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Comparison Anticancer Effects of Platinum Ribavirin with Platinum Azidothymidine via Telomerase, Bcl-2, miRNA-21 and miRNA-122 Biomarkers Genes Expression on HepG2 Cancer Cell Lines

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Abstract

Background: Anticancer medication assessment is performed by numerous approaches including biomarkers gene expression. This study aimed to investigate the anticancer effects of Pt-Rb and Pt-AZTon HepG2 cells.

Method: In this case-control study, four groups of cells were examined. Group A was the control group, group B was untreated cancer cells, and Groups C and D were treated with Pt-AZT and Pt-Rb, respectively. Using the MTT test, LC_{50} was determined, and the relative gene expression of the biomarkers was assessed by RNA extraction, cDNA synthesis and RT-PCR. Through histopathlogical study apoptotic regions of cells were compared. Data analysis was done using ANOVA and Turkey's post hoc test.

Results: The results showed a significant increase for the proapoptotic gene miRNA-122 (19.97 ± 0 .04) in group D compared with group C (10.36 ± 0.007**)**. Also, there was a significant decrease in the antiapoptotic genes in group D, including miRNA-21 (0.10 ± 0.014), telomerase (0.56 ± 0.480), and Bcl-2 (0.41 ± 0.276), compared with group C (miRNA-21: 2.0 ± 0.145, telomerase: 2.49 ± 0.231, and Bcl-2: 2.93 ± 0.276). There were significant differences between the nearly all studied groups ($P < 0.05$). There were more extensive apoptotic regions in group D compared with group C.

Conclusion: Using Pt-Rb has more benefits in terms of stronger anticancer effects than Pt-AZT on cancer cells. Also, lower drug resistance and lower side effects in Pt-Rb were considered compared with Pt-AZT, indicating that it can be more effective in anti-cancer therapy.
Keywords: miRNAs, Platinum compounds, HepG2 cells, Telomerase

Introduction

One of the major mortality causes in the world is cancer with an annual mortality rate of more than 10 million.[1] The molecular basis of cancers includes two factors: protooncogens and tumor suppressor genes (TSG). Protooncogenes are regulatory factors in biological processes and have many roles, such as signal transductions in the form of growth factors. When protooncogene are converted to oncogenes by several processes such as point mutation, gene amplification, and chromosomal translocation, cancer cells are formed. TSGs are involved in normal cell growth and prevents the development of cancers.[2] TSG mutations through gene deletion or inactivation, of alleles lead to TSG inactivation and finally cancers develop.[3] One of the methods for cancer treatment is chemotherapeutic drugs; moreover, biomarkers are used for the prognosis and diagnosis of many cancer diseases. Using clinical biomarkers in oncology and chemotherapy of cancers has made extensive progress, including tumor prognosis and diagnosis and patients screening and monitoring response to drugs, etc. Therefore, they can help to get correct decision in medicine.[4] Cancer biomarkers are classified in two major groups: antiapoptotic factors such as Micro RNA-21 (miRNA-21), telomerase, and Bcl-2 that their expressions are increased in untreated cancer cells; thus, in anticancer drug treatment their expression is reduced. On the other hand, proapoptotic factors such as Mico RNA-122 (miRNA-122), Bcl-2-like protein 4 (BAX), and FAS ligand (FASLG) that their expression decreases in untreated cancer cells; however, in anticancer treated cancer cells their expression is increased. Telomeres are the terminal portions of each chromosome that contain both protein and DNA. Among the previously mentioned biomarkers, telomerase prevents telomere shortening,[5] and its activity varies depending on the cells that affect them. For example, its activity is very low in somatic cells but very high in germ line cells and cancer cells, which can lead to the production of immortal cells; therefore, telomerase is an attractive target for cancer therapy.[6] The Bcl-2 family is a regulatory protein class in the apoptotic pathway, and aberrant function can cause autoimmune illness, cancer, and neurological disease.[7] MiRNAs are non-coding RNAs with an average length of 19–25 nucleotides that regulate gene expression post-transcriptionally, including the formation and development of cancer.[8, 9] Some miRNAs are proapoptotic factors, like miRNA-122, and some are antiapoptotic, like miRNA-21. Some miRNAs from plasma, like miRNA-21 and miRNA-155, act as biomarkers in the diagnosis and prognosis of certain cancers, such as hepatocellular carcinoma (HCC).[10]

Among the anticancer drugs, platinum-based drugs, such as cisplatin, carboplatin, and nedaplatin, have significant effects on cancer cells. Among the previously mentioned drugs, cisplatin is the most acceptable drug; it is widely used in solid cancers and its mechanism of action is by creating lesions in the DNA of cancer cells, especially with a purine base that cannot be repaired and leads to apoptosis.[11] In recent years, newly synthesized compounds such as platinum ribavirin (Pt-Rb) and platinum azidothymidine (Pt-AZT) and platinum levetiracetam (Pt-Lev) have received serious attention.[12, 13, 14]

In our study, the anticancer power of two newly synthetic compounds, Pt-AZT and Pt-Rb are examined and compared. Also, we aimed to evaluate potential of the expression of these genes as biomarkers in cancer.

Materials and Methods

Cell culture

In this case-control study, two different cell lines were assessed, including Hepatocyte G2 cell (HepG2) and Human dermal fibroblast cell lines (HDF), as cancer and healthy cell lines, respectively. Hepatoma G2 cell (HepG2) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 2 mM glutamine, bovine fetal serum, streptomycin (100 mg/ml), and penicillin (100 IU/ml). The cultured flasks were located in an incubator under conditions of atmospheric humidity, 37 °C, and 5% CO_2.

Classification of studied cell

The cells in the study were classified into four groups, including group A as a negative healthy control group (HDF cells), group B was untreated HepG2 cancer cells, and group C and D were treated cancer cells with Pt-AZT and Pt-Rb, respectively. Using MTT test, LC_{50} for the previous mentioned drugs was determined. Moreover, using real-time polymerase chain reaction (qPCR), the quantity genes expression of miRNA-122, miRNA-21, telomerase, and Bcl-2 were determined.

Treatment with Rb and AZT

The sterile platinum forms of Rb and AZT were synthesized by the Department of Chemistry, Faculty of Basic Sciences, Razi University of Kermanshah, Iran.

MTT assay

Basis of MTT

A set of dehydrogenases in living mitochondria cells reduces a yellow water-soluble tetrazolium dye to insoluble purple formazan crystals. By dissolving crystals in Dimethyl sulfoxide (DMSO), purple dye appears. In dead cells, there is no production of purple dye due to the lack of dehydrogenases. After that the intensity of color production is measured at a wavelength of 540 to 630 nm, which has a direct correlation with the number of living cells. This is the basis of the MTT assay, which measures mitochondrial activity as a means of identifying living cells.

MTT procedure

After trypsinizing the studied cells (HDF and HepG2), cell monolayer was formed for creating a uniform suspension of the cells. Subsequently, 10000 cells were added to each 96-well plate and incubated with doses of 100, 200, 300, 400, and 800 µg/ml of Pt-AZT and Pt-Rb for 24, 48, and 72 hours. Following discarding the supernatant liquid from each well, 60 microliters of MTT solution were added and incubated for 3-4 hours. By removing the supernatant solution and adding DMSO, purple formazon crystals appeared. Then, optical density was read using a Nanodrop at 570-620 nm and LC_{50} was obtained.

Histopathological experiment

The studied cells, including HDF and HepG2 cells, were fixed in a 25T flask with buffered formalin (10:1) for 24-48 hours. Dehydration and cleaning were done for one hour with 90% ethanol and xylene, respectively. The cells were mixed with paraffin wax and were cooled in cryogenic plates. Using a microtome, the cells in paraffin wax were cut to obtain 5 µm slides, and finally staining of the slides was done with 10 µl of trypan blue.

Molecular study

We conducted this phase of the study in the following way:

RNA extraction

RNA extraction from cultured cells was done by using a commercially available kit (QIAzol Lysis Reagent) (Qiagen, Frankfurt, Germany).

cDNA synthesis of extracted RNA

cDNA synthesis was done using a commercially available kit (Transcriptor

First Strand cDNA Synthesis Kit, Roche, Bavaria, Germany). The protocol was according to the manufacturer's instruction.

Real-time PCR for the study of the quantity of gene expressions

Real time PCR was performed using an ABI 7500 qPCR system and Taqman reagents, according to the kit's instructions. The sequences of target genes including miRNA-122, miRNA-21 and RNU6 (a housekeeping gene as a reference gene) and telomerase, Bcl-2, and β-actin (a housekeeping gene as a reference gene) are indicated in table 1. The Δct of target genes including miRNA-21 and miRNA-122 were calculated by subtracting the threshold cycle (ct) of the mentioned target genes from ct of RNU6 (a housekeeping gene). Also, for telomerase and Bcl-2, the ct of mentioned genes were subtracted from ct of β-actin (a housekeeping gene). Then, by subtracting the Δct of each gene from the Δct of the control group, ΔΔct was obtained. Using the formula, fold change was calculated: $F.C=2^{-\Delta\Delta ct}$

Statistical analysis

The statistical analyses were performed using SPSS software version 26. A *P* value less than 0.05 was determined to be statistically significant. Also, Tukey's post hoc test and one-way analysis of variance (ANOVA) test were used for pairwise comparisons between groups and data analysis, respectively.

Ethical Statement

This study was performed in line with the principles of the Declaration of Helsinki and ethically approved and granted by department of Biochemistry and Biophysics, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran (IR.IAU.PS.REC.1399.055).

Results

MTT results

The MTT test was done at concentrations of 100, 200, 300, 400, and 800 µg/mL Pt-AZT or Pt-Rb on HepG2 and HDF cells at three period of times of 24, 48, and 72 hours. The results showed the LC_{50} for Pt-AZT was 400 µg/ml and for Pt-Rb was 300 µg/ml in 48 hours. At zero concentration all HepG2 cells were viable, and at a higher concentration than the LC_{50}, many cancer cells were killed through apoptosis (Figure 1). The results of MTT tests for Pt-Rb on HDF cells showed slight necrosis effects in concentration 800 µg/ml at 72 hours, but the effects of Pt-AZT on HDF cells showed more necrosis than Pt-Rb at the same mentioned concentration at 48, 72 hours. Furthermore, neither of the two recently developed anticancer medications affected HDF cells at doses of 100, 200, 300, or 400 µg/ml (Figure 2). The findings demonstrated that Pt-AZT had higher adverse effects on HepG2 cells than Pt-Rb.

Gene expression results

The results of the study showed that the expression level of proapoptotic gene, i.e., miRNA-122, was higher in group D (19.97 ± 0.04) than group C (10.36 ± 0.007), and antiapoptotic gene expression levels in group D, including telomerase (0.56 ± 0.480), Bcl-2(0.41 ± 0.276), and miRNA-21(0.10 ± 0.014), were lower than the same gene expression level in group C, including telomerase (2.49 ± 0.231), Bcl-2 (2.93 ± 0.276), and miRNA-21 (2.0 ± 0.145). However, the expression level of miRNA-122 as a proapoptotic gene in group B (0.12 ± 0.01) was lower than group C or group D, but the expression level for antiapoptotic genes in group B, including telomerase (5.76 ± 0.339), Bcl-2 (3.52 ± 0.678), and miRNA-21 (5.65 ± 0.07), was the highest in all other groups (Table 2). Figure 1 makes it simple to see these circumstances. Significant differences were observed between the group majorities ($P < 0.05$). (Figure 3) (Table 2).

Histopathological results

The study results showed that in the untreated cancer group (group B), there were cancer cells with several enlargement nucleuses due

to the increasing synthesis phase in the cell cycle of HepG2 cancer cells. There were no apoptotic regions in the cells (Figure 4). In treated cancer groups, several apoptotic regions can be observed, but the extension of these areas in the Pt-Rb treatment was more as compared with the treated cells with Pt-AZT (Figures 5 and 6).

Discussion

In the study, the anticancer effects of Pt-AZT and Pt-Rb were evaluated by measuring the expression levels of miRNA-21, miRNA-122, telomerase, and Bcl-2 in four studied groups. Our finding showed that antiapoptotic factors such as telomerase, Bcl-2, and miRNA-21 were more decreased in group D (the cancer-treated group with Pt-Rb) compared with group C (the cancer-treated group with Pt-AZT). Furthermore, it was indicated, miRNA-122 as proapoptotic factor in group D was higher than group C, and there were significant differences between almost all majority mentioned groups ($P < 0.05$), but there was no significant difference between group A (healthy negative control group) and group D (the cancer-treated group with Pt-Rb) for antiapoptotic genes which means the level of biomarkers expression in group D was as same as group A. Therefore, there were no difference between healthy and cancer cells in terms of biomarkers level genes expression. Accordingly, treatment of cancer cells with Pt-Rb was more effective than Pt-AZT. Interesting, there were no similar conditions between groups C and A ($P < 0.001$), leading to a higher anticancer power for Pt-Rb than Pt-AZT. Also, our findings showed that the mentioned antiapoptotic factors in cancer-treated groups (C and D groups) were lower than those in the cancer-untreated group (B group). In the study, there were significant differences between groups C and D ($P < 0.001$).

Among the three steps of protein synthesis, regulation of mRNA translation for protein synthesis has an important role in controlling gene expression. Any disturbance in translation can lead to cancer development. The capping process by 7-methylguanosine through a 5′ to 5′ phsphodiester bond is a critical step in the regulation of translation. The previous mentioned bond is resistant to 5′-exonucleases, prevents the degradation of the 5′-end of mRNA, and makes the polypeptide chain of this mRNA resistant to the change of amino acids in proteins. Thus, it can be said that the translation process in higher eukaryotes such as humans relies on capping. The capping process is done by a complex called the eukaryotic initiation factor (eIF) complex. One of its subunits is eIF4E, which is involved in capping. By capping the 5' primary transcript of mRNA, the splisosome complex can be carried to phosphorylate RNA polymerase. Then, this complex transfers to the cap site of the nascent premRNA for splicing of premRNA to mature RNA. The mentioned factor is converted from protooncogene eIF4E to oncogenic form by the uncontrollably overexpression of eIF4E. Eukaryotic initiation factor 4E binding proteins (4E-BPs) phosphorylation by the MAPK pathway can cause the availability of eIF4E, the mentioned factor, is placed in the chaperone through mRNA maturation and transfer from the nucleus to the cytoplasm to create a spatial conformation.[15] Because eIF4E may bind the cap region of eukaryotic mRNA, it can promote the translation and export of cancer-causing proteins from the nucleus to the cytoplasm, which is why it has been linked to cancer. The eIF4E factor is also proposed as a therapeutic target for cancer treatment.[16] According to previous research, in cap site of eukaryotic cells, substituting N6-methyl guanosine with N6-methyladenosine can alter mRNA and

eventually result in the appearance of cancer cells.[17]

Nearly 40 years ago, ribavirin was identified as a broad-spectrum antiviral medication. More recent research indicates that ribavirin may potentially be a useful cancer treatment. Here, ribavirin targets the eukaryotic translation initiation factor (eIF4E), an oncogene that is increased in around 30% of malignancies, including a large number of lymphomas and leukemias. Particularly, ribavirin functions as an eIF4E inhibitor to reduce its expression and prevent eIF4E-mediated oncogenic transformation.[18] Also, studies showed that ribavirin inhibits cancer cells by suppressing translation of eIF4E and reduce the synthesis it as oncoprotein.[19] Ribavirin can cause less translocation of oncogenic factor eIF4E from the nucleus to the cytoplasm.[20] This medication blocks translation of oncogenic proteins, as well as eukaryotic initiation factor 4E (eIF4E), prevents it from developing from a proto- to -oncogenic state. Research by Xu et al. showed that ribavirin targets the mentioned factor through Rb-reduced drug resistance to chemotherapy and reduces eIF4E activity and oncogenic protein translation in chemoresistant nasopharyngeal carcinoma cells.[21] Similar to our work, which demonstrated that ribavirin attenuates antiapoptotic factors, another study showed that ribavirin attenuates carcinogenesis by down regulating IL-6 and IL-8 (inflammatory factors) in human lung adenocarcinomas in vitro.[22]

Azidothymidine (AZT, 3′-azido-3′-deoxythymidine, zidovudine) is a chain terminator due to the presence of an azide (N_3) group that replaces the 3′-hydroxyl group of thymidine, consequently, adding any nucleotide in the 3' position is blocked without the formation of a phsphodiester bond by DNA polymerases.[23] In cancer cell treatment with AZT, the S phase of the cell cycle is arrested by destroying DNA due to the chain terminator properties of AZT, and followed by reducing the activity of antiapoptotic biomarkers such as telomerase; therefore, many cancer cells undergo apoptosis.[24]

Large volumes of the platinum component of the previously discussed medications were taken up by cancer cells and deposited in the cytoplasm, where they react chemically with DNA to form irreversible intra-stranded and inter-stranded crosslinks. The guanine at position N7 in the main groove of DNA has the highest nucleophilic property. It quickly platinates to produce adduct compounds such as 1, 3 inter-stranded guanines, which can induce deformities in DNA duplexes that are irreversible and ultimately cause cells to experience apoptosis.[25] AZT by itself has limited anticancer effects due to conformational changes in DNA that cannot be repaired but when combined with platin, platinum's synergistic actions can provide greater anticancer effects than similar non-platinum substances.[26] A number of studies back up our opinion. One study found that the platinum compound levetiracetam had a greater anticancer effect than levetiracetam.[14] Another study found that the platinum compound cisplatin had an anticancer effect primarily because it forms a crosslink with purine bases, particularly with guanine bases, which platinum parts of cancer cells cannot repair, causing the cells undergoing apoptosis.[27] Another study showed the great anticancer potential of Pt-Rb by reducing the expression level of antiapoptotic factor and boosting the expression level of proapoptotic genes higher than that of ribavirin, so Pt-Rb has greater anticancer effect than Rb.[12]

The biomarker variation in our study is consistent with previous research; for instance, the levels of antiapoptotic biomarkers, such as telomerase, Bcl-2, and miRNA-21, were higher in group B (untreated cancer cells) compared with groups C and D (cancer-treated groups).

Additionally, miRNA-122 as a proapoptotic factor in group B was lower than in the treated cancer groups (C and D). Numerous studies support our opinion: research has shown that adding cisplatin and another factor to treated cell lines might increase the proapoptotic factors BAX, BAK, MCL1, and decreases BCL2, which in turn can trigger apoptosis.[28] According to a study, the anti-cancer effects of cisplatin are associated with down-regulation of miRNA-21 expression and up-regulation of miR-122.[29] The decreasing of telomerase leads to telomere shortening and apoptosis of cancer cells; some studies confirm this finding. The level of telomerase expression depends on cell types. In somatic normal cells its expression level is very low and are tightly controlled but in cancer cells and germ cells are very high.[30] In our study, there were significant differences between groups C and D for telomerase and Bcl-2. Therefore, the levels of telomerase and Bcl-2 expression were more reduced in group D than C, which means more effect on cancer cells and more extending apoptosis by Pt-Rb than Pt-AZT. Also, it is reported that miRNA-21 and miRNA-122 expression levels are predictive biomarkers in many cancers, including HCC, indicating that the expression level of miRNA-21 is increased and miRNA-122 is reduced,[31] similar to what we found in our study.

Nevertheless, one benefit of using Pt-Rb instead of Pt-AZT was that it had fewer side effects, as shown by HDF-healthy cell lines that were treated with Pt-Rb instead of Pt-AZT. When Pt-Rb is added at a high concentration (800 mg/µl) for 72 hours, it only causes minor necrosis in healthy cells; however, Pt-AZT causes considerable necrosis at the same dose (800 mg/µl) after 48- and 72-hours treatment (Figure 3).

One of the methods for drug resistance is via MDR (multidrug resistance), which is regulated by biomarkers so that proapoptotic factors such as FASLG, miRNA-122, and BAX inhibit the Wnt/βcatenin pathway and enhance sensitivity to drugs. On the other hand, antiapoptotic factors such as miRNA-21, telomerase, and Bcl-2 stimulate MDR expression via the Wnt/βcatenin pathway and enhance drug resistance. It was simple to find the difference about MDR resistance between the two studied groups, C (cancer cells treated with Pt-AZT) and D (cancer cells treated with Pt-Rb), because in our study, anti-apoptotic gene expression in group D was more decreased compared to group C. Also, pro-apoptotic gene expression in group D was more increased than in group C. As a result, MDR gene expression was more decreased, and resistance to Pt-Rb was lower than that of Pt-AZT. Research that treated cell lines with oxaliplatin (OXA) supports our opinion. It found that a proapoptotic factor such as miRNA-122 by suppressing Wnt/β-catenin pathway in HCC cells elevated the sensitivity to OXA, which in turn reduced MDR1 expression. Therefore, miRNA-122 is novel target for HCC [32]. In another study, the high level of miRNA129-2 causes downregulation of the progesterone receptor and as a result increase in the amount of progesterone hormone in the body, which is associated with breast cancer, so suppressing the expression of this molecule can help preventing cancer.[33] In a study, it is demonstrated that expression of hTERT mRNA may be related to those of MDR1.[34] In a study it was shown treated cancer cells with some drugs such as cisplatin, 5-fluorouracil reduced expression or inhibited activity of telomerase that was accompanied with increased sensitivity. Possible reasons for increased sensitivity and decreased drug resistance can be due to telomere shortening, expression of ABC family, genomic instability, telomerase translocation.[35] In another research, it is shown that in osteosarcoma cancer cells treated with cisplatin, increasing hTERT of telomerase

was along with chemotherapy resistance to cisplatin; therefore, reducing telomerase expression has an important role to decreasing drug resistance.[36] Also, based on figures 5 and 6, it was concluded that Pt-Rb had a greater extent of apoptosis in cancer cells than Pt-AZT.

Some of the limitations of this study were different in vivo condition medium of cell lines with cancer cells in human body. These differences can affect the response of cells to drugs, another limitation was the use of one type of cancer cell (HepG2cells), and failure to consider the tumor microenvironment also synergistic or antagonist effects with each other are not investigated in this study.

Conclusion

Our findings showed that antiapoptotic factors such as telomerase, Bcl-2, and miRNA-21 decreased in group D (the cancer-treated group with Pt-Rb) compared with group C (the cancer-treated group with Pt-AZT). Also, it was indicated that miRNA-122 as a proapoptotic factor in group D was higher than group C, and there were significant differences between almost all the majorities mentioned groups ($P < 0.05$). But there was no significant difference between group A (the healthy negative control group) and group D (the cancer-treated group with Pt-Rb), which means the level of biomarkers in group D was as same as group A; therefore, there was no difference between healthy and cancer cells in terms of biomarker level. Accordingly, a higher anticancer power for Pt-Rb was found as compared with Pt-AZT.

Using Pt-Rb has more advantages in terms of stronger anticancer effects than Pt-AZT on cancer cell lines through biomarkers by stimulating proapoptotic and inhibiting antiapoptotic factors. Also, lower drug resistance and lower side effects in Pt-Rb than Pt-AZT can be considered more effective in anti-cancer chemotherapy.

We suggest future studies for a better investigation of these two compounds on different cancer cell lines and animal models and clinical trials on humans. Also, more tumor markers from both anti-apoptotic and pro-apoptotic groups should be used. Moreover, the synthesis of new anticancer drugs based on platinum or other metals such as zinc and copper can help to develop anticancer drugs with less side effects and better efficiency.

Data Availability

The author will provide the data from this study upon request by the editor.

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Authors' Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Abdolreza Sabokrouh], [Nasim Ghaffari], [Soheila Hajivand] [Freshteh Atabi], [Reza Saghiri] and [Zohreh Sharifi]. The first draft of the manuscript was written by [Abdolreza Sabokrouh] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript."

Conflict of Interest

None declared.

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Table. 1 Primers sequences of target genes in sense and antisense with qPCR conditions

Name	Sequences	Direction	Expected product size	Stages of qPCR
has-miR-21-F	GTTCTTCAGTGGCAAGC	Sense	55 bp	Stage1: 95°C-2 min Repeat 1 cycle Stage 2: Step1: 95°C-5sec Step2:60°C-30sec Repeat 40 Cycles
has-miR-21-R	GAACATGTCTGCGTATCTC	Antisense		
H-miR-122-F	GGAGTGTGACAATGGTG	Sense	63 bp	
H-miR-122-R	GAACATGTCTGCGTATCTC	Antisense	63bp	
RNU6-F	AAGGATGACACGCAAA	Sense	58 bp	
RNU6-R	AACGCTTCACGAATTTGCGT	Antisense	58bp	
Bcl-2 -S	AGATGAAGACTCCGCGCCCCT CAGG	Sense	566bp	Stage 1 : 50ºC-2min Repeat1 cycle Stage 2: 95ºC-10min Repeat 1 cycle Stage 3: 95ºC-15sec 58ºC-1min Repeat 50 cycles
Bcl-2- AS	CCAGGTATGCACCCAGAGTGA TG	Antisense	566bp	
Telomerase-S	GACATGGAGAACAAGCTGTTT GC	Sense	185bp	
Telomerase-AS	ACAGGGAAGTTCACCACTGTC	Antisense	185bp	
B-Actin-S	CGTGCGTGACATTAAGAAG	Sense	305bp	
B-Actin-AS	GGCATAGAGGTCTTAGATG	Antisense	305bp	

Antiapoptotic genes including telomerase, Bcl-2, miRNA-21; Proapoptotic gene was miRNA-122; RNU6 as internal control gene for miRNAs assessment; B-Actin as internal control gene for telomerase, Bcl-2 assessment; qPCR: Quantity polymerase chain reaction

Table 2. Comparative statistical results of miRNA-21 and miRNA-122 Telomerase and Bcl-2 genes expression in the four studied groups.

	Groups				***P*-value**					
Dependent variable	**A**	**B**	**C**	**D**	**A vs. B**	**A vs. C**	**A vs. D**	**B vs. C**	**B vs. D**	**C vs. D**
miRNA-21	0.11 ± 0.007	5.65 ± 0.07	2.0 ± 0.145	0.10 ± 0.014	<0.001	<0.001	0.880	<0.001	<0.001	<0.001
miRNA-122	9.12 ± 0.01	0.12 ± 0.01	10.36 ± 0.007	19.97 ±0 .04	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Telomerase	0.17 ± 0.004	5.76 ± 0.339	2.49 ± 0.231	0.56 ± 0.480	<0.001	<0.001	0.07	<0.001	<0.001	<0.001
Bcl-2	0.2 ±0.014	3.52 ± 0.678	2.93 ± 0.276	0.41 ± 0.276	<0.001	<0.001	0.520	<0.190	<0.001	<0.001

Studied groups: A: HDF cells as control group; B: untreated HepG2 cancer cells; C: Pt-AZT treated HepG2 cancer cells; D: Pt-Rb treated HepG2cancer cells; $P < 0.05$ represents significant difference between two groups. miRNA-21: microRNA21; miRNA-122: microRNA-122; Bcl-2: B-Cell Lymphoma 2

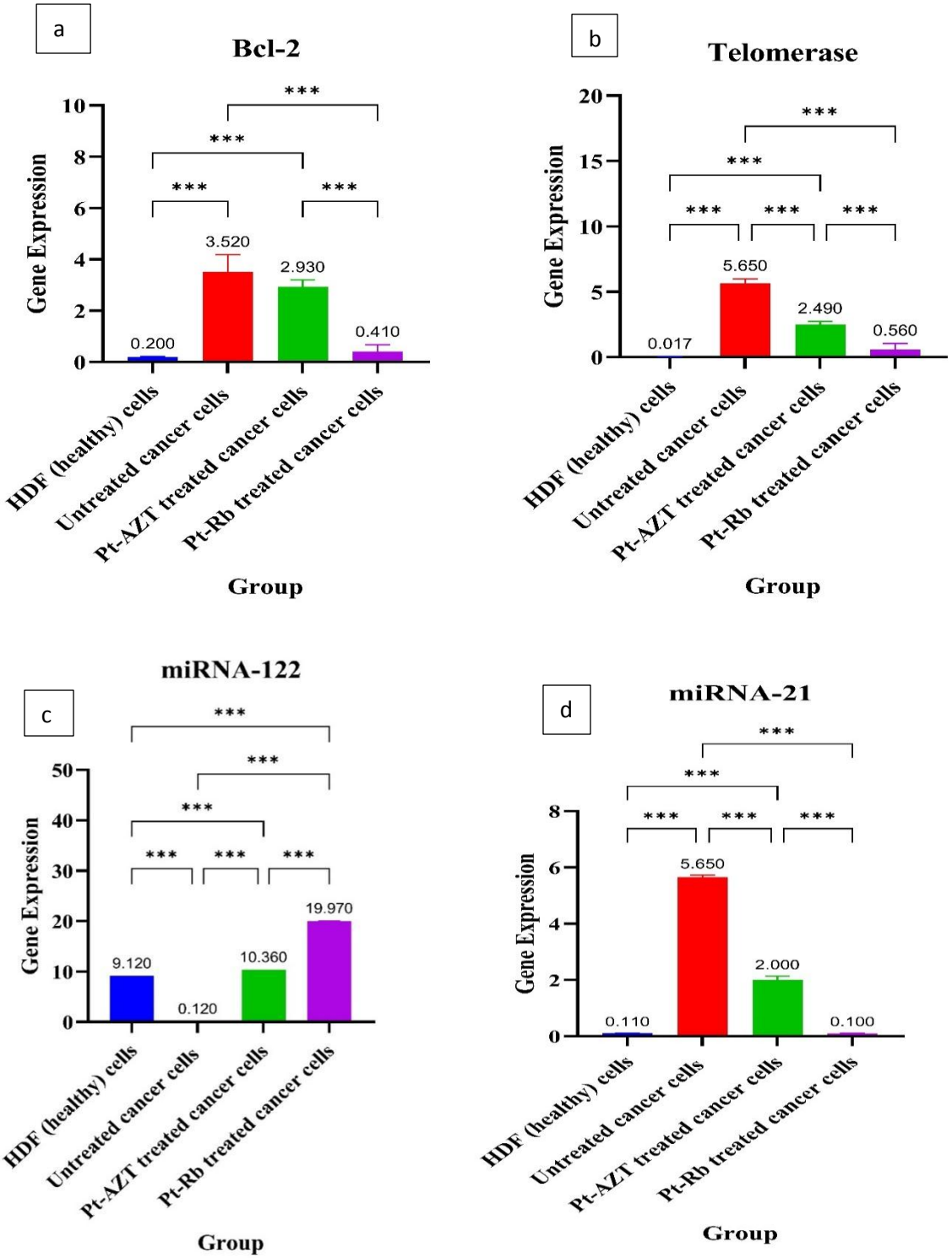

Figure 3. The biomarkers gene expression in four studied groups. In miRNA-122 as a proapoptotic gene the level of the gene expression in treated groups (groups C, D) was increased but in untreated cancer group (group B) was reduced. In antiapoptotic genes such as telomerase and Bcl-2 and miRNA-21, the genes expression level in the treated groups (C and D) were reduced but in untreated group (group B) was increased.

Pt-AZT: Platinum Azidothymidine; Pt-Rb: Platinum Ribavirin

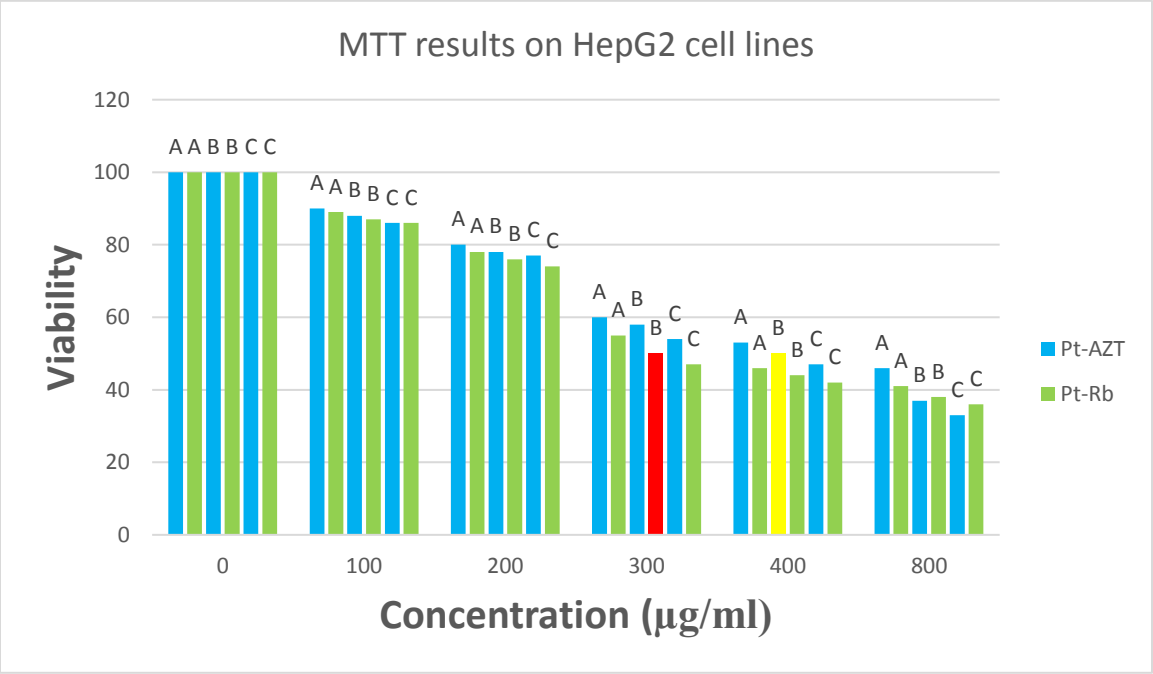

Figure 1. Results of MTT test at different concentrations of the drugs. The red column showed that LC_{50} for Pt-Rb and was at concentration 300 µg/ml in 48 hours and yellow column indicates LC_{50} for Pt-AZT was 400 µg/ml in 48 hours. There were no apoptotic cells at zero concentration but with increasing level of the drugs the viability of cells was reduced (A: 24 h treatment; B: 48 h treatment; C: 72 h treatment).

Pt-AZT: Platinum azidothymidine; Pt-Rb: Platinum ribavirin; MTT test: 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide test

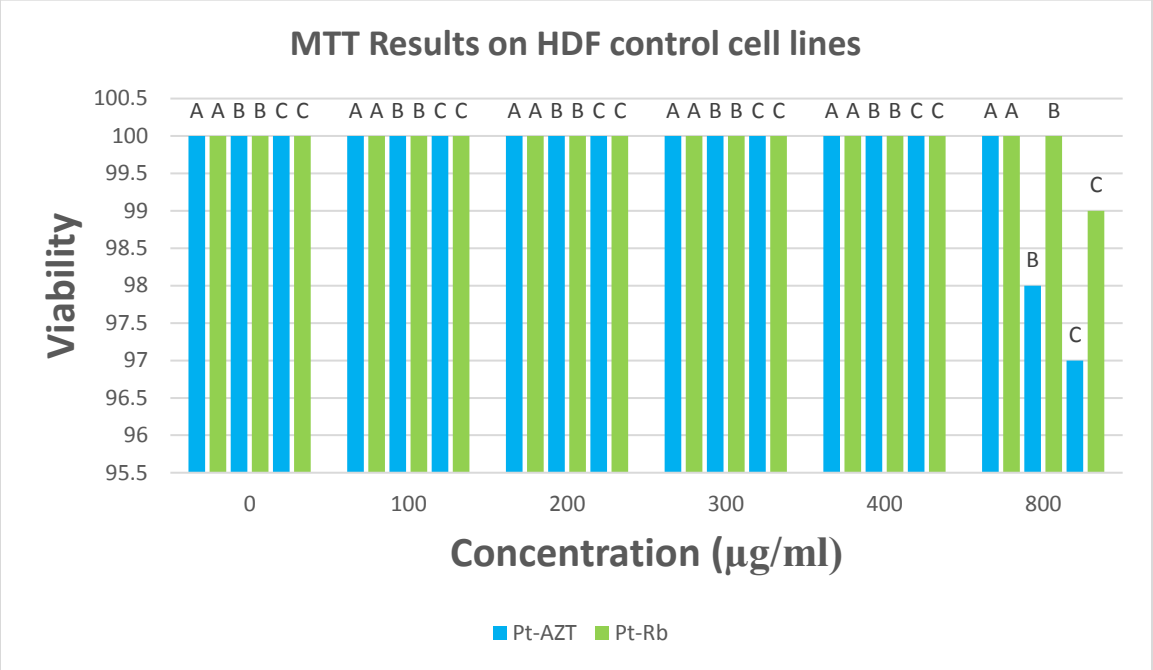

Figure 2. Results of MTT test at different concentrations of drugs on HDF control cell lines. The results showed that none of cells underwent apoptosis, but there was necrosis in 800µg/ml and 48, 72 hours concentrations for Pt-AZT. Also, there was slight necrosis in 800µg/ml in 72 hour for Pt-Rb. This test showed that the drugs had no effect on HDF control cells at LC_{50} concentration area but in higher concentrations necrotic cells were observed (A: 24h treatment; B: 48h treatment; C: 72h treatment).

HDF cell lines: Human dermal fibroblast cell lines; MTT test: 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide test

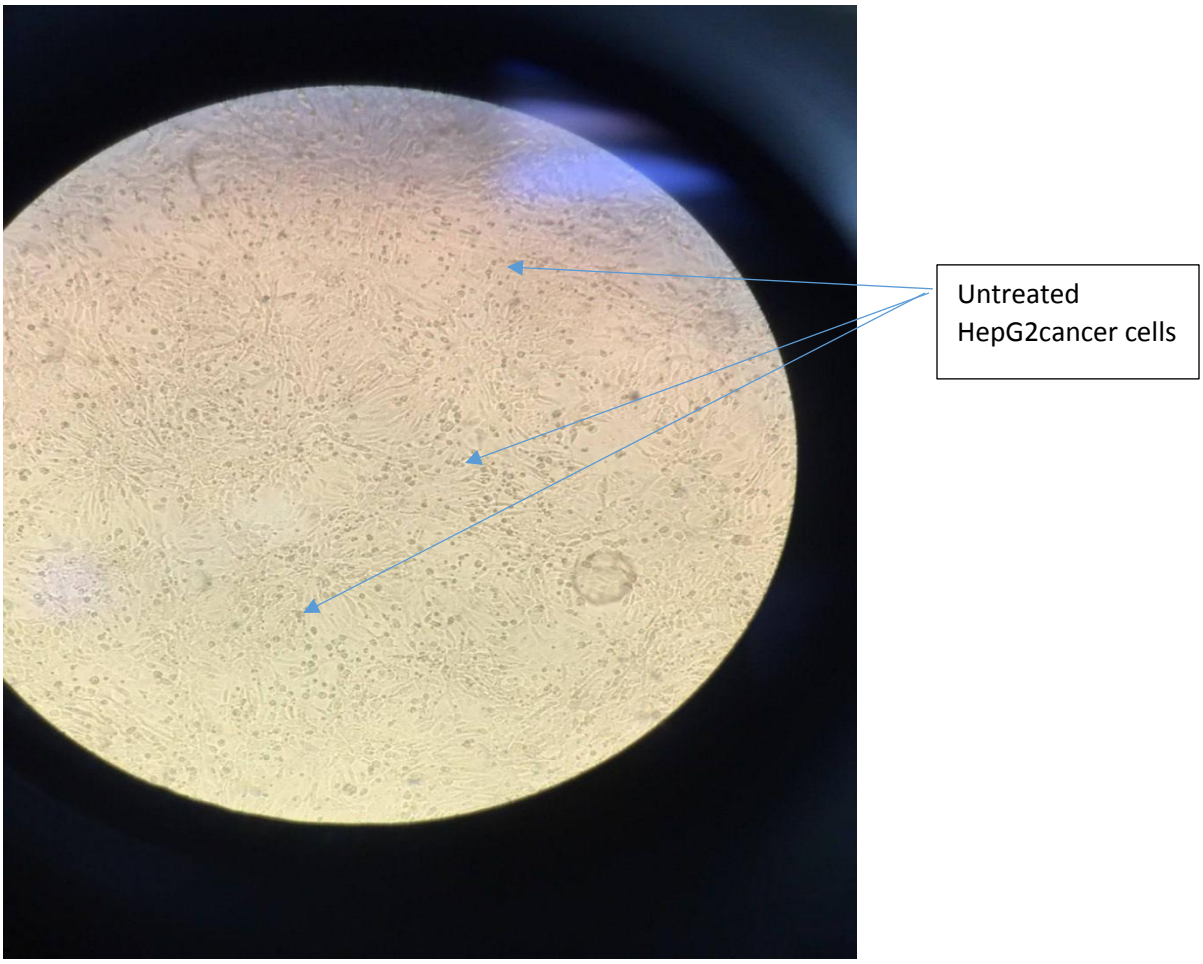

Figure 4. The HepG2 cells before drugs treatments. There were many HepG2 cancer cells. There were many HepG2 cancer cells in the figure. HepG2 cell lines: Hepatoma G2 cell lines

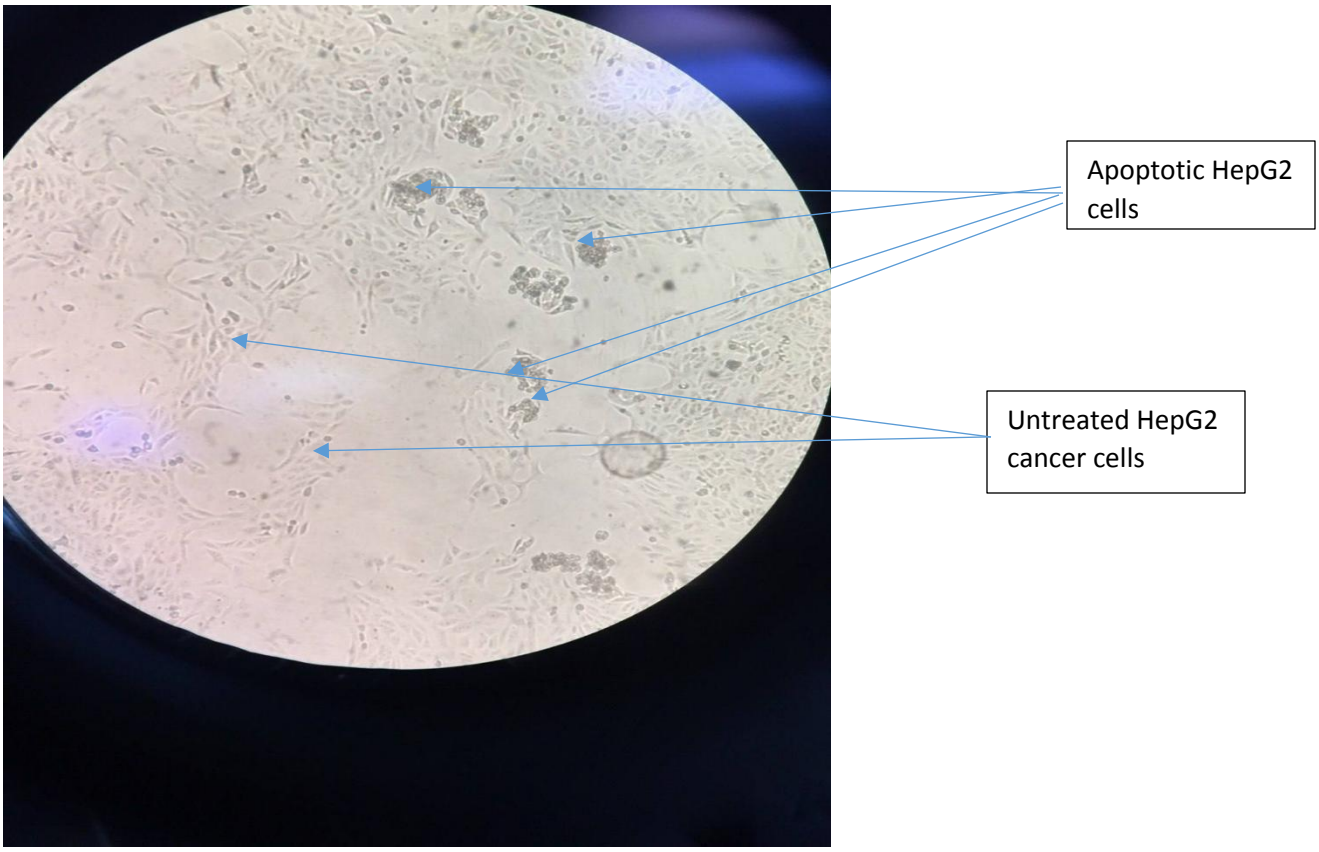

Figure 5. Pt- AZT treatment of HepG2 cells, there were a few apoptotic regions but the density of untreated cancer cells is higher than apoptotic cells.
Pt-AZT: Platinum azidothymidine

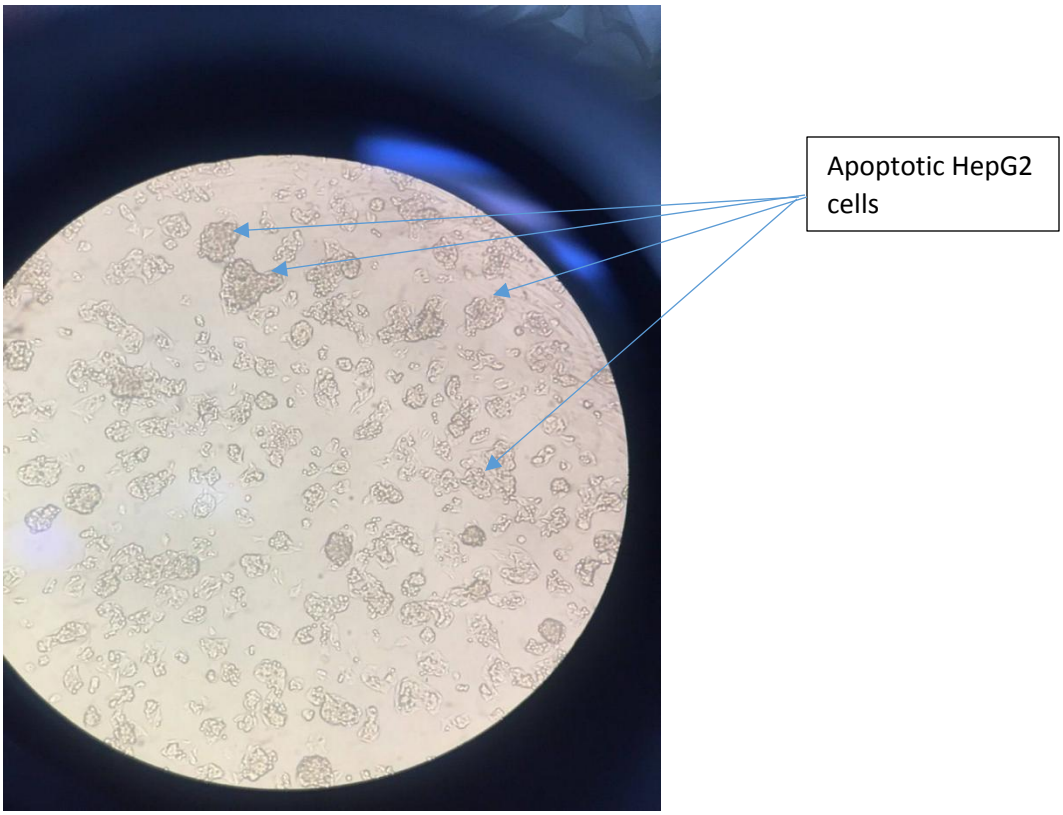

Figure 6. Pt- Rb treatment of HepG2 cells, there were extensive apoptotic regions. In this figure, almost all cells are apoptotic.
Pt-Rb; Platinum ribavirin