

## CYTOTOXICITY AND EXPRESSION STUDIES OF ANGIOGENESIS-PROMOTING GENES IN CANCER CELL LINES UNDER THE TREATMENT OF CANCER CANDIDATE DRUGS

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### ABSTRACT

**Objective:** Human antigen R (HuR) is a member of the RNA-binding proteins which is a critical factor in mediating the proangiogenic factors resulting in angiogenesis among the tumors. During inflammation and anoxic conditions, cytokine interleukin-1 (IL-1) was found to activate the HuR which, in turn, aids in upregulation or downregulation of the hypoxia-inducible factors (HIF) 1 $\alpha$ . The present study was designed to study the correlative effect of the cancer drug docetaxel (Taxotere) on the expression levels of HuR and HIF1 $\alpha$  using HeLa cell lines.

**Materials and Methods:** The drug was tested for cytotoxicity among the cancer cell lines using the lactate dehydrogenase and 3-(4)5-Dimethylthiazol-yl)-2, 5, biphenyl tetrazolium bromide assay. Gene expression and correlation were found by the real-time expression of the gene members under the treatment. The same study was also confirmed using the molecular docking tools to screen for the possible potential anticancer target.

**Results:** The drug was found to decrease the expression levels of the cytokine IL-1 along with the HuR proteins. This was also found to show a positive inhibition on the expression of HIF1 $\alpha$ . The study was also confirmed using the molecular docking studies which are in accordance to the wet lab studies.

**Conclusion:** The present study confirmed that the two receptors might act as potential anticancer drug targets which might aid in designing more novel drugs.

**Keywords:** Human antigen R, Anticancer targets, Hypoxia-inducible factors 1 $\alpha$ , Docetaxel.

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### INTRODUCTION

Cancer remains a major health problem in the world, both in developed and developing countries. The World Health Organization and World Bank estimate that 12 million people worldwide suffer from cancer annually and 7.6 million of them died [1]. The high incidence and mortality of cancer patients are currently due to low rate of the success of treatments. Various methods against cancer have been developed including surgery, radiation, immunotherapy, and chemotherapy. However, the success rate of the methods is still low due to its disadvantages [2]. This situation encourages the efforts to discover and develop novel anticancer drugs that more effective and sensitive without serious side effects [3]. Cancer cell proliferation occurs beyond normal limits, and it is uncontrolled. This is due to the disruption of cell cycle, the increase of angiogenesis, and suppression of apoptosis. Apoptosis is a form of cell death designed to eliminate unwanted host cells [4,5]. The process of apoptosis is programmed through a series of events involving a set of gene products. Apoptosis is responsible for physiological and pathological processes. In the pathological process, apoptosis plays a major role in tumor cell death [6].

Human antigen R (HuR), a ubiquitously expressed member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, exhibits specific affinities for AU-rich elements-containing RNA sequences [7] *in vitro* which correlate with their *in vivo* decay rates, thereby implicating HuR in the proangiogenic factors mediated angiogenesis. Generally speaking, angiogenesis is initiated by hypoxic or inflammatory conditions [8,9]. Hypoxia and inflammation activate HuR function through modulation of HuR subcellular localization and RNA-binding activity [6]. Furthermore, another inflammation-related cytokine interleukin-1 [10] could also activate the function of HuR to stabilize a set of target mRNAs for the fulfillment of a certain process [11]. In turn, activated HuR stabilizes TNF $\alpha$  or promotes the translation

of hypoxia-inducible factors (HIF) 1 $\alpha$  through the ARE located in the 3' UTR, forming a positive feedback [12,13].

Considering the multiple roles of HIF-1 in tumor progression and metastasis, there have been great interests in developing inhibitors targeting this pathway. It is clear that HIF-1 $\alpha$  regulation pathway is a highly complex network involving several signaling cascades and overlapping mechanisms, each of which could serve as a promising target or step to intervene selectively [14]. It is not surprising that most of the reported HIF-1 $\alpha$  inhibitors were originally discovered for targeting other endogenous molecules and later their HIF-1 $\alpha$  inhibitory activity was recognized through some empirical testing. For this reason, there are no specific HIF-1 $\alpha$  inhibitors [15] currently and developing specific HIF-1 $\alpha$  inhibitors in hypoxic tumors represent a challenge. Taxotere is the trade name for docetaxel. In some cases, health-care professionals may use the trade name Taxotere when referring to the generic drug name docetaxel. Docetaxel is an anticancer ("antineoplastic" or "cytotoxic") chemotherapy drug [16]. It has been approved in the treatment of breast cancer, non-small cell lung cancer, advanced stomach cancer, head and neck cancer, and metastatic prostate cancer [17]. Currently it is being studied to treat small cell lung, ovarian, bladder, and pancreatic cancers, soft tissue sarcoma and melanoma [19].

In the present study we aimed to screen for the potency of the drug Docetaxel against the hypothetical drug targets for pancreatic cancer. The study was conducted on the HeLa cell lines by estimating the gene expression levels of the possible drug targets under the drug treatment. The study was also confirmed using the *in silico* studies.

### MATERIALS AND METHODS

The cell culture medium (Dulbecco's Modified Eagle's medium [DMEM]), fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic

acid (EDTA), penicillin and streptomycin were purchased from HiMedia labs. Human cervical carcinoma HeLa cells were obtained from Indian Academy degree college, Bangalore, India. The cell lines were cultured in the DMEM medium, supplemented with FBS (10%) and streptomycin (100 µg/mL). The cells were grown in CO<sub>2</sub> incubator (Mettler, Germany) at about 37°C with 90% humidity and 5% CO<sub>2</sub>. Cells were subcultured when confluent using trypsin/EDTA. Docetaxel (Taxotere) CAS Number 114977-28-5 was purchased from Sigma-Aldrich. The drug was soluble in methanol and used at varying concentrations for the study.

**Primers design**

Primer pairs were designed using Primer 3 software according to the protocol described by Sudhakar *et al.* [18] and were purchased from Sigma Aldrich. The specificity of the polymerase chain reaction (PCR) products and the primer pairs was checked *in silico* using BLAST (National Centre for Biotechnology Information) alignment tools.

**3-(4)5-Dimethyl-thiazol-yl)-2, 5, biphenyl tetrazolium bromide (MTT) assay**

The current study was investigated to study the cytotoxic activity of the methanolic suspension of the selected drug on the HeLa using MTT assay [20]. The cells were treated with different concentrations (10, 20, 40, 60, 80, and 100 µg/ml) of the drug suspension to estimate the percentage cytotoxicity and half maximal inhibitory concentration (IC<sub>50</sub>) (50% growth inhibition). Colchicine (2.5 nM) was used as positive control throughout the study. The cells were plated at a cell density of about 5000–8000 cells/well and seeded in 96-well plates (HiMedia). After 24 h of incubation in the incubator, the cells were treated with different concentrations of the drug suspension. First, well without treatment was maintained as control. Following treatment, the cells were added with 20 µL of MTT solution (5 mg/mL in phosphate-buffered saline [PBS]), and after incubation for 4–5 h, the media were removed and the cells were washed thrice with PBS. The plate was then vibrated for about 15 min before measuring the absorbance at 570 nm in each well using an enzyme-linked immunosorbent assay plate reader (Genetix, Bengaluru). Cytotoxicity was expressed as a percentage of growth inhibition and the concentration needed to inhibit the cell growth by 50% (IC<sub>50</sub>) was calculated. Percentage growth inhibition was calculated using the formula  $(1 - [\text{Optical density (OD) of treated} / \text{OD of control}]) \times 100$ . Each of the experiment was done using six replicates for each of the drug concentration and repeated in triplicates.

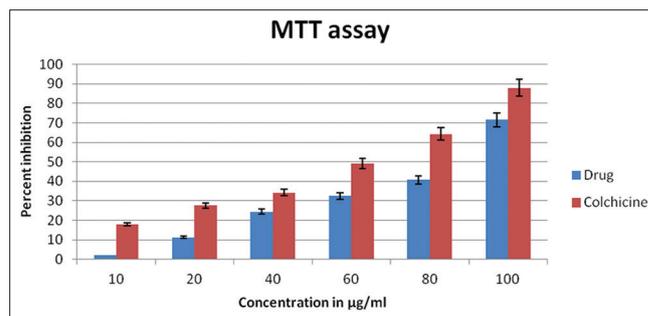
**Lactate dehydrogenase (LDH) assay**

Toxicity of the suspension was also assayed by estimating the concentration of the cytosolic enzyme LDH which is usually released into the media when the cell membrane gets damaged.[21] The cells were seeded according to the protocol as described in the previous section, and the drug suspensions at respective concentrations (0.5, 1, 1.5, 2, 2.5, and 3 µg/ml) were added for treatment. Wells without treatment were served as control. Following incubation, after 24 h, the contents from each well were removed and estimated for the release of LDH into the media. The cytotoxicity was measured using the plate reader (Genetix) at 540 nm.

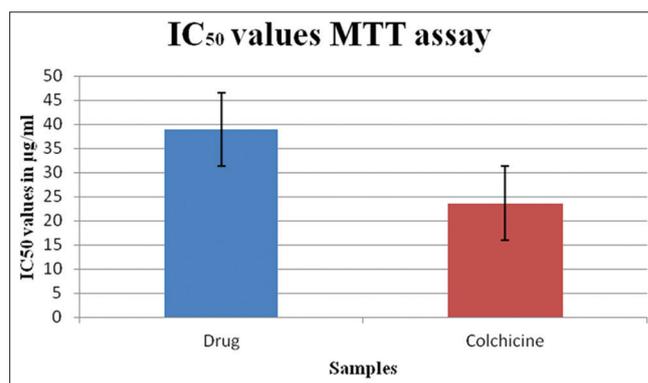
**RNA extraction**

The over confluent cell cultures (both treated 1.5 and 3 µg/ml and controls) were used in the extraction process. Total RNA was then extracted using an RNeasy Mini Kit (Qiagen 74104). The experiment was conducted based on the instructions mentioned in the kit.[22]

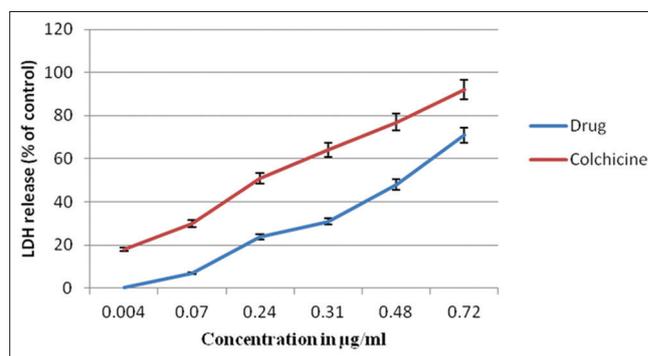
The trypsinized cells after suspending in 2 ml of PBS were pelleted down by centrifuging at 6000 rpm for 5 min. The pellet obtained was



**Fig. 1:** The percent inhibition values in 3-(4)5-Dimethyl-thiazol-yl)-2, 5, biphenyl tetrazolium bromide assay. All the values were average of triplicates and the values are expressed as value±standard error. All the measurements were normalized to control (0%)



**Fig. 2:** The half maximal inhibitory concentration values in 3-(4)5-Dimethyl-thiazol-yl)-2, 5, biphenyl tetrazolium bromide assay. All the values were average of triplicates and the values are expressed as value±standard error



**Fig. 3:** The percentage of lactate dehydrogenase (LDH) released from HeLa cells following 24 h exposure in LDH assay. All the values were average of triplicates and the values are expressed as percentage control values±standard error. All the measurements were normalized to control (0%)

**Table 1:** The details of primers used in the RT-PCR

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	

HuR: Human antigen R, HIF: Hypoxia-inducible factors, RT: Reverse transcription, PCR: Polymerase chain reaction, FW: Forward, RV: Reverse

then resuspended in 560 µl AVL buffer and 560 µl chilled ethanol. The contents were mixed thoroughly and centrifuged again at 8000 rpm for about 1 min to facilitate the RNA to bind to the column. The column was then washed with 700 µl of wash buffer and centrifuged at 15 s followed by incubation with 60 µl of AVE buffer. The RNA eluted was now collected in the 2 ml of collection tubes and stored at -20°C. The RNA quality was checked by the ultraviolet spectrophotometer and used for complementary DNA (cDNA) synthesis.

**Reverse transcription (RT) PCR: cDNA synthesis**

The cDNA synthesis was carried using the RT PCR kit using SuperScript TMII Reverse Transcriptase, 200 U/µl (HiMedia). In brief, 2 µg of the RNA obtained in the previous section was used as the starting reaction. The RNA concentration obtained was about 1.97 µg/µl. Hence, about 1.12 µl of the total RNA was used in the reaction. Random primers and

1 µl of RT enzyme were added mixed thoroughly, and the contents were incubated at 25°C for 10 min. Followed by the incubation at 70°C for 45 min, the cDNA obtained was then stored and used for real-time PCR analysis.

**Real-time PCR**

Primers for real time (Table 1) were designed using primer3 software and were purchased from Sigma-Aldrich. The real-time PCR assay was then performed according to Deepak *et al.* using the iQTM SYBR Green Supermix (HiMedia). The primers (600 nM) and 1 µl of the RT products were used in the PCR assay, and the reaction was done in a total volume of 12.5 µl. All the reactions were carried out in duplicates and also run in parallel with its respective negative control, to confirm the positive amplification.

**Expression of HuR and HIF1-A members in samples**

Real-time quantification was carried on the sample (both control and treatment) in the Corbett Research cycler (Bio-Rad). The HuR primers (HUR) (forward: 5' GGGGGCATTCTAAGTCCAGTGA 3' and reverse: 5' TATCCCGCTATCCC CCTACATTAT 3'; 509bp) and HIF1-A (HIFA) of about 600 nM concentration were used for the amplification program. 1.1 µl of the RNA products were initially used, and the program was run for about 40 cycles at 92°C for 50 s, 64°C for 40 s, and with an elongation at 72°C for 60 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5' TGGAAAGCTGTGGCGTGAT 3' and reverse: 5' CCCTGTTGCTGTAGCCGTAT 3'; 395bp) was also amplified along with the respective gene of interest for a comparative analysis of the mRNA expression. The comparative analysis of the relative levels of mRNA of the test samples (including control) was quantified using  $\Delta\Delta C_t$  method. The  $C_t$  values obtained for the gene of interest were normalized to its housekeeping gene.

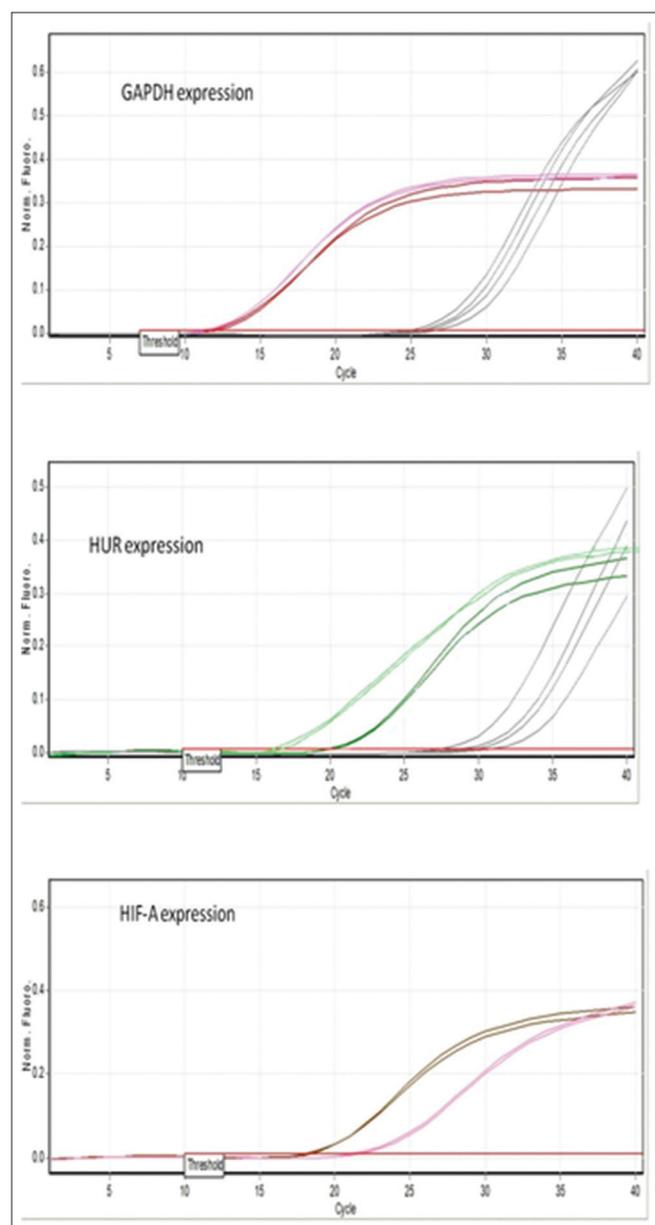
**Docking studies**

Software such as SWISH Model, swish PDB viewer, ACD ChemSketch, Rampage, Argus Lab 4.0.1, and PATCHDOCK were used in the docking studies. HUR and HIF1-A receptors were used in the present context, and a model study to dock or inhibit the receptors were aimed at. Human-like antigen R and hypoxia-inducible factor 1 alpha (NC\_000014.9) were taken from the National Center for Biotechnology Information Entrez database. Homology modeling was done using SWISH Model freeware and the best template was chosen for the docking study. The models selected were further analyzed on PatchDock for docking studies.

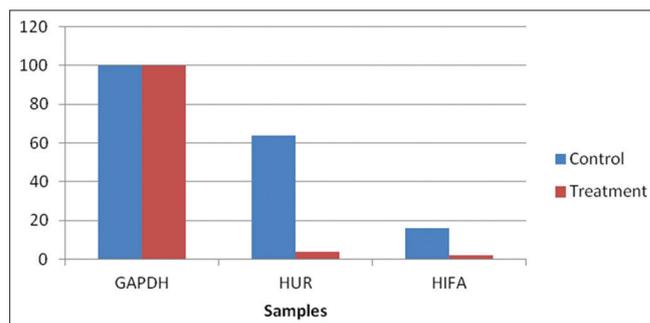
**RESULTS AND DISCUSSION**

**MTT assay**

All the measurements were normalized to control (without treatment) and were considered as 0%. Our study confirmed that the treatment of the cells with the drug suspension at 10µg/mL showed proliferation activity but was very negligible when compared to the positive control and was found to be dose dependent. The activity of the drug was also found to positive when compared to colchicine and negative control. These values indicate that the drug suspension was equally potent to



**Fig. 4: Images of quantification curves of the expression of gene members. Left: Glyceraldehyde-3-phosphate dehydrogenase housekeeping gene; Middle: Human antigen R gene expression (dark line: Treatment and light line: Control); Right: Hypoxia-inducible factor A gene expression (Dark line: Control and light line: Treatment).  $C_t$  values are average of duplicates**



**Fig. 5: The  $2^{-\Delta\Delta C_t}$  values for the gene members obtained from the real-time polymerase chain reaction. All the experiments were average of duplicates**

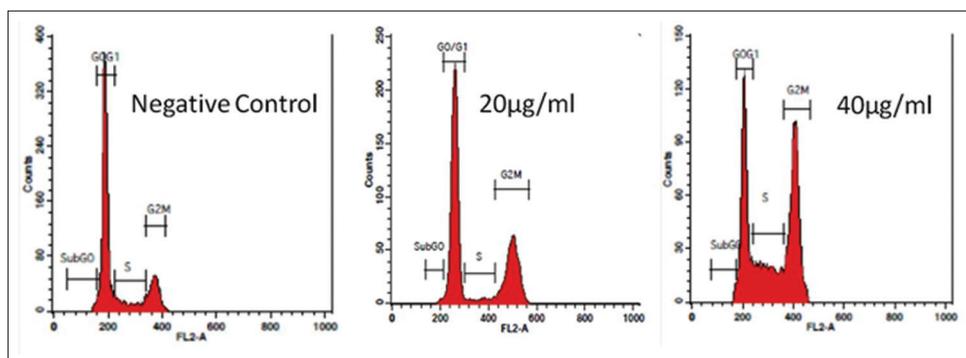


Fig. 6: The cell cycle analysis results. 1% dimethyl sulfoxide was used as a negative control. All the values are average of triplicates

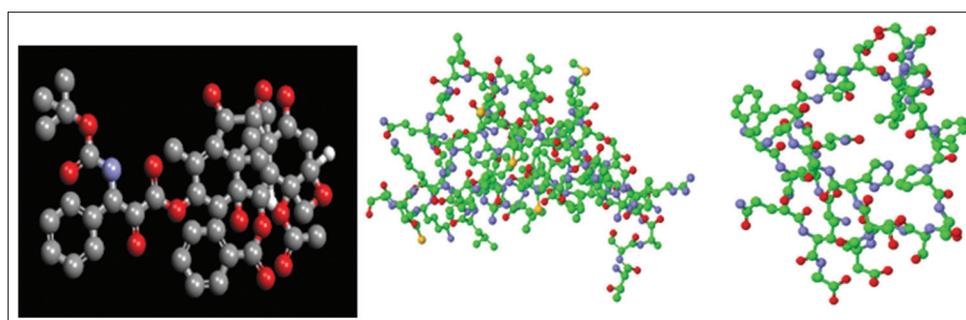


Fig. 7: Images of the drug and receptors. Images were as viewed from Arguslab. Left: Drug molecule; middle: Human antigen R 1 receptor; right: Hypoxia-inducible factors 1A receptor

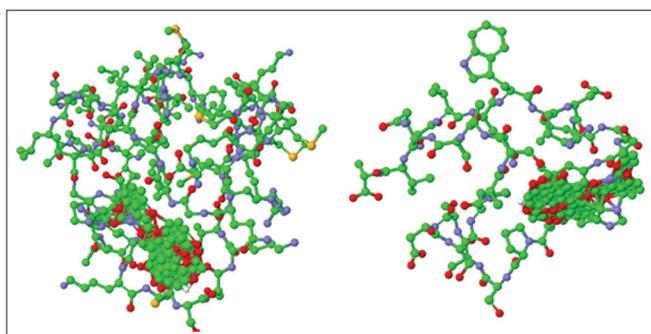


Fig. 8: Images of the solutions obtained from the PatchDock. Images were as viewed under Arguslab

positive control. The  $IC_{50}$  values were found to 38.8 and 23.56 for the drug and positive control, respectively (Fig. 1 and Fig. 2).

#### LDH assay

Drug-induced membrane damage on HeLa cell lines was assayed by LDH release. After incubation, the amount of LDH release was calculated in comparison to the negative control (OD 0.01). Our results reported that, relative to untreated cells, the release was found to be dose dependent and the results were in accordance to MTT assay (Fig. 3). At an initial concentration, the release was very negligible when compared to colchicine and negative control. After 24 h of incubation, the drug suspension showed a significant increase in the LDH release relative to the control after 1  $\mu$ g/ml. At a concentration of 3  $\mu$ g/ml, the percentage release was found to  $71 \pm 19.4$  and  $92 \pm 13.4\%$  for drug and positive control, respectively ( $p < 0.05$ ).

#### Expression of gene members

All the results obtained are normalized to housekeeping gene (GAPDH), to reduce the differences, that usually occurs between the samples. The level of mRNA expression of each gene member (GAPDH, HUR, and

HIFA) was studied separately. The sample with the lower  $\Delta\Delta Ct$  values was taken as calibrator, and the remaining samples were relatively compared with that calibrator. The Ct values of the housekeeping gene, HUR, and HIFA were found to be 11, 21, and 18, respectively. Based on the Ct values, the Ct values of the samples (treatment with the drug) were normalized to the housekeeping gene (GAPDH). The GAPDH gene expression was considered as 100%. From the studies and calculated  $2^{-\Delta\Delta Ct}$  values, it was observed that the HUR gene was under expressed when compared to the control and HIFA. Both HUR and HIFA were under expressed in the treatment than the control (Fig. 4 and Fig. 5). HUR gene was 64 times under expressed in the treatment and HIFA was 16 times under expressed on treatment.

#### Cell cycle distribution

Cell cycle distribution by fluorescence-activated cell sorting (FACS) analysis was done using HeLa cell lines to find out whether the drug suspension exerted any anticancerous effect by inducing the cell cycle arrest. The experiment was followed according to Kaur *et al.* In brief, approximately  $1 \times 10^6$  cells (HeLa) were plated in a 6-well plate containing 2 ml of complete DMEM. Following incubation for 24 h, cells were treated with or without (control) drug suspension at varying concentrations of 20 and 40  $\mu$ g/ml. 20  $\mu$ M of colchicine was used as positive control and 1% dimethyl sulfoxide as negative control (1% in DMEM media), the cells were collected and centrifuged at maximum speed of 5 min at RT, and the pellet was collected. The pellet was resuspended in 1 $\times$ PBS and fixed overnight at 4 $^{\circ}$ C in a 2 ml of fixing solution (20% PBS in 70% ethanol). The cells were then pelleted by centrifuging at 4000 rpm for 10 min (RT). The pellet was washed with cold 1 $\times$ PBS and incubated for 15 min (RT) in 500  $\mu$ l of propidium iodide (PI) solution containing 0.05 mg/ml PI and 0.05 mg/ml RNaseA in PBS. The percentage of cells or viability in various stages of the cell cycle in both treatment and controls was determined using FACS Caliber (BD Biosciences, San Jose, CA).

#### Cell cycle analysis

Our results of cell cycle analysis by flow cytometry in case of HeLa cell lines indicate that the drug suspension has significantly arrested the

G2M phase of cell cycle. The cell cycle arrest in G2M phase was found to be  $33.82 \pm 0.05\%$  and  $42.86 \pm 0.03\%$  at 20 and 40  $\mu\text{g/ml}$ , respectively, when compared to positive control ( $80.83 \pm 0.02\%$ ). The arrest was found to be  $11.45 \pm 0.33\%$ . The results confirm that the drug is a potent inhibitor for cancer and can arrest the cell division and stops cell proliferation very effectively (Fig. 6).

#### Docking studies

From the PatchDock results, the potent action of the drug toward the drug targets was confirmed. About four models were designed using SWISS model for each target. Of all the 8, only one target was selected, for which the patch dock score was high. The scores of model HUR and model HIFA were 4872 and 4716, respectively (Figs. 7 and 8). These two seems to be better fit than the other models.

#### CONCLUSION

Based on the significant findings from this work, it was confirmed that the drug was as potent as colchicines on the HeLa cell lines. Although the drug was being used in the clinical settings, no data were reported of its molecular validation. The two drug targets in the present study also confirm their possible role as anticancer drug targets which can be studied further. Even from *in silico* studies, it was found of the possible role of the receptors as novel drug targets and likely the drug Docetaxel might act on them.

#### AUTHORS' CONTRIBUTIONS

The first author (Salam) initiated and conducted the research and prepared the methods and results in the manuscript. The second author (Sudhakar) guided the first author in laboratory protocols and execution.

#### CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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