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THESIS IN BIOMEDICIN

Effect of Oxaliplatin on the HCT116 P53+/- Colon cancer cells

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Abstract

Colorectal cancer is considered as one of the common widely-spread cancer, especially in the western countries. Oxaliplatin as an effective chemotherapeutic agent in FOLFOX regimens is used to treat colorectal cancer. In this study, we investigate cytotoxicity of Oxaliplatin as single chemotherapeutic agent on HCT116 P53+/- is investigated to identify molecular mechanism of Oxaliplatin action in induction of apoptosis pathway. Effective Oxaliplatin concentrations, which inhibit 50% of cell viability, were determined using XTT method. To confirm the effective concentration Standard curve and time-dependent assay are performed. The exposure of Oxaliplatin to HCT116 P53+/- colon cells with deficiency of mismatch repair characteristic resulted to decrease the number of viable cells through apoptosis. It can be concluded that the effect of Oxaliplatin is dose- and time-dependent. Also, the mechanism behind the drug effect should be investigated through detecting of protein, RNA, and DNA levels. Western blot analysis demonstrated relocalization of Bax to mitochondria and induction of intrinsic apoptosis pathway resulted Oxaliplatin exposure. Furthermore, inactivation of Bax in HCT116 P53+/- will result resistance to treatment with Oxaliplatin.

Keywords: Apoptosis, Bax, HCT116 P53+/-, Oxaliplatin, XTT.

Introduction

Colorectal cancer

Cancer is the abnormal growths of cells, which lead to uncontrolled proliferation; subsequently cancer cells invade nearby tissues, and metastasis will occur when cancer cells spread to distant organs. type of cancer will be defined -on the basis of origine of organ that cancer started in or type of cells from which they are derived

Colorectal cancer (CRC) is a type of cancer from the colon and rectum, the mixed terminology is used as colon and rectum cancers have many common features and in many cases they are studied together. Colorectal cancer is the third most common cancer, and fourth most common cause of cancer death worlwide (Cancer Research UK,2008). American Cancer Society has estimated about 49380 death caused by colorectal cacer during 2011.

CRC consists of a heterogenouse complex of diseases; depend on their specific genetic or epigenetic background each patient has exclusive disease that is differ from other patients (Ogino and Goel, 2008). Global molecular classification is classified CRC to chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). Molecular classification is considered due to its benefit in predicting the response of targeted therapy (Ogino and Goel, 2008).

CIN: Tumors with CIN have abnormalities in karyotype and show chromosomal gains and losses (Grady., 2004). Allelic loses is commen in CRC (Vogelstein *et al*., 1989). CIN promot carcinogenesis in CRC through gain in oncogenes or loss of tumor suppresors (Ogino and Goel., 2008).

MSI: Microsatellite are short repeated sequence of DNA. The most common microsatelite in human is CACA.. which occurs tense of thousands across genome. Instability or change in length of microsatelite is a sign in DNA of tumors compared with DNA of normal cells (Ogino and Goel., 2008). All CRC show MSI when screening is composed of large number of markers (Tomlinson *et al*., 2002).

CIMP: In CRC promotor methylation effect on tumor supressor genes in which make them silence (Aqrawal *et al*., 2007).

The first keyfactor For development of colorectal cancer is to disable the normal DNA repair mismatch, subsequently genetic instability are provided, then cells can gradually collect sufficeint number of mutation to provide malignancy phenotype, and one of the complusory phenotype which is leading to CRC is the ability to avoid apoptosis (Watson., 2004). Apoptosis can prevent and manage tumorgenesis (Huerta *et al*., 2006). Epithelial cells with colorectal origine demonstrated the tendency to undergo apoptosis after DNA damage (Alastair *et al.,* 2000).

HCT116 P53+/-

Human Colon carcinoma cell lines are divided in three categories:

-Microsatellite-stablelines like HT29,C70,..

-Microsatellite-unstable lines like HCT116,HCT15,..

-Microsatellite-status unknown like VACO400,VACO429 (Cancer Research UK,2011).

The human colon tumor cell line (HCT116) with epithelial-like morpgology are derived from human colon cancer (figure 1). Homozygous mutation in the mismatch repair gene *hMLH1* on human chromosome 3 can provide microsatelite instability in HCT116 cell lines (Koi *et al*., 1994). HCT 116 P53 +/- are cell lines with heterozygous charecteristic on *P53*.

P53, a tumor suppressor gene has an important role in cellular response to DNA damage (Smith *et al*., 2000). It regulates mostly the gense whose involving in DNA repair and cell survival (Seo *et al*., 2002). Human cancer cell lines with mutation in *P53* has been shown reduction in DNA repair (Smith *et al*., 2000). Since more than 50% of colon tumors have mutation in *P53* , and as *P53* mutation plays a known pivotal role in colon cancer development, its status will be considered and investigated more (Baker *et al.*,1990).

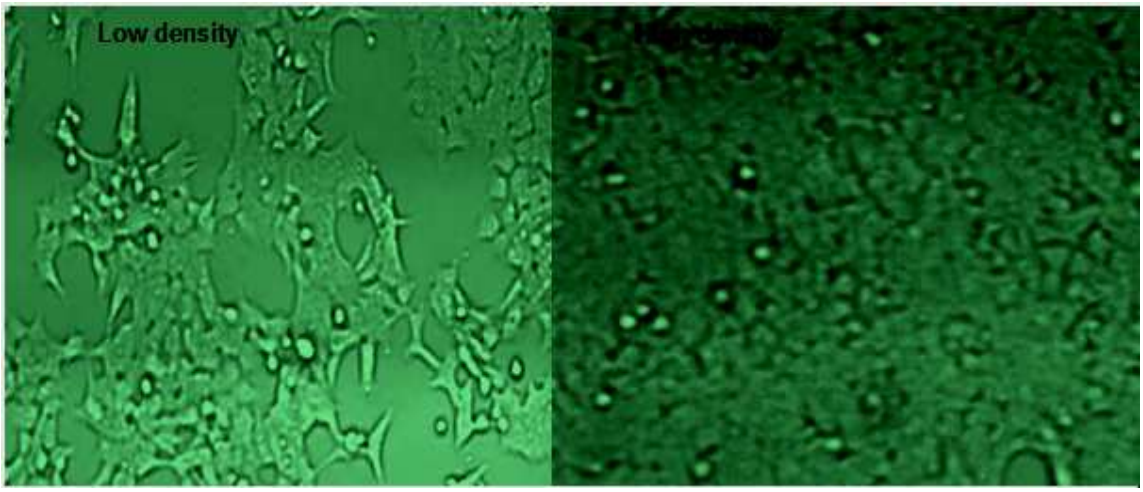

Figure1. HCT 116 P53 +/- seeding in flask T-75.

Chemotherapy

Swedish Cancer Society has recently revealed that mortality in colorectal cancers has decreased in recent decade due to the improvement in treatment. Paul Ehrlich, German chemist defined the term of chemotherapy in the early 1900s.Treatment including chemical agents is known as chemotherapy. Chemical agents are chosen to stop the uncontrolled growth of cancer cells (Vincent *et al*., 2008). Because Chemotherapy can reduce cancer cells in original organ either in distant organ, it is considered as systemic treatment. Almost more than half of patient diagnosed cancer will receive chemotherapy and until now, chemotherapy is an effective cure method.

Drugs with platinum core that are using in combined regimen as chemotherapeutic agent are binding covalent with DNA bases; as a result they inhibit DNA synthesis (Zlatanova *et al.*, 1998). Moreover, evidence has showed that cancerous cells whose using apoptosis as immediate and predominant response to DNA damage had a good response to chemotherapy (Seo *et al*., 2002). Apoptosis was shown as a potential target in cancer treatment (Huerta *et al*., 2006). Oxaliplatin combination with 5-Fluorouracil and Folinic acid as potential chemotherapy agent is using in treatment of metastatic colorectal cancer, and showed 50% response rate (Hata *et al*., 2005).

A little change in cisplatin as a first generation of platinum-base anticancer can produce a new more active anticancer. If the two amine moieties in cisplatin substitute with DACH, third generation platinum-base anticancer drug will create that is more active and does not have cross-resistance with cisplatin. On the other hand this substitution lead to less water solubility, according solving this problem the chloride moieties will substitute and in result of this further modification Oxaliplatin as third generation platinum–base agent will create (figure 2) (Graham *et al*., 2004).

Oxaliplatin

Oxaliplatin or trance-1-diaminocyclohexane oxalatoplatinum or L-OHP is one of anticancer platinum compounds with DACH (diaminocyclohexane) as its carrier ligand (Robert and Pelley, 2001). Platinum-base drugs are the most efficient between anticancer agents (Raymond *et al*., 2002).

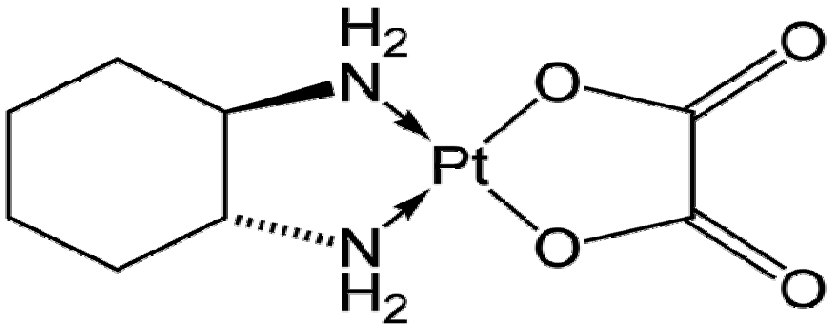

Figure 2.Oxaliplatin; Modification in Cisplatin can create an active anticancer agent with DACH; binding of DACH to a d (GG) site within double-stranded oligonucleotides induces binding of the oligomer (Boudny *et al*., 1991).

Oxaliplatin has a wide anticancer activity spectrum and it showed lack of cross-resistance with cisplatin or even carboplatin during several trials (Raymond *et al*., 2002). The US food and drug administration (FDA) reviewed application for introducing Oxaliplatin as first line therapy in metastatic colorectal cancer patients in March 2000 (Robbert and Pelley, 2001).

Oxaliplatin showed wide cytotoxic effect on different cell lines like colon, ovarian and lung cancer cell lines (Raymond *et al*., 2002). Its anticancer effect will be optimizing when it will be used in combination with 5-fluoracil (5-FU), gemcitabine, cisplatin or carboplatin (Raymond et al., 2002). Oxaliplatin has synergistic activity with 5-fluoracil and there is no cross-resistance with 5-FU and CPT-11 and other platinum-base anticancer agents (Robbert and Pelley, 2001).

Oxaliplatin through its DACH can produce type of DNA adduct which would not recognized by Mismatch Repair system (Sergent *et al*., 2001). Besides it has potential to induce intrinsic pathway of apoptosis (Hata *et al*., 2005). Understanding of modifications at intrinsic apoptosis pathway, which lead to colorectal cancer, can introduce the treatment options (Huerta *et al*., 2006).

Aim

In present study, we investigate Oxaliplatin cytotoxicity to HCT116 P53+/-; effect of Oxaliplatin was assessed as single chemotherapeutic agent. In addition to confirm effect of Oxaliplatin we investigate if it is dose or time dependent.

In an effort to clarify the mechanism associated with Oxaliplatin, we examined efficacy of Oxaliplatin using HCT116 P53+/- colon carcinoma cells with special attention to the status of *P53* gene. The study focused to identify the effect of Oxaliplatin on Bax expression.

Material and methods

Chemicals

Oxaliplatin or [SP-4-2- (1R-trans)]-(1, 2-Cyclohexanediamine-N, N′) [ethanedioata (2)-O, O’] platinum, with chemical formula C8H14N2O4Pt and Molecular Weight: 397.29 were purchased from Sigma-Aldrich (09512, Sigma-Aldrich, Sweden). Oxaliplatin diluted 1mg/mldH_2O, aliquot in ependorfs and used as drug stock; drug stock stored at -20°C and warm in room temperature before use.

XTT 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt with 1% phenazine methosulfate (PMS) (TOX2, Sigma-Aldrich, USA) reconstituted with 5ml Medium in dark and stored in -20 °C, before use warm in 56°C water bath.

Cell line

HCT116 P53+/- (American type culture collection (ATCC), Bethesda, MD; ATCC#CCL 247) was seeding in flask T-75 (2x10 ^{6}cells per ml). Subculture was done every 2-3 days using Trypsin-EDTA 1X, 5 ml per flask T-75 (L11-004, PAA Laboratories GmbH; Austria).

Medium

Liquid cell culture media, McCoy’s 5A Medium (Exx-022, PAA Laboratories GmbH, Austria and also SIGMA-ALDRICH, Sweden) without L-glutamine supplemented with 50ml fetal bovine serum 10% (FBS), 3.75ml L-glutamine 200Mm (G7513, Sigma-Aldrich, UK), and 5ml 1X PEST (Penicillin/Streptomycine100X, P11-010, PAA laboratories GmbH, Austria). Prepared medium was stored in 2-8°C and before use warm in 37°C.

Cell culture

HCT116 P53+/- was received in passage 24 and until end of experiment subculture continued to passage 49. In passage 24 the cells split to two T-75 flasks, one 0.5 ml cell suspension and other 1 ml cell suspension plus 20ml medium. This work helped us to have cells every day, following they were sub cultured in only one T-75 flask in different day. Subculture start with washing with 5ml PBS, add 5ml trypsin, incubation about 7 minutes and then add 5ml medium to trypsin and centrifuge 1000 rpm 5minites, discard the supernatant and suspend cells in 5ml fresh medium. Cells kept in incubator with 5%CO2 and 37°C.

Cell viability measurement

During subculture cell suspension with 5 ml medium were kept in ice until performing cell counting, cell counting were done to make 96 well-plate (83.183, Flat bottom with lid, SARSTED, Inc., Newton, USA); according cell counting 30,000 cells per well was seeded in triplicate. 96 well-plates was incubated 24 hours, subsequently treatment were performed during 48 hours, after 24 hours the drug, which was diluted with medium, changed according half-life of drug. After 48 hours treatment 100µl of

medium was discarded and in dark XTT was warmed at 56 °C and 20µl XTT were added to every well. 96 well-plates were covered with aluminum foil and incubate for 4 hours with 5% CO2 and 37°C. Absorbance was read in 405nm following 595nm as background with FLUOstar Galaxy (BMG labtechnology).

Treatment

The first range of concentration 4, 8, 12, 16, 20 µg /ml performed with serial dilution (Table 1), and also Second range of concentration 1, 2, 4, 6, 8 µg /ml was performed with serial dilution (Table 2).

Table1.Preparation of diluted drug

Final drug concentration (µg/ml)	Volume of diluent (medium) µl	Volume and source of drug
20	9800	**200 µl**
16	1600	**6400 µl of20 µg/ml**
12	1500	**4500 µl of 16 µg/ml**
8	1333	**2666 µl of 12 µg/ml**
4	1000	**1000 µl of 8 µg/ml**

Table2. Preparation of diluted

Final drug concentration (µg/ml)	Volume of diluent (medium) µl	Volume and source of drug
8	9920	**80 µl**
6	2000	**6000 µl of 8µg/ml**
4	2000	**4000 µl of 6µg/ml**
2	2000	**2000 µl of 4µg/ml**
1	1000	**1000 µl of 2µg/ml**

Standard curve

Following 48 hours treatment just before adding XTT, subculture was performed and three different cell suspensions was prepared 5000, 25000, and 125000 cell per ml (Table 3). According layout 1 appendix 100µl of them was added to standards wells, subsequently XTT performed.

Table3.Standards preparation

Standards (cell/ml)	Cell suspension (µl)	Fresh medium (µl)
5000	10	**1990**
25000	50	**1950**
12500	200	**1800**

Time-dependent assay

According effective concentration, time-dependent assay was started; Four 96 well-plates was prepared as layout 2 appendix, 24hours, 48 hours, 72 hours, and 96 hours. Time-dependent assay was performed two times with 2µg/ml and one time with 1µg/ml Oxaliplatin. After 24 hours seeding treatment was started for four, the day after XTT was measured for 24 hours plate and three was treated, at second day XTT was measured for 48 hours and treatment was done for two remained plate, cell viability measurement continued until 96 hours after treatment with effective concentration.

Western blotting

Protein extraction

Two 6 well-plates (92006, TPP, Europe, Switzerland) were prepared according layout 3, 24 hours treatment and 48 hours treatment. Protein isolated at two following days after treatment completed for each plates. Cells were washed with 1.5 to 2 ml PBS per well gently, and trypsin was added 1ml per well, after 5 minutes incubation the same amount of medium were added to wells and cells pipette to falcon tubes, falcon tubes were centrifuged 5 minutes 1000 rpm, mixed medium and trypsin were discarded and 1ml fresh medium was added, cells with medium was pipette up and down and the place change to ependorf tubes and centrifuged 13000 rpm 15 minutes at 4°C. Medium was discarded gently and pellet were washed two times with cold PBS and centrifuged 13000 rpm 15 minutes at 4°C; PBS was removed; one protease inhibitor tablet (11836170001, complete Mini EDTA-free, Roche Diagnostic GmbH, Germany) was solved in 10 ml RIPA Buffer ; 100 ml RIPA Buffer was prepared with 25 mM Tris. HCL pH 7.6; 150mM NaCl; 1% NP-40; 1% sodium deoxycholate; 0.1% SDS (89900, Pierce Biotechnology catalogue), and stored in cold room at 4°C. 600 µl of RIPA Buffer for every two wells were added, and pellet mixed with RIPA Buffer. Ependorfs were placed in ice and for 15 minutes shake in ice, then centrifuge 13000 rpm 15 minutes at 4°C. Supernatant separated accurately and place in new ependorfs, RIPA Buffer contain protein were stored at -20°C. Whole process of protein extraction was done on ice.

Protein measurement

Nine Standards were prepared with 1ml bovine serum albumin 2 mg/ml according table 1 at 1296.6 instruction of pierce BCA protein Assay Kit (23225, pierce biotechnology). 500µl of reagent B was added to 25ml of reagent A to prepare the working reagent. 96 well-plates was prepared according layout 4 appendix (Table 4).

96 well-plates were incubated 30 minutes inside FLUOstar Galaxy, and then 10 minutes cooled in room temperature; absorbance was measured at 550 nm.

Table4. Preparation of protein samples and standards

	Sample (µl)	Standards (µl)	RIPA Buffer (µl)	DH2O (µl)	Working reagent (µl)
Standards	-	25	10	-	**200**
2µl proteins	2	-	8	25	**200**
10µl proteins	10	-	-	25	**200**

Western blotting

Reagents and stock solution for western blot was prepared according manual of SDS-PAGE (Laemmle) buffer system including 10% (w/v) SDS; 1.5M Tris-HCL-PH8.8; 0.5M Tris-HCL-PH6.8; Sample buffer (SDS Reducing Buffer); 10x electrode (Running) buffer-PH8.3; 10% APS, 4% (stacking) and 12% (resolving) gel; 10x washing buffer (TBS) and TBS/T (165-3301, Mini-PROTEAN 3 Cell, BIO-RAD).

According ladder (161-0374,Kaleidoscope ladder; Bio Rad) and molecular weight of the protein of interest, 12% SDS-PAGE as resolving gel and 4% SDS-PAGE as stacking gel was prepared (165-3301,Mini-Protean 3 cell instruction manual). 10 well combs with 1.5mm spacer were chosen to provide more volume (66µl) per well. Proteins volumes were calculated to provide 60 µg as concentration for every protein samples (Table 5). Samples diluted with sample buffer in 1:1 ratio. Samples were heated at 95°C for 4 minutes before loading in wells.

Table5. Preparation of loading sample

	Protein volume (µl)	Sample Buffer (µl)	Total volume (µl)
Control 24 hours	25.41≈25	25	**50**
Treated 24 hours	26.96≈27	27	**54**
Control 48 hours	15.83≈16	16	**32**
Treated 48 hours	28.32≈28	28	**56**

Electrophoresis was run with 200 volts constant as it is recommended, the time depend on the blue line of samples was approximately 40 minutes. Four filter papers, membrane, gel and four filter papers were the order of gel sandwich. Semi dry transfer was run at 15 volts for 35 minutes. Non-specific binding was blocked with incubation in TBS/T with 5% skim milk for 1 hour at room temperature on shaker.

Primary Antibodies, Bax mouse monoclonal antibody (IgG_1) (6A7: Sc-23959, Santa Cruz Biotechnology, Inc., Europe), and β-actin, mouse monoclonal antibody (IgG_1) (C4: Sc-47778, Santa Cruz Biotechnology, Inc., Europe) were diluted in 5% skim milk in TBS/T with 1:200 ratio. Membranes were incubated with primary antibodies in cold room 4°C on shaker overnight.

Goat anti-mouse IgG-AP as secondary antibodies (Sc-2008, Santa Cruz biotechnology, Inc., Europe) was diluted in 5% skim milk in TBS/T with 1:500. Membrane was incubated 45 minutes at room temperature on shaker. One BCIP/NBT (B565525TAB, Sigma-Aldrich, Sweden) was solved in 50ml-deionized water by vortex and were poured on membrane, membrane was shaking about 1 hour.

Statistical analysis

XTT results were analyzed with student T-test.

Nitrocellulose membrane scanned by Bio-Rad image software using Coomassi scan program (165-9770, Proteomweaver 4.0 Professional 2-D analysis software; Bio-Rad laboratories, Inc. USA).

Consequently Imaged membrane was analyzed using Image J software (Image J 1.44p, Java 1.6.0_20; Wayne Rasband, National Institute of Health, USA).

Results

Morphology and pathology of HCT116 P53+/-

HCT116 P53+/- originated from colon carcinoma with mutator phenotype is an adherent epithelial cell, which grew plain that attached easily to culture flask. Cells were allowed to grow until reaching 80-90% confluence about 3 days, and then they were split and serially passaged. At every passage monolayer of cells were cultured uniformly in T-75 flask surface. Cells after two days incubation that their confluence was about 50-60% and they were in their exponential phase, were used in experiment. After treatment with Oxaliplatin, cell deformation was observed besides decreasing in their number.

Optimum Oxaliplatin concentration measurement

Before adding XTT to cells, cultured cells were observed through microscope (figure 3). When cells compared with control which did not get any drug, The treated culture with 1µg/ml concentration of Oxaliplatin showed decrease in number of alive cells and 8µg/ml or higher (12, 16, 20 µg/ml) concentration which previously used for treatment, showed notable decrease in number of alive cells, also cells lose their origin shape. Therefore, 1µg/ml and 2µg/ml was used in time-dependent experiment because they were covering IC50 concentration.

Cytotoxicity of Oxaliplatin for HCT116 P53+/- was measured using XTT method. 48 hours after treatment for each concentration with Oxaliplatin, XTT was added to cells, and percentage of cell viability was measured. IC50 concentration, which demonstrates the cytotoxicity of Oxaliplatin for 50% of cells, or concentration in which 50% of cells are viable, was observed between 1µg/ml and 2µg/ml dose of treatment (figure 4). Results depend on three independent experiments. Each sample with different concentration of Oxaliplatin was compared with control sample using student t-test which indicates samples with P>0, 01.

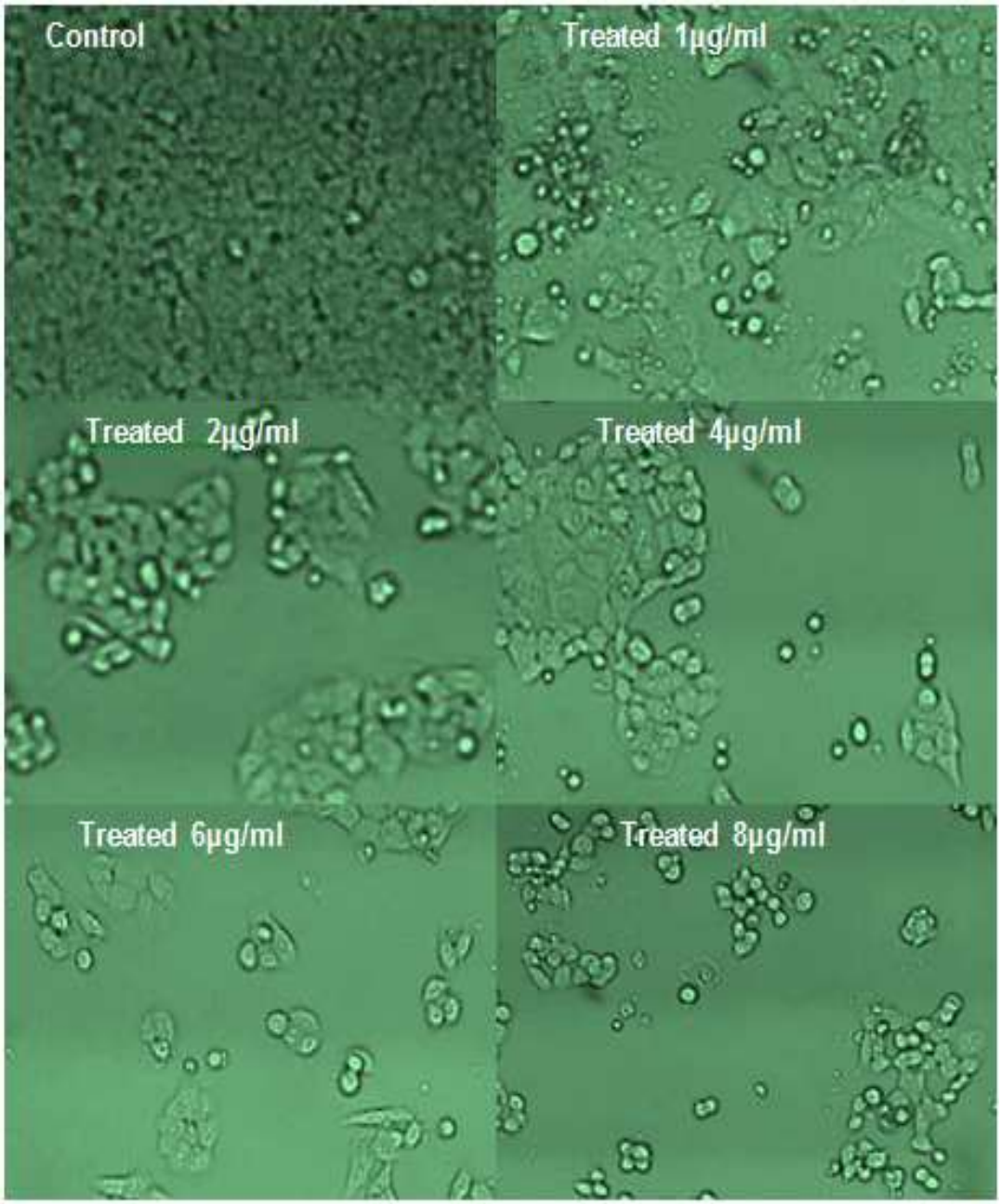

Figure3. HCT116 P53+/- after incubation with various concentration of Oxaliplatin for 48 hours (10X Inverted-microscope image)

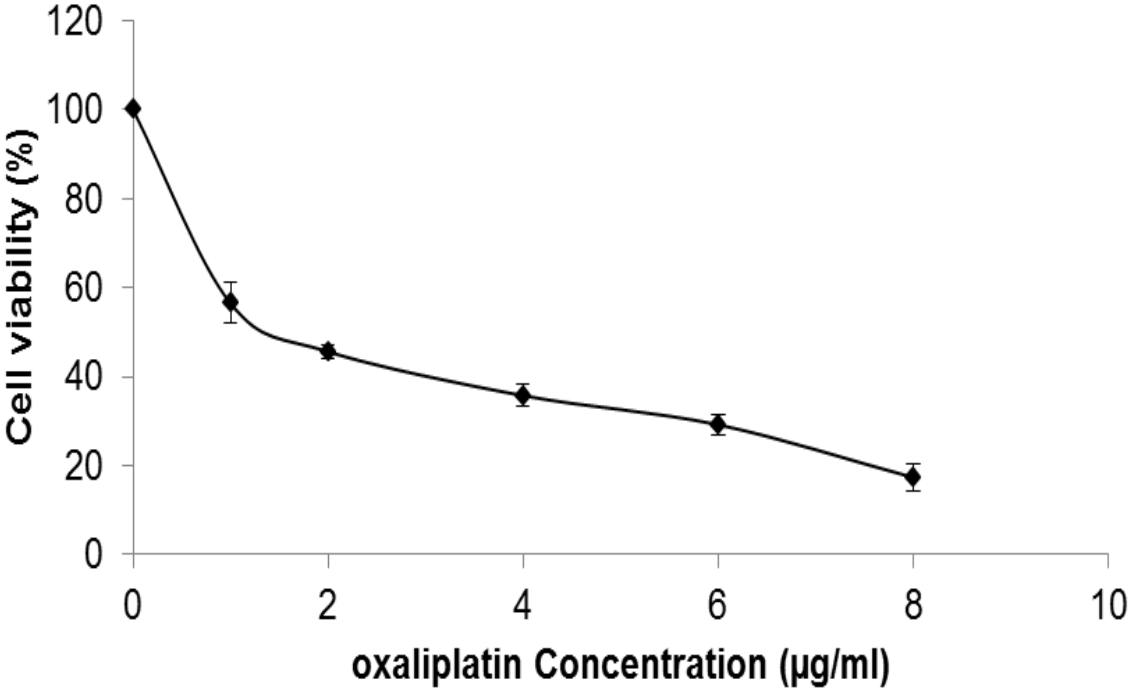

Figure4**.** Effect of various Oxaliplatin concentrations on HCT116 P53+/- cell viability

Number of viable cells after treatment according standard curve

In order to find the number of cells after treatment with Oxaliplatin, 5000, 25000 and 125000 cell/ml of cell suspension prepared and poured to wells whose during 48 hours treatment contained just medium like Blank wells before adding XTT. Standard curve was created according absorbance of different cell suspension, *m* value was calculated from standard curve, consequently number of cells in different range of Oxaliplatin treatment concentration was obtained through $y=kx+m$ (figure 5). According results increase in Oxaliplatin concentration caused decrease in the number of viable cells.

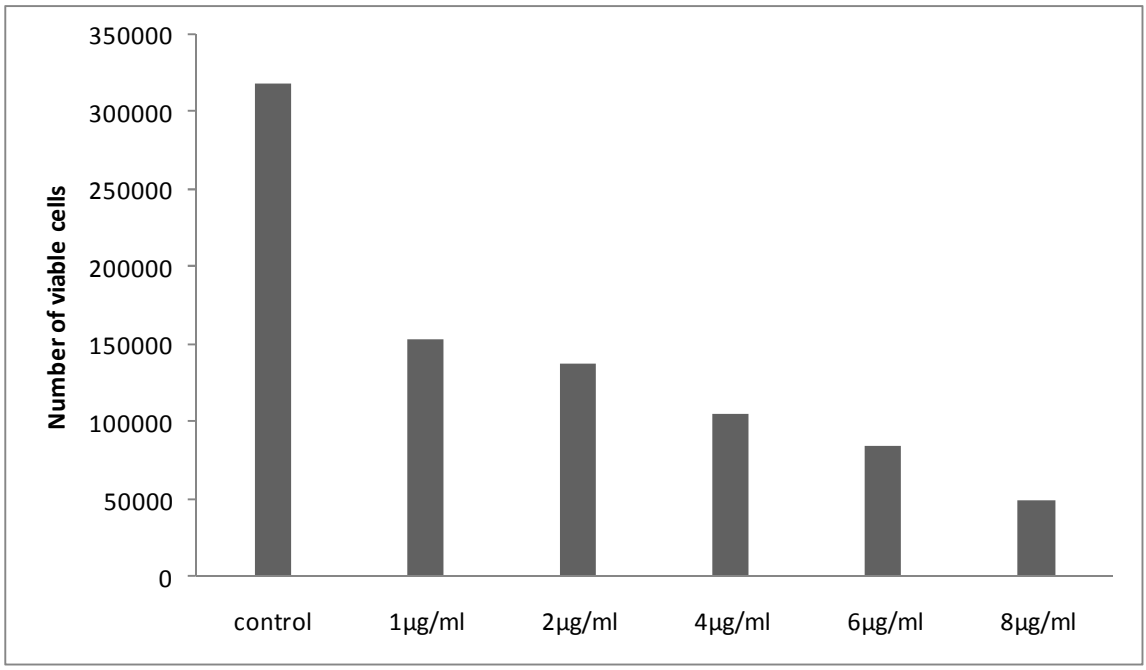

Figure5. Number of viable cells per well after 48 hours treatment with Oxaliplatin

Number of viable cells showed decrease dramatically with increasing treatment dose. Number of remained viable cells in wells with 1µg/ml and 2µg/ml Oxaliplatin treatment were about half of alive cells that observed in control.

Time-dependent Assay

To further analyze time-dependent assay was performed, HCT116 P53 +/- was incubated with 1µg/ml and 2µg/ml separately over 96 hours (figure 6). 2µg/ml was found as more effective dose, therefore experiment continued with 2µg/ml to assess the cytotoxicity of Oxaliplatin over time. XTT colorimetric assay was performed four times for one time-dependent assay. Percentage of cell viability for 24 hours plate was measured after 24 hours Incubation, 48 hours plate after 48 hours incubation, 72 hours plate after 72 hours incubation, and 96 hours plate after 96 hours incubation with Oxaliplatin (figure 7). The result confirmed the time between 24 and 48 hours incubation with Oxaliplatin as effective time to provide IC50 concentration. The results are based on two independent experiments.

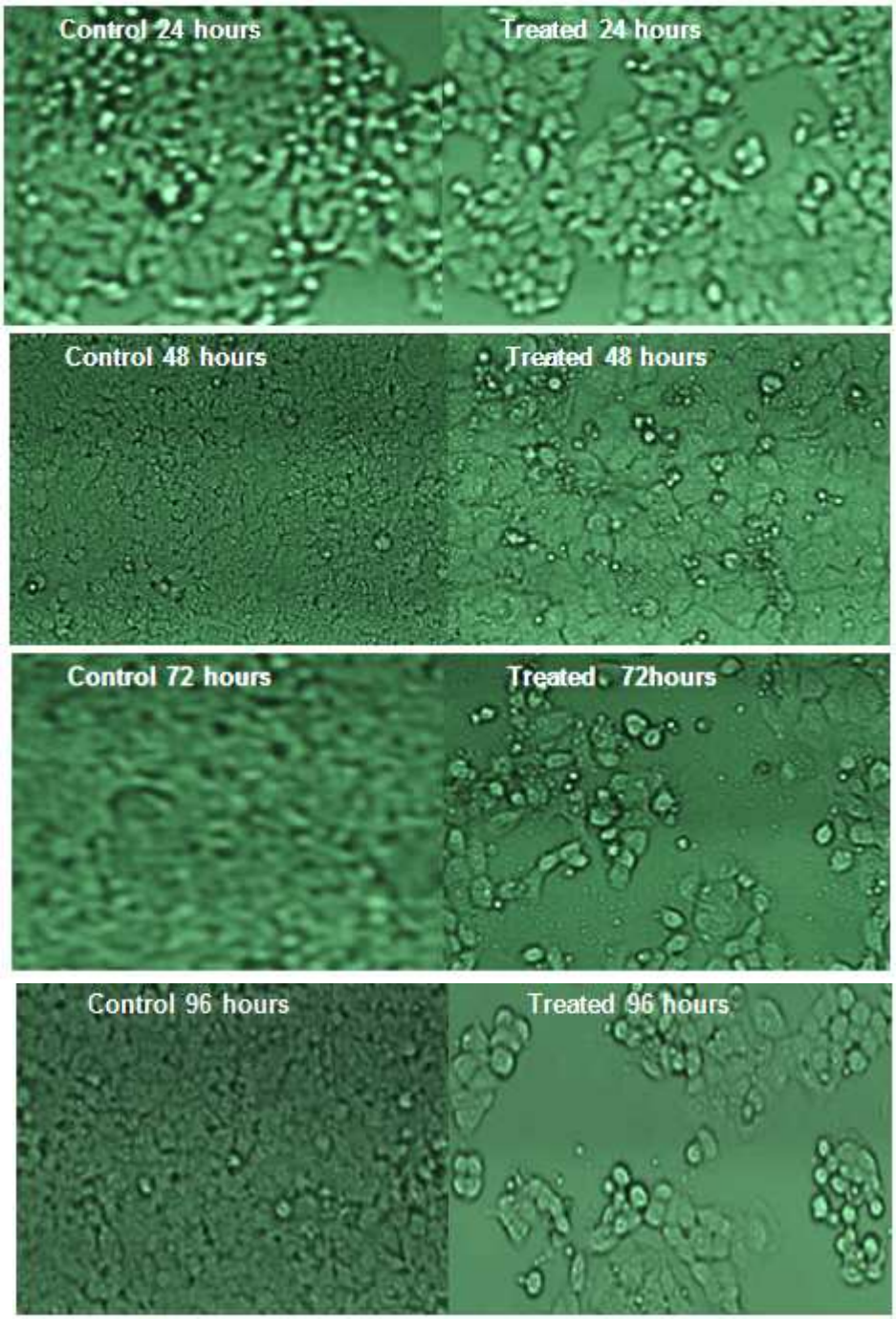

Figure6. HCT116 P53 +/- incubated with 2µg/ml Oxaliplatin over time (10X Inverted-microscope image)

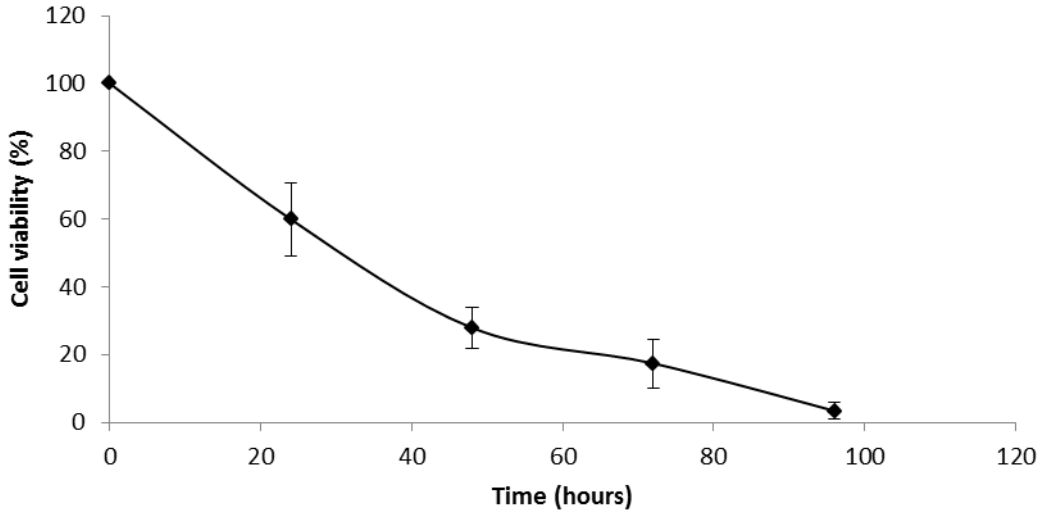

Figure7. Effect of 2µg/ml Oxaliplatin is shown on HCT116 P53+/- over time.

Percentage of viable cells decreased over incubation with Oxaliplatin during 96 hours treatment. Cell viability percentage was close to zero at 96 hours incubation. It demonstrated that selected dose which was 2µg/ml had efficacy.

Quantitative determination of protein concentration

Micoplate procedure was performed because of smaller protein volume requirement (max 10µl). Based on series of dilution of known concentration standards curve were made, accordingly unknown concentration was determined. Control 24 hours protein sample was extracted from cells without oxaliplatin exposure incubated 24 hours; Treated 24 hours protein sample was extracted from cells incubated 24 hours with oxaliplatin; Control 48 hours protein sample incubated 48 hours without oxaliplatin exposure; and Treated 48 hours protein sample derived from cells incubated 48 hours with oxaliplatin. According standard curve , protein concentration were calculated for every samples (figure 8). Highest concentration belonged to Control 48hrs while the lowest concentration was for Treated 24hrs. Treated 48hrs showed 1.5 times less concentration comparison with its control.

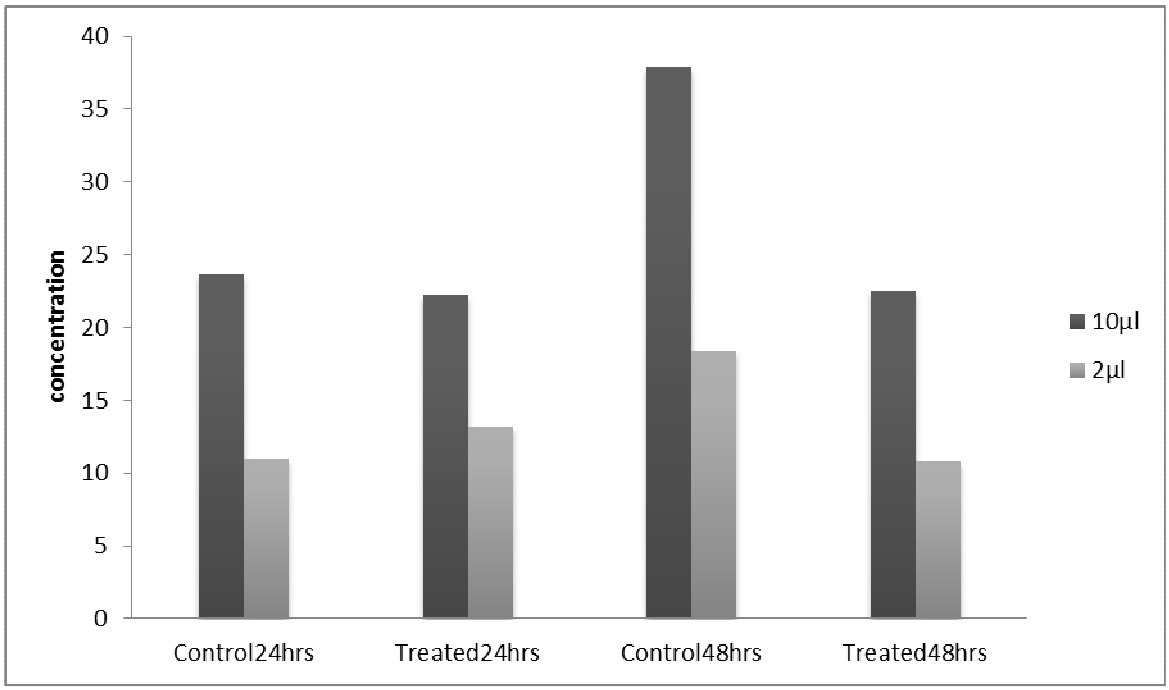

Figure8.Total protein concentration per 10 and 2µl in different samples.

Expression of Bax

To investigate the apoptotic cascade induced by Oxaliplatin exposure, expression of Bax was assessed by western blotting (figure 9). For internal control β-actin used to normalize level of protein expression.

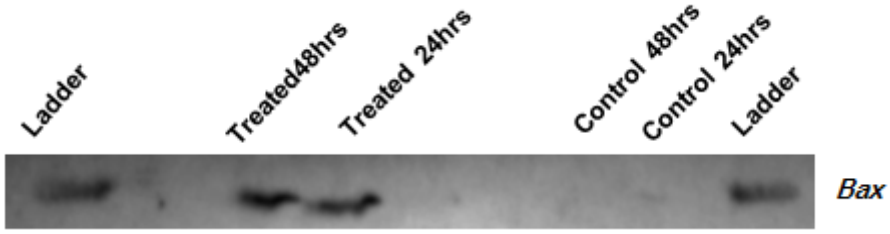

Figure9. Bax expression in HCT116 P53+/- after Oxaliplatin exposure

Membrane was cut between pink (25 kDa) and green (37 kDa) ladder band. After development, membrane was scanned and image was analyzed through Image J (Figure 10). Results are based two independent western blots. Untreated cells or controls were used to predict response of HCT116 P53+/- cells to Oxaliplatin. The results demonstrated that treatment with Oxaliplatin at IC50 concentration induced Bax expression.

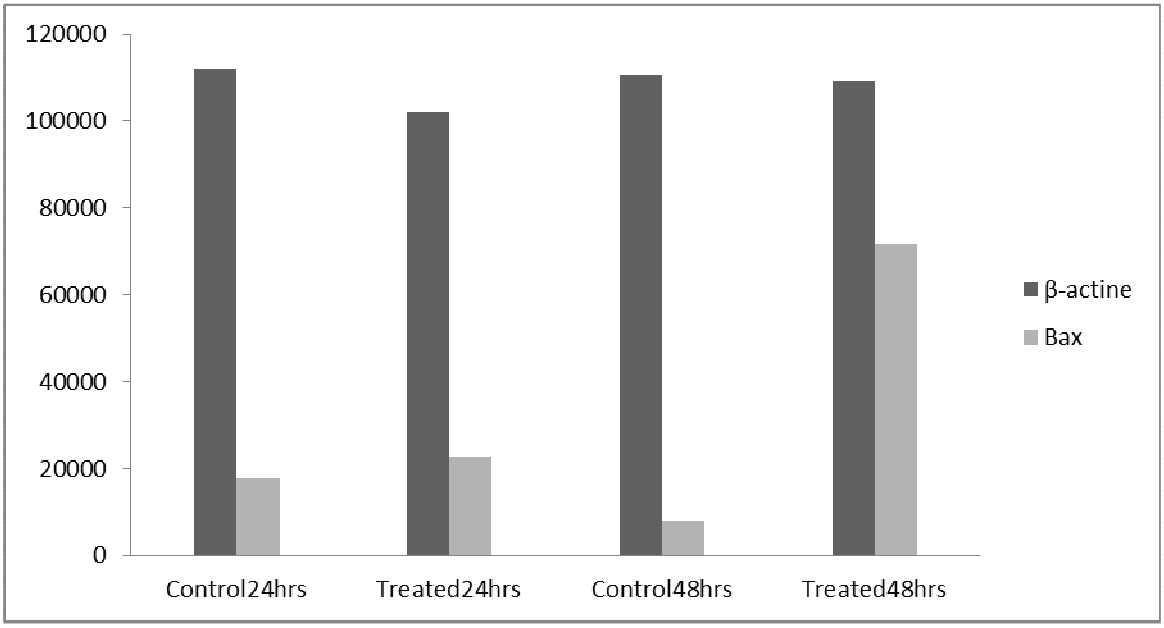

Figure10. Expression level of Bax and β-actin

Controls band were so weak, and control 48hrs showed less expression of Bax comparison with control 24hrs. Bax expression in treated 48hrs was almost nine times more than control 48hrs.

Discussion

Colorectal cancer was observed as resistance tumor to chemotherapy regimens (Blijham, 1991). In 2000, new attractive regimen was introduced for CRC treatment, FOLFOX regimen in which Oxaliplatin used in combination with 5-Fluorouracil and Leucoverin; this regimen showed higher clinical efficacy about 51% (Gramont *et al*., 2000). In this study, Cytotoxicity of Oxaliplatin was evaluated to HCT116P53+/- as a single chemotherapeutic agent. Effective concentration was found between 1µg/ml and 2µg/ml, which inhibited 50% of cell viability during 48 hours treatment (Dahan *et al*., 2009). Standard curve that had been provided from different cell suspension confirmed IC50 concentration. 30000 cells per well were seeded in 96 well-plate in triplicate and according standard curve half amount of control cells provided in wells incubated with 1µg/ml and 2µg/ml Oxaliplatin.

We predicted to have no resistant in HCT116 P53 +/- due to its deficiency in mismatch repair (Sergent *et al*., 2002); HCT116 P53+/- that is heterozygous in P53 was sensitive to Oxaliplatin during our experiment (Seo *et al*., 2002). Exposure of HCT116 P53+/- to effective concentration of Oxaliplatin reduced number of cells through apoptosis induction.

Expression of Bax was assessed due to discover the way of apoptosis induced by Oxaliplatin. The result demonstrated functional role of Bax in intrinsic apoptosis cascade following exposure to Oxaliplatin (Gourdier *et al.*, 2002; Hayward *et al*., 2004; Arango *et al*., 2004). Intrinsic apoptosis pathway will be activating by relocalization of Bax to mitochondria, cytochrome releasing to cytosol and activation of caspase 3. To initiate apoptosis, Bax expression should show increase and the result of western blot in this study, demonstrated increase in Bax expression in treated samples, which had exposure to Oxaliplatin about 48 hours. Following amount of cytosolic cytochrome C can confirm this evidence. Consideration deficiency in one allele of P53 in HCT116 P53+/-, Oxaliplatin was powerful enough to recruitment Bax and induces apoptosis via Bax-dependent pathway. The recent study showed Oxaliplatin had potential to induce intrinsic apoptosis pathways on HCT116 P53+/- cells, while there is evidence that Oxaliplatin had minor induction rate in apoptosis via Bax-dependent pathway (Hata *et al*., 2005).

Conclusion

Information about molecular mechanism involved in induction of apoptosis after treatment with chemotherapeutic agents can provide us improvement in treatment efficacy and introduce markers, which are helpful to predict response of treatment. It is confirmed that Oxaliplatin can induce forming intrastrand DNA adducts which cause interrupt DNA replication and transcription, but downstream molecular event have not been known as well. 1μg/ml and 2μg/ml Oxaliplatin can provide effective treatment on HCT116 P53+/-, and cells need to expose over 24 to 48 hours to show effective cytotoxicity. As shown in this experiment effect of Bax expression on HCT116 P53+/- after exposure to Oxaliplatin can clarify the effect of Oxaliplatin on intrinsic apoptosis pathway. Apoptosis Induction can be one of the effective treatments in cancer therapy. Inactivation of Bax in HCT116 P53+/- can lead to resistance to Oxaliplatin.

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Appendix

Layout 1:

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	C	1µg/ml	2µg/ml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	C	1µg/ml	2µg/ml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	C	1µg/ml	2µgml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	C	1µg/ml	2µg/ml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	C	1µg/ml	2µgml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	C	1µg/ml	2µgml	4µg/ml	6µg/ml	8µg/ml	B	S1	S2	S3	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Layout2:

PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	B		C		2µg/ml		2µg/ml			B	PBS
PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Layout 3:

C24/48	Treated 2µg/ml 24/48	Treated 2µg/ml 24/48
C24/48	Treated 2µg/ml 24/48	Treated 2µg/ml 24/48

Layout4:

A (S1)	B (S2)	C (S3)	D (S4)	E (S5)	F (S6)	G (S7)	H (S8)	I (S9)			
A	B	C	D	E	F	G	H	I			
C242µl	T242µl	C482µl	T482µl								
C24	T24	C48	T48								
C2410µl	T2410µl	C4810µl	T4810µl								
C24	T24	C48	T48								