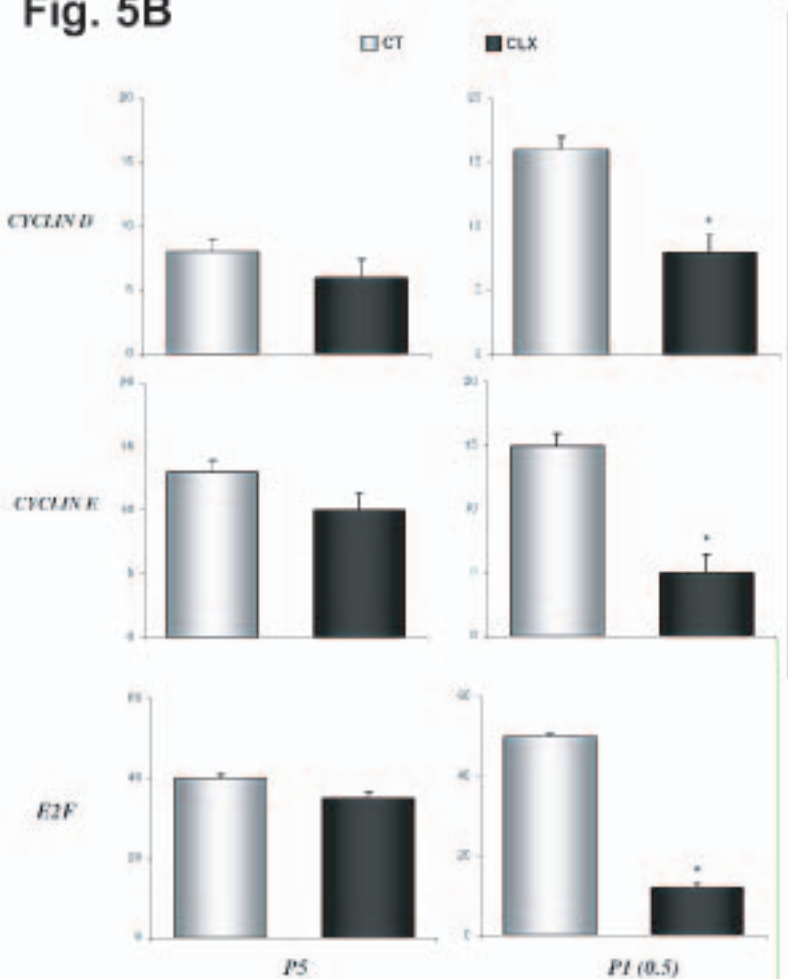
Fig. 5A**Fig. 5B**

Fig. 6A

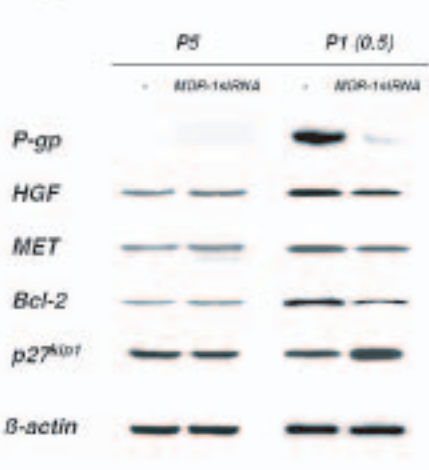


Fig. 6B

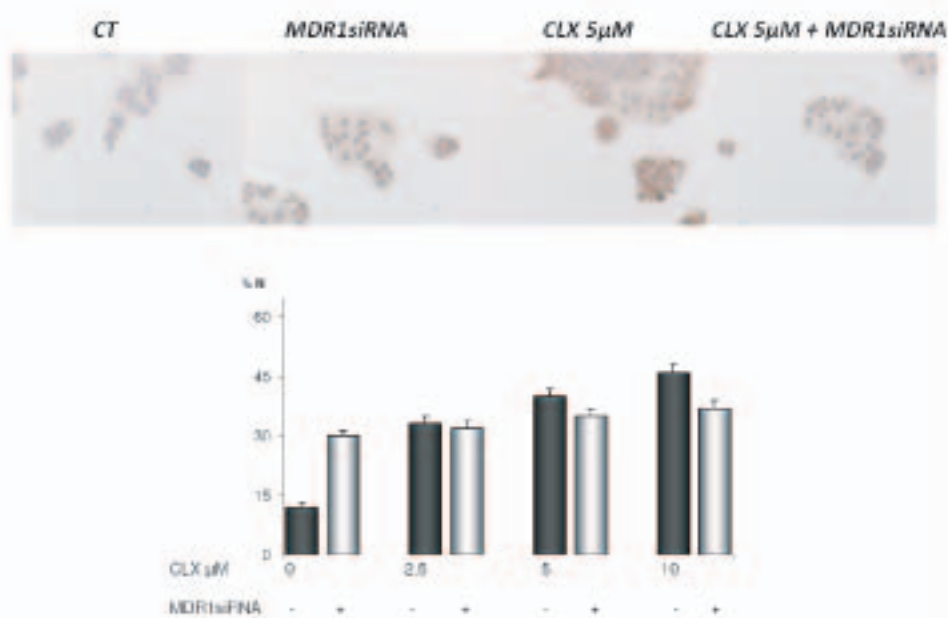


Fig. 6C

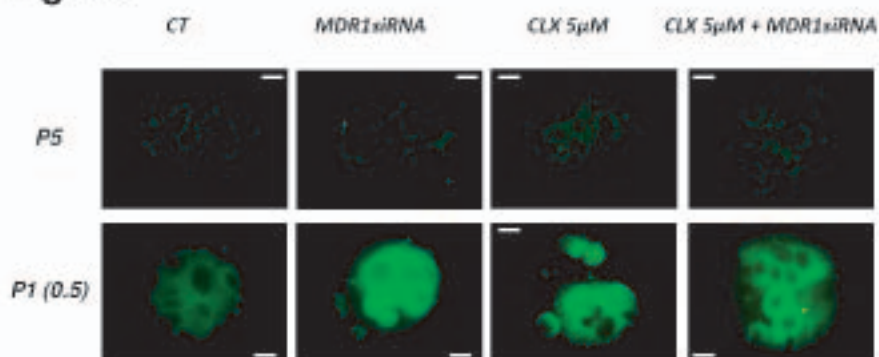


Fig. 6D

	MDR1-siRNA		MDR1-siRNA		MDR1-siRNA		MDR1-siRNA	
	-	+	-	+	-	+	-	+
CLX (μM)	0		2.5		5		10	
sub G1 peak (%)	4.2±1.2	4.8±0.9	5.9±2.2	5.5±2.0	6.2±2.2	5.1±2.3	16.9±4.2	6.5±3.2*
G0/G1 cells (%)	45±6.2	49±5.3	72±1.0	51±2.6*	64±5.6	47±5.9*	58±6.2	46±4.2*

