Expression Studies of the Cancer Drug and Its Effect on Angiogenesis-Promoting Genes in Cancer Cell Lines

Salam Abbas[1], Noor Harith AL-Hamid[2]

[1]Assi. Lect., Nursing technique Dept. Kufa Technical Institute, 31003 AAl-Furat Al-Awsat Technical University, kufa, Iraq, [2]Assist. Lect. University of Baghdad -College of Science - Biotechnology Department/Iraq

Abstract

Objective: Being a member of RNA binding proteins, Human Antigen R (HuR) is considered as an acute factor to mediate the proangiogenic factors causing the angiogenesis amongst tumor cells. All through the anoxic conditions and inflammation, the cytokine IL-1 activated the HuR which further downregulated or upregulated the hypoxia inducible factors (HIF1α). Current study was conducted to observe the correlative impact of Decetexal (Taxotere) cancer drug on HIFα and HuR expression levels while utilizing the cell line HeLa.

Key words: *HuR, Anticancer targets, HIF1 α, Decetexal*

Introduction

Cancer is a main health issue in both developing and developed countries of the world. World Bank and WHO (World health organization) assess that about 12 million worldwide individuals agonize with caner while 7.6 million of them die per year [1]. Lower success rates of treatments lead to the higher mortality and incidence rates among cancer patients. Thus, numerous procedures have been formulated against cancer such as chemotherapy, immunotherapy, radiation and surgery. Though, the rate of success for these methods is still lower because of their drawbacks [2]. Such condition inspires the struggles to develop and discover the more impactful and profound anticancer properties devoid of severe side effects [3-6]. Moreover, the proliferation of cancerous cells is uncontrolled and happens far away from normal limits. This occurs because of the cell cycle disruption, suppression of apoptosis and increased angiogenesis [7-10]. As the apoptosis is a procedure of cell death intended for the elimination of undesired host cells [4, 5]. This apoptosis process is automated with a series of events comprising of a set of products of genes. Moreover, apoptosis is liable for several pathological & physiological procedures and pathological process comprise a major role of apoptosis in the death of tumor cells [6].

Being a widely expressed member of RNA binding proteins ELAV's family, HuR displays precise similarities to the sequences of ARE-containing RNA in vitro [7]. It associates to their in vivo rates of decay, thus, involving HuR in the angiogenesis of proangiogenic factors. Commonly, initiation of angiogenesis takes place through the inflammatory or hypoxic settings [8, 9]. Additionally, hypoxia & inflammation stimulate the function of HuR by modulating the binding activity of RNA and subcellular localization of HuR [6]. Moreover, other cytokine IL-1linked inflammation [10] can initiate the HuR functions for stabilizing the target mRNAs' set to accomplish the certain procedure [11]. As a result, the activated HuR further stabilize TNFα or stimulate the HIF1α translation by ARE situated in 3' UTR while creating a positive feedback [12, 13]. while several healthcare authorities might utilize the taxtotere name in order to refer to the docetaxel name generic drug. As docetaxel is anti-cancerous (cytotoxic or antineoplastic) chemotherapy drug [14-16], thus, permissions have been made for its usage in metastatic prostate cancer,

Corresponding author:
Salam Abbas
kin.slm22@atu.edu.iq

advanced stomach cancer, non-small cell lung cancer and breast cancer [17]. Recent studies indicate its usage for the treatment of melanoma, soft tissue sarcoma, pancreatic cancer, ovarian bladder and small cell lung [19].

Materials and Methods

The streptomycin, penicillin, fetal bovine serum (FBS) and cell culture medium (DMEM) were acquired from the labs of HiMedia. The HeLa cells of human cervical carcinoma were attained from the Indian Academy Degree College of Banglore, India. Moreover, the culturing of cell lines was carried out in the medium of DMNEM enhanced with streptomycin (100µg/mL) & FBS (10%). Likewise, the growth of cells took place in the incubator of CO_2 (Memmert, Germany) at 5% CO_2 & 90% of 37ºC temperature. Usage of the confluent trypsin/EDTA was done during the sub-culturing of cells. Further, the Decetexal (Taxotere) CAS # 114977-28-5 was bought from the Sigma Aldrich and this drug was soluble in methanol and sued at fluctuating concentrations for the research.

Primers design: The pairs of the primers purchased from the Sigma Aldrich are described in table (1). These were designed using formerly designated Primer3 software by Sudhakar *et al* [18]. Furthermore, the alignment tools from BLAST (National Centre for Biotechnology Information) were used to check the *in silico* primer pairs and PCR product's specificity.

RT PCR: cDNA synthesis: The 200U/µl Super script TMII Reverse Transcriptase (HiMedia) was utilized along with RT PCR kit to carry out the cDNA synthesis. To start the reaction, the small amount of RNA about 2µg attained in the former section was utilized. The 1.97µg/µl concentrations of RNA was acquired, thus, 1.12µl of the total RNA was utilized in reaction. Further, the 1µl RT enzyme and random primers were mixed together properly followed by the 10 minutes incubation at 25ºC. After the 45 mins incubation at 70ºC, the obtained cDNA was stored for further usage in the analysis of RTPCR process.

Table 1: Table presents the primers details used in RTPCR.

Gene		Sequence (5'->3')	Length	GC%	Product
HuR	FW	GAGGTAGGGACCACCAGGAT	20	60.03	888
	RV	ACTTGCTCTTTTCTCTTGGCAG	23	59.68	
HIF-1A	FW	TGACCTGCTTGGTGCTGATT	20	59.89	208
	RV	GCGCTGAATCTTTGCTATGG	20	57.6	

Real time PCR: The table (1) primers were bought from Sigma Aldrich and prepared using software of primer 3. The usage of iQTM SYBR Green Supermix (HiMedia) allowed to perform the real time PCR conferring to the method described by **Deepak V. *et al* (2018)[19]. The 1µl RT products and 600nm primers were utilized in the assay of PCR with reaction using 12.5µl of total volume. To confirm the positive amplification, the whole reactions were run in duplicates and also in parallel to their negative control.**

Docking studies: Various soft wares such as PATCHDOCK, ArgusLab 4.0.1, Rampage, ACD ChemSketch, Swish PDB viewer and SWISH Model were utilized for docking studies. In present context, the HIF-1A and HUR receptors were used while among the model study to dock or inhibit receptors. The Hypoxia inducible factor 1 alpha (NC_000014.9) and human like R antigen were obtained from enterz database of NCBI. SWISH Model freeware was used for homology modelling while choosing best templates for the studies of docking. Moreover, the selected models were additionally analysed on the patchdock.

Results and Discussion

MTT assay: For this assay all measurements were control normalized (without treatment) and considered as 0 in percentage. The primary observations indicated the cell treatment with 10µg/mL drug suspension presented an anti-proliferation activity. This was extremely negligible activity when compared to the dose dependent positive control. However, the drug's activity was observed to be positive on comparing to the negative control and colchicine. All such values show equally potent drug suspension to the positive control. Thus, the values of IC_{50} were 23.56 & 38.8 for positive control and drug correspondingly.

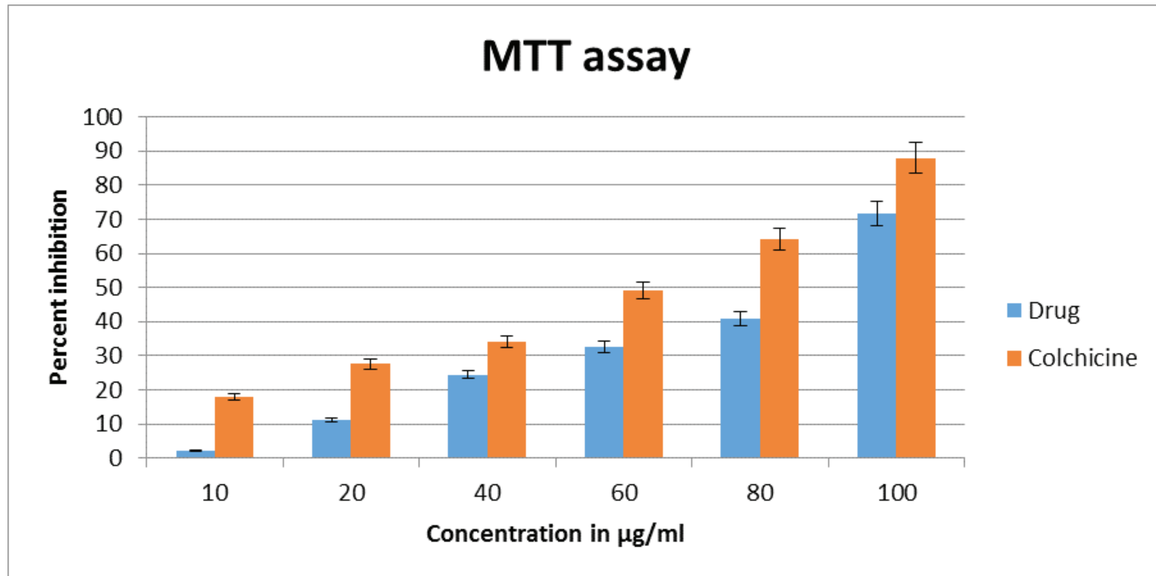

Figure 1: Graph indicates the values of percent inhibition the assay of MTT. All values are articulated as ±s.e value and values were triplicates' average and all measurements were control (0%) normalized.

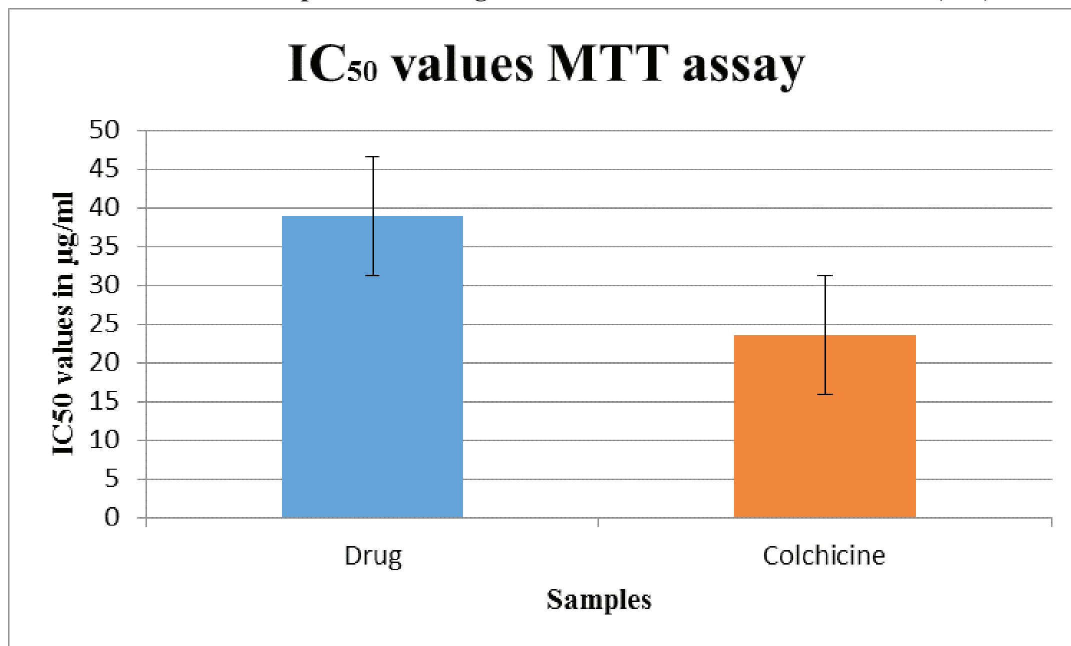

Figure 2: Graph is indicating the values of IC_{50} in assay of MTT. All values were presented as vale ±s.e and were average of triplicates.

LDH assay: The assay of the drug induced damage of membrane was done on due to the release of LDH. Subsequently to the incubation, the quantity of the released LDH was further calculated while comparing to the (OD0.01) negative control. The outcomes showed that comparative to the untreated cells, the release was dose

dependent and according to the assay of MTT. Initial concentration presented a very negligible release on comparing to the negative control and colchicine. However, the 24 hours incubation, the suspension of drug indicated a substantial upsurge in the release of LDH comparative to the control afterwards 1µg/ml amount. While, at 3µg/ml concentration, the percent release observed was 71±19.4%for drug & 92±13.4% for positive control with a significant difference of ($p < 0.05$).

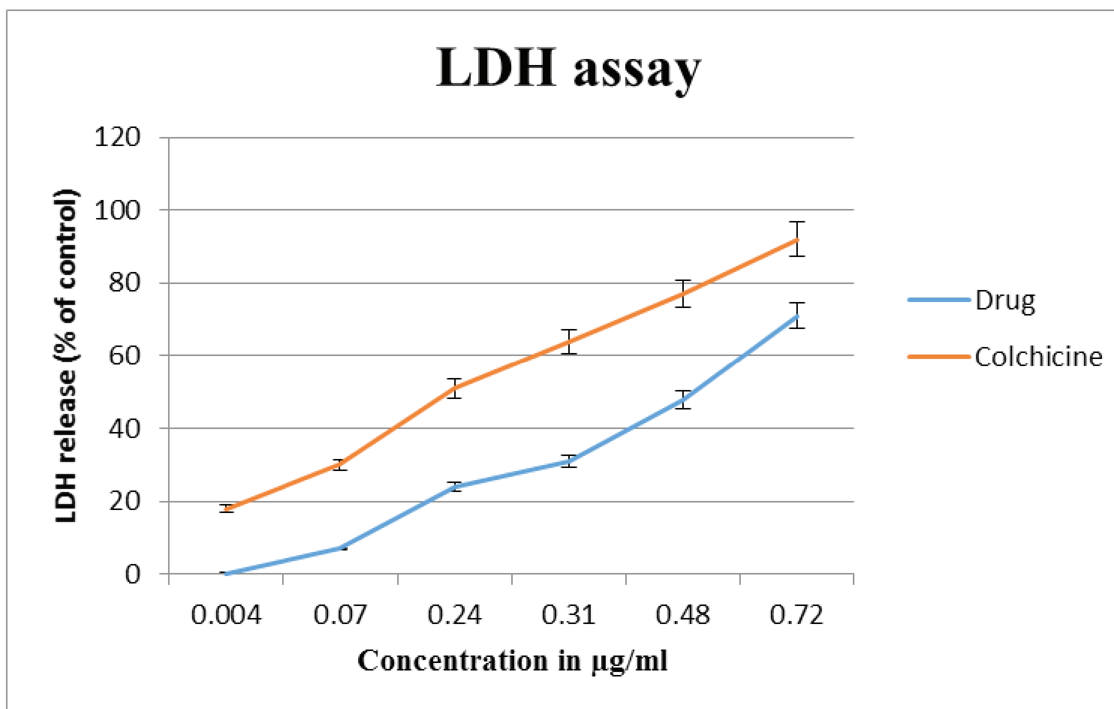

Figure 3: This graph represents the LDH release percentage from the HeLa cells succeeding to the 24 hours LDH assay exposure. The triplicates' values were average and were expressed as the values of percentage control (values± s.e.) and all measurements were normalized to (0%) control.

Expression of gene members: The variance is reduced amongst the samples by normalizing all the obtained outcomes to the housekeeping (GAPDH) gene. The mRNA expression levels for each gene (HIFA, HUR & GAPDH) member were studied individually. From this, the samples having lower values of $_{\Delta\Delta}$Ct was precieved as calibator whereas, the remaining samples were rather comparative to the calibator. Thus, the Ct values of the housekeeping genes, HIFA & HUR were 11, 18 and 21 correspondingly. Reliying the Ct values of samples that have been treated with drugs were normalized to the GADPH housekeeping gene. The expression of GAPDH gene was deliberated as 100% in value. Hence, from the calculated values of $2^{-\Delta\Delta Ct}$ and several researches, the observations indicated the underexpression of HUR gene in comparison to the HIFA and control. Therefore, both HIFA & HUR were underexpressed in the treatment in comaprison to control group. HIFA showed 16 times underexpression in treatment while HUR gene was underexpressed 64 times on treatment.

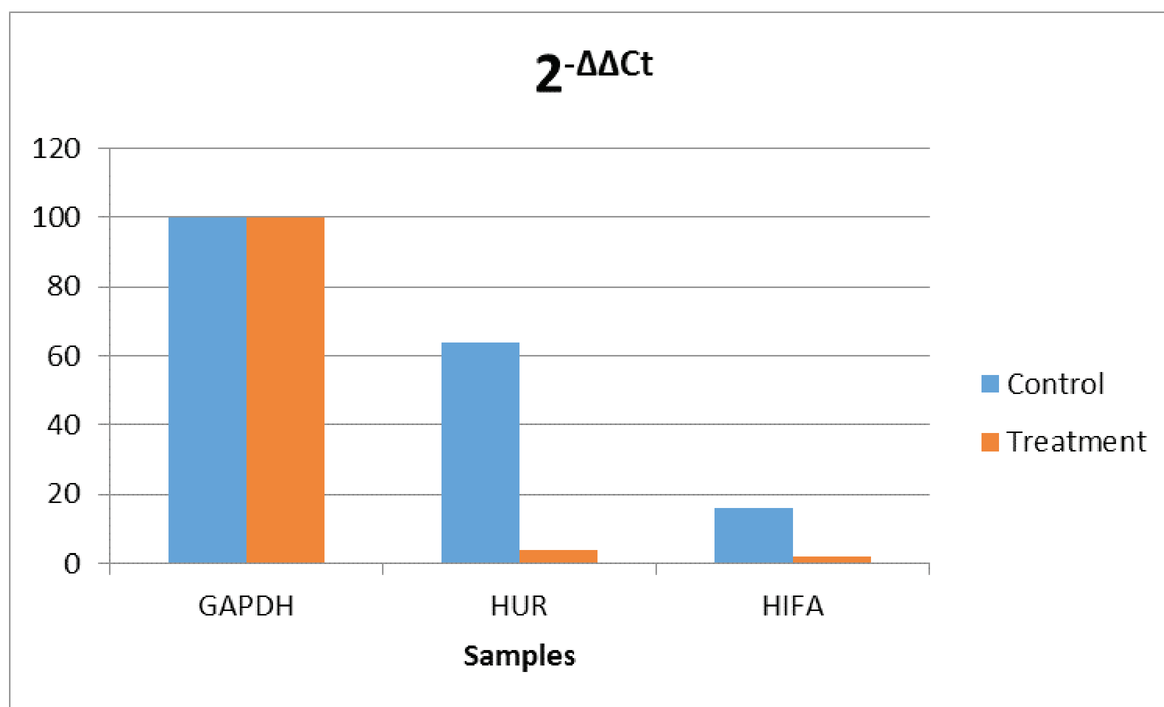

Figure 4: This graph represents the values of $2^{-\Delta\Delta Ct}$ for members of genes attained from the RT PCR. Thus all experiments were the average of the duplicates.

Cell cycle Analysis: HeLa cell lines were used to analyze cell cycle distribution with FACs analysis. This was carried out to observed either any of the anti-cancer drug suspension was applied through the arrest of cell cycle induction. This experiment was carried out as (Kaur, M., et al 2008)[20]. Shortly, approximate plating of 1 x 10^6 HeLa cells was done in 6 well plates that comprised of 2ml complete DMEM. After 24 hours incubation, cells were further treated without or with (control) suspension of drug at fluctuating concentrations of 40 and 20µg/ml. From which DMSO of 1% was utilized as negative control as 1% in the media of DMEM and 20µM of colchicine as a positive control. The cell were assembled and centrifugation was done for 5 minutes at a RT maximum speed and pellets were gathered. The collected pellets were re-suspended in solution of 1XPBS & a 2ml fixing solution of 20% PBS in 70% ethanol was used to fix overnight at 4°C. Further cells were pelleted through 10 minutes (RT) centrifuge at 4000rpm. These pellets were cold wash with 1XPBS and then incubation was done for fifteen minutes (RT) in the solution of 500µl propidium iodide (PI) that comprised of 0.05mg/ml RNase A and 0.05mg/ml in PBS solution. Moreover, the FACS Caliber from BD Biosciences (San Jose, CA) was used for the determination of cell percentage & their viability in several cell cycle's stages in control and treatment as well. Th usage of FACS Caliber from BD Biosciences (San Jose, CA) assist in determination of cells' percentage and their viability in several cell cycle's stage of both control and treatment.

Cell cycle analysis: The analysis of the conclusions of cell cycle through flow cytometry exhibits that in HeLa cells case, a significant arrest was observed in the cell cycle's G2M phase due to drug suspension. Upon comparing to the positive control (80.83±0.02%), the G2M phase cell cycle arrest was observed to be 42.86±0.03% & 33.82±0.05% at 40 and 20µg/ml correspondingly. Thus, the arrest observed was 11.45±0.33% and conclusions approved that drug is a potential cancer inhibitor as it can efficiently stops the proliferation of cell and arrest the division of cell.

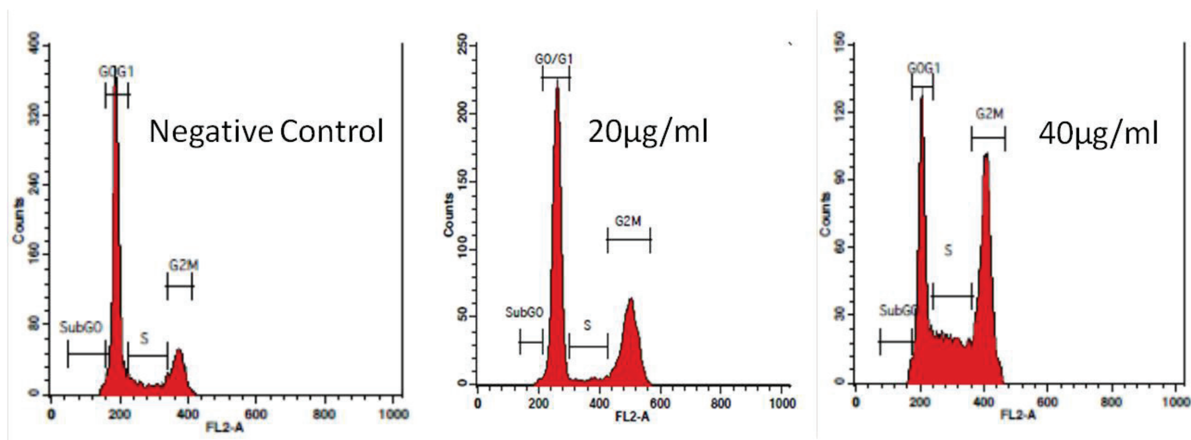

Figure 5: The outcomes of analysis of cell cycle are presented in the images. In which DMSO in 1% was utilized as a negative control whereas, all other values triplicate's average.

Docking studies: The outcomes of the patch Dock confirmed the potential drug action for the targets of drug. For each target, about four models were created while using SWISS model. Therefore, out of the eight merely 1 target got chosen with a higher score of patch Dock. Hence, the HIFA model score was 4716 whereas for HUR model was observed to be 4872 and both of these appeared to be appropriate as compared to the other models.

Conclusion

Relying on the substantial outcomes of the research, the potency of drug was confirmed on the cell lines of HeLa as colchicines. Although, the usage of drug in clinical settings reported no data for its molecular level validation. The two targets of drug in current research approves their potential function as an anticancer drug target and that can further be researched. However for the *insilico* studies, the receptors showed a potent role a fresh drug targets and most probably Dectexal drug may act on these.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

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