International Journal of Pharmacology and Toxicology, 4 (2) (2016) 201-207

International Journal of Pharmacology and Toxicology

Website: www.sciencepubco.com/index.php/IJPT
doi: 10.14419/ijpt.v4i2.6644
Research paper

ATM kinase inhibitor KU-55933 contribution in cisplatin mediated HeLa proliferation

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Abstract

Several approaches including chemotherapy and radiation therapies are being at the forefront to treat various types of cancer including cervical cancer. However, the success and failure of genotoxic based therapy is attributed to aberrant ability of carcinoma to patch up genomic breaks. Here, we have used cisplatin as a genotoxic drug model and HeLa as in vitro carcinoma model due to less responsiveness and resistance of HeLa against cisplatin. Here, attempts are made to investigate the effects of DNA double strand break inhibitor KU-55933 against the cisplatin cell growth and cytotoxicity. Following experiments namely in vitro plasmid DNA metabolizing, Trypan blue dye exclusion, MTT, and PI based Flow cytometery PI assays were conducted to study cell growth and cytotoxicity effects. Based on the cell viability and PI based staining data, results remarked that KU-55933 combined with cisplatin could bring convincing cell growth arrest in HeLa. The reduction in HeLa proliferation was noticed from 70% to 30% in case of KU-55933 added with cisplatin over cisplatin alone. However, we noticed none apoptosis based cell cytotoxicity in case of cisplatin alone or combined with the inhibitors. We also observed significant DNA instability in case of KU-55933 treated HeLa lysates added to plasmid DNA substrate over HeLa lysate without KU-55933 treatment. In conclusion, KU-55933 can potentiate low dose of cisplatin response against HeLa. The effect of KU-55933 may not be attributed due to its enhancing the apoptosis way, rather than through cell growth arrest mechanism due to extensive DNA breaks.

Keywords: *Genotoxic Drug; Double Strand Break; Inhibitor; Carcinoma; DNA Break.*

1. Introduction

Every year, cervical cancer affects ~500,000 women worldwide, and ~275,000 patients die of this disease. Currently, several approaches including chemotherapy and radiation therapies are being at the forefront to treat various types of cancer including cervical cancer (Hanahan and Weinberg, 2000; Schnitt, 2010; Kimbung et al., 2015; Braunstein et al., 2016). Among the potential genotoxic drugs, the use of platin-based drugs have rapidly increased and faced with several challenges including drug resistance and side effects (Inoue et al., 2014; Ha et al., 2014; Tonsing-Carter et al., 2015). The cis-Diaminedichloroplatinum II (cisplatin), an agent commonly used in chemo-radiation, acts by producing DNA inter-strand and intra-strand adducts (Inoue et al., 2014; Ha et al., 2014; Tonsing-Carter et al., 2015; Toulany et al., 2015; Ceccaldi et al., 2015). These crosslinks appear to impair replication, transcription of DNA and potential double-strand DNA breaks in the genome (Zhang et al., 2015; Liu et al., 2015; Ratner et al., 2016).

There are several culprits behind cisplatin drug failure including aberrant DNA repair mechanisms in carcinoma (De Bont and van Larebeke, 2004; Jackson and Bartek, 2009; Curtin, 2012; Kelley et al., 2014; Khanna, 2015; Velic et al., 2015; O'Connor et al., 2015; Gavande et al., 2016; Liu et al., 2016; Puigvert et al., 2016). Therefore, sensitization of tumor cells towards chemo-radiotherapy is considered via inhibition of the DNA damage response (DDR) and attempted pre-clinically (Jackson, 2002; Lieber, 2010; Chapman et al., 2012; Kavanagh et al., 2013; Aparicio et al., 2014; Álvarez-Quilón et al., 2014; Jeggo and Löbrich, 2015; Ceccaldi et al., 2016; Hitrik et al., 2016; Mladenov et al., 2016; Samadder et al., 2016).

The DNA double-strand break (DSB) is the principle cytotoxic lesion for ionizing radiation but can also be caused by mechanical stress replication fork halt or other type of DNA lesion due to genotoxic drugs (Albarakati et al., 2015; Andrs et al., 2016; Flores-Pérez et al., 2014; Hong et al., 2015; Huang et al., 2016; Jekimovs et al., 2014; Rajamanickam et al., 2016). Improper repair of a DSB can lead to mutations or to larger-scale genomic instability to induce apoptosis in carcinoma inflicted with genotoxic drug. In the present scenario, to reduce the drug doses and bolster the drug response are prime concern. The noticeable attempts are reported to test certain inhibitors drugs dedicated against double strand break repair proteins including KU-55933, SCR-7, SCR-17 and L189 along with the existing genotoxic drug regimen (Hickson et al., 2004; Srivastava et al., 2012; Surovtseva et al., 2016; Srivastava and Raghavan, 2015; Vecchio and Frosina, 2016; Weterings et al., 2016). Herein, we have attempted to evaluate the modulatory role of KU-55933 towards Cisplatin based HeLa cell cytotoxicity.

2. Materials and methods

2.1. Materials

The chemicals including Cisplatin (Catalogue Number-13119), KU-55933 (Catalogue Number-16336), were purchased from

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Cayman Chemical. All other cell culture media, reagents, serum, chemicals, plasmid pBR322 etc. were purchased from Merck India limited and Himedia Labs Pvt. Ltd. Mumbai, India.

2.2. Cell culture, maintenance and cryopreservation of HeLa

Cryopreserved HeLa were obtained from NCCS, Pune, India. These cells were thawed at 37°C and grown in DMEM (Dulbecco's Modified Eagles Medium) with 10% FBS (Fetal Bovine Serum), antibiotic solution containing 10000 units of penicillin/10mg streptomycin with 5.0% CO_2. Cells were constantly examined for signs of deterioration (change in pH, morphology and density). HeLa were trypsinized from 75cm^2 culture flask and were centrifuged to form a uniform cell suspension. For the total cell count 10µl of suspended cells were observed on a hemocytometer. For viability checking 10 µl of cell suspension was mixed in equal amount of filter sterilized Trypan blue dye. Cell count was performed.

2.3. Preparation of drugs/inhibitors

Drug cisplatin was initially prepared as stock solution by dissolving 10 mg in 500 µl DMSO and stored at -20°C. Final working concentration of cisplatin (33 µM) was prepared and used in cell based assay. The KU-55933 inhibitor was first prepared as stock concentration of 5 mM by dissolving 1mg powder in 500 µl DMSO and stored at -20 °C. The final working solution of KU-55933 was used in all cell-based assays at 2.5 µM concentration.

2.4. Trypan blue dye exclusion assay to determine effects of ku-55933 towards cisplatin toxicity

The HeLa were grown up to 80-90% confluenecy was harvested and plated on xix well plate at the seeding density of 200,000 cells per well. The media volume was kept at 2 ml and was allowed to grow for next 16-18 h. Next day, overnight grown wells were treated in triplicates as DMSO control, cisplatin (33 µM), and cisplatin (33 µM) + KU-55933 (2.5 µM) in complete DMEM media. The drug and inhibitor were incubated for 48 h at 37°C in CO_2 incubator. After incubation, media were removed and stored if any floating cells are recovered in the aspirated media. Then, wash with PBS, added with 0.3-0.5 ml 0.25% trypsin/EDTA for 2-3 min in incubator. Further, added 2 ml media to inactivate trypsin and collect in 2 ml Eppendorf tube and centrifuge for 2min at 8000 rpm. Ten µl of Trypan blue dye is mixed with 10 µl of cell suspension. Then, cells were counted using standard protocol of hemocytometer assay for viable and dead cells.

2.5. MTT based cytotoxicity of cisplatin in combination with ku-55933

The 80-90% confluent HeLa were plated on 96 well plate with the seeding density of 5000 cells per well. After 16-18 h of seeding, drug Cisplatin / inhibitors (SCR7, KU-55933) along with 200 µl complete MEM media were added and incubate for 48 h at 37°C in CO_2 incubator. Volume and concentration of drug and inhibitors should be predetermined and premixed with the media. For MTT assay standard protocol was followed with slight modifications as fresh RPMI (without phenol red) was used. After the purple formazan crystal was precipitated, the microscopy photograph was taken using inverted microscope at 10X objective.

2.6. DNA metabolizing activity of protein lysates from inhibitor/ drug treated HeLa

The plasmid DNA degradation assay was performed to assess the DNA break ability of cisplatin drug and effect of HeLa lysates. One µl of pBR322 plasmid DNA (100 ng /µl) and one µg of plasmid DNA pBR322 were mixed with 2 µl each of TAE buffer (Tri-acetate/EDTA 10 mM, pH 7.4). Then after, different concentration of cisplatin drug ranging from 10 µM to 500 µM (final concentration) was added to the reaction mixture. Final volume of each reaction mixture was brought to 25 µl by addition of nuclease free water in a microcentrifuge tube. Reaction mixtures were incubated for 24 h at 37°C. In a separate experiment, with 50 µM cisplatin treated plasmid DNA sample, we also included whole cell protein lysates obtained from treated HeLa as DMSO control, cisplatin (33 µM), cisplatin (33 µM) + KU-55933 (2.5 µM) from cell based experiment. At the end of experiment, the standard ethidium bromide stained gel was used to assess the DNA shearing or ligation activity due to cell lysates presence.

2.7. The Flow cytometry based propidium iodide based cell viability assay

HeLa were grown in three replicates of 35 mm^2 culture dishes at a seeding density of 200,000 cells/dishes. After the treatment period, 2 ml 500 µl Trypsin-EDTA (Gibco) was added to each dish for 3-4 min until all cells had detached. The cell suspension was then added to 15 ml Falcon tubes and centrifuged at 6000×g for 3 min. The supernatant was removed and the pellet washed with HBSS solution. Cells were centrifuged and the supernatant was removed. Further, 10 µl PI added from a stock of 50 mg/ml to final concentration of 10 mg/ml. Further, incubated for 30 min and then centrifuged to get the pellet. The pellet was again washed using PBS two times. Then, pellets were suspended in BD staining buffer and analyzed on the flow cytometer (BD FACSJazz). A minimum of 10,000 events were collected and analyzed using a 488 nm laser and 610LP, 616/23BP emission filters. PI inclusion signified loss in membrane integrity and cell death. Values were represented as percentage with reference to control.

2.8. Statistical analysis

The experiments were independently conducted three times. Results are expressed as mean ± SD. Data from the different assays were statistically compared using one pair t-test as Microsoft office excel 2010 statistical package. Statistical significance was acceptable to a level of $p < 0.05$.

3. Results and discussion

Nowadays, chemotherapeutic treatment for surmounting cancerous cell using some chemicals, drugs and inhibitors are designed to achieve better success by generating DNA lesions in carcinoma genome (Hanahan and Weinberg, 2000; Jackson, 2002; Lieber, 2010; Schnitt, 2010; Chapman et al., 2012; Kavanagh et al., 2013; Aparicio et al., 2014; Álvarez-Quilón et al., 2014; Jeggo and Löbrich, 2015; Ceccaldi et al., 2016). Even if DNA damage is 100% as in case of certain chemotherapy drug regimen but the outcome and prognosis are not as per the expectations. Cancer generally generates acquired resistance to almost all chemotherapeutic drugs via a variety of various mechanisms and pathways also related with side effects (Jackson, 2002; Lieber, 2010; Álvarez-Quilón et al., 2014). Chemotherapeutic resistance whether it is acquired or intrinsic is sustained by alteration in drug targets and signal transduction molecules, increased repair of drug-induced DNA damage (Jackson, 2002; Lieber, 2010; Álvarez-Quilón et al., 2014).

3.1. Trypan blue dye exclusion and MTT assay to determine effects of ku-55933 towards cisplatin toxicity

The photomicrograph presented in Figure 1A show total cell count for HeLa cell during different drugs/inhibitors treatment condition. The corresponding total cell count using hemocytometer was performed and data is given in Figure 1B. Data indicated that cisplatin responds to arrest HeLa growth some extent. Simultaneous treatment of KU-55933 resulted into accentuated reduction in total HeLa cell count. For the same in vitro HeLa drug treatment assay,

we conducted Trypan blue exclusion assay to observe the extent of dead and viable cell. The microscopy photographs presented in Figure 2A depicts the Trypan blue dye uptake from the growing HeLa treated with or without cisplatin/KU-55933. We also estimated the dead cell count using hemocytometer and data are presented as bar graph in Figure 2B. The Trypan blue dye exclusion assay is widely accepted to understand the anticancer activity including anti-proliferative and cytotoxic activity (Strober, 2001, Kristine et al., 2011).

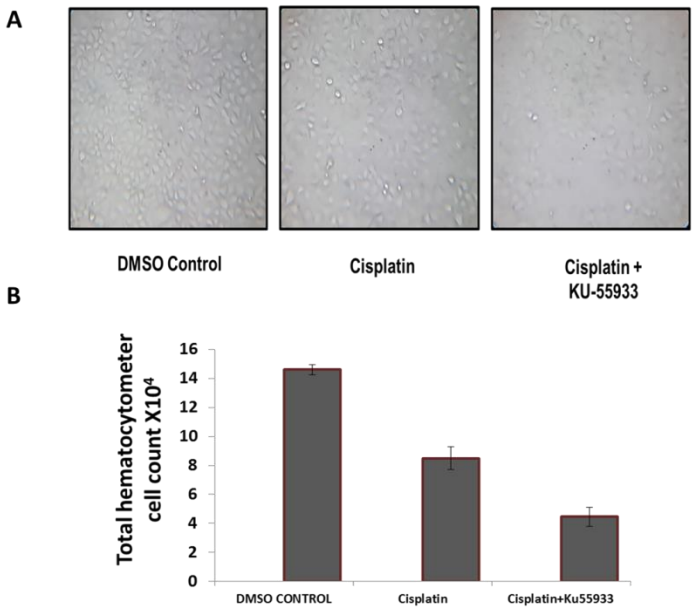


Fig. 1: Hemocytometer and Microscopy Based HeLa cell Proliferation Data Is Presented. (A). This Diagram Represents the Photomicrograph Taken at 10X for HeLa Growing in Six Well Plate with Different Treatment Condition Whereas DMSO Control, Cisplatin 33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM). The Microscopy Photographs were taken at the End of Drugs/Inhibitors Treatment for 48 H. at the End of Treatment; The Microscopy Photograph was Captured Using Light Inverted Microscope. The Data are represented as Mean ± SD. Each Experiment Was Conducted Independently Three Times. (B). This Diagram Is A Representative Bar Graph Data for HeLa Cell Growing in Six Well Plate with Different Treatment Condition Whereas DMSO Control, Cisplatin 33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM). The Total Cells were Recorded Using Hemocytometer and Data Is Presented as Percentage of Total Cell Count X10^5 In Each Treatment Conditions.

To our surprise, we found none significant HeLa cytotoxicity as determined from dead cell count data and microscopy data. Herein, Trypan blue dye exclusion data indicated that KU-55933 can efficiently synergies and accentuate the effect of cisplatin anti-proliferative effect against HeLa.

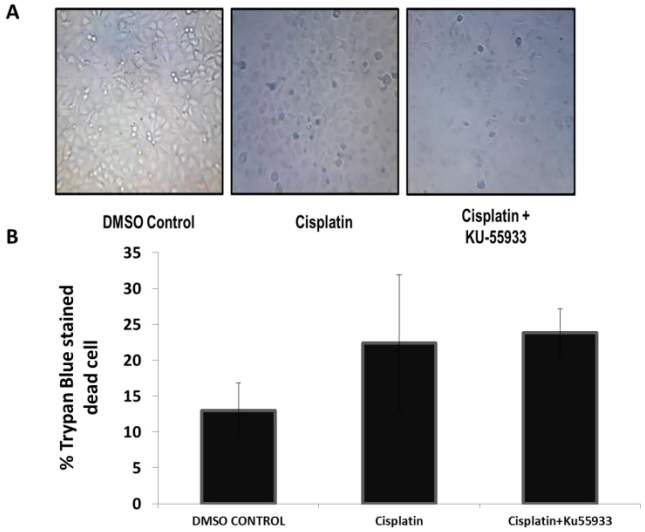


Fig. 2: This Data Represented the Trypan Blue Dye Exclusion Assay Results Obtained from HeLa Drug Toxicity Assay. (A) This Is A Representative Photomicrograph Taken at 10X for HeLa Cell Growing in 96 Well Plate with Different Treatment Condition Whereas DMSO Control, Cisplatin 33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM). The Microscopy Photograph were Taken after Trypan Blue Dye Exclusion Assay Staining in the Well, Where Cells Were Growing under Different Treatment Conditions. The Data are represented as Mean ± SD. Each Experiment was Conducted Independently Three Times. (B). This Diagram Is A Representative Bar Graph Data for HeLa Cell Growing in Six Well Plate with Different Treatment Condition Whereas DMSO Control, Cisplatin 33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM). The Total Cell and Viable Cell Count were Recorded Using Hemocytometer and Data Is Presented as Percentage of Viable Cell in Each Treatment Condition. The Data are represented as Mean ± SD. Each Experiment was Conducted Independently Three Times.

The results presented in Figure 3 represent the microscopy photographs taken at the end of MTT based cell growth and viability assay. The analysis of microscopy data point out that addition of KU-55933 along with cisplatin could not elicit cell cytotoxicity as intensity of MTT based colored formazan crystal production showed no difference. Hence, MTT based assay data supports Trypan blue dye exclusion assay results showing no discernible increase in the cell cytotoxicity due to combined effects of cisplatin and KU-55933.

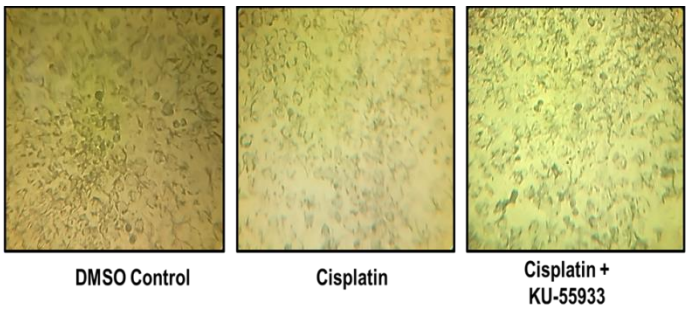


Fig. 3: The Photograph Depicts the Photomicrograph Taken at 10X for HeLa Cell Growing in 96 Well Plate with Different Treatment Condition Whereas DMSO Control, Cisplatin 33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM). The Microscopy Photographs were Taken at the End of MTT Based Assay with Formazan Colored Using 10 X Objective.

3.2. In vitro DNA metabolizing activity of protein lysates

The in vitro DNA damage ability of cisplatin was assessed using plasmid DNA pBR322 based nicking/shearing. The results as ethidium bromide stained gel photographs are depicted in Figure 4A. However, we did not observe clear nicking or shearing of plasmid DNA substrate ranging from 20 µM to 100 µM, but at 500 µM some shearing damage is observed. We conclude that our results are in consonance with earlier reports that at low concentration cisplatin may not bring clear nicking or damage to DNA (Inoue et al., 2014; Ha et al., 2014; Tonsing-Carter et al., 2015; Toulany et al., 2015). Therefore, we performed in vitro cell based experiment at 33 µM cisplatin concentration which is less genotoxic and possibly with fewer side effects. Further, we asked whether cell lysates obtained in vitro HeLa cell based treatment could possess differential DNA metabolizing activity towards plasmid DNA pBR322 substrate treated with cisplatin at 50 µM. The agarose DNA ethidium bromide stained gel is presented in Figure 4B. Data indicated that HeLa whole cell lysates possessed significant DNA metabolizing activity compared to cisplatin drug treated control. To our notice, the data demonstrated that in case of cell lysates from cisplatin plus KU-55933 produced more nicking of plasmid DNA substrate over only cisplatin treated HeLa lysates. Therefore, we tried to explain that cisplatin plus KU-55933 treated HeLa lysates may have less active pool of DNA double strand break repair proteins over the cisplatin alone treated HeLa lysates. Such probable difference might lead to the clear difference in the DNA damage /shearing pattern in the plasmid DNA pBR322.

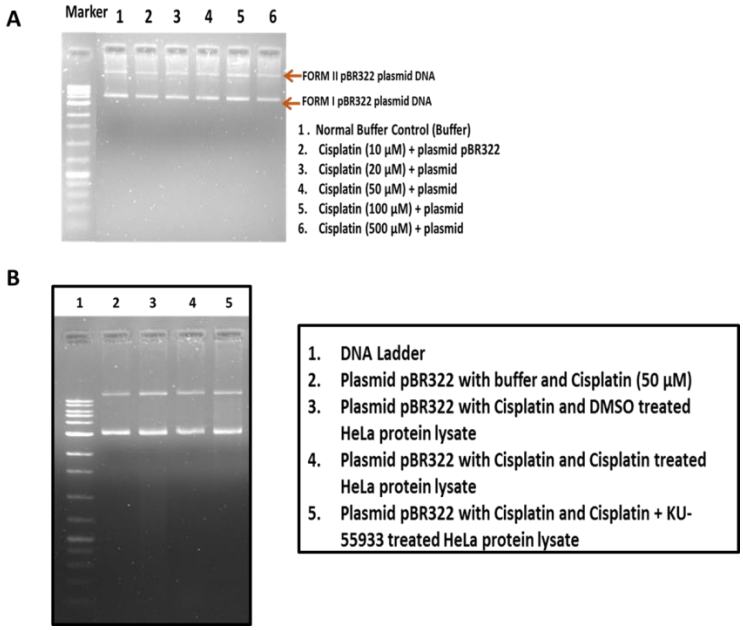


Fig. 4: (A) This Agarose Gel Photograph Shows the Plasmid DNA Damage Assay Result against Cisplatin Treatment Ranging from 20 µM to 500 µM. The Reaction was performed at 16°C in TAE Buffer and Its Composition Is Described in Method Section. The Data are represented as Mean ± SD. Each Experiment was Conducted Independently Three Times. Figure 4 (B). This Gel Photograph is A Representative Agarose DNA Gel Stained for Pbr322 Plasmid Treated with HeLa Whole Cell Lysates Extracted from in Vitro Treated Cell Based Assay as DMSO Control, Cisplatin (33 µM) and Cisplatin (33 µM) + KU-55933 (2.5 µM) as Mentioned in the Method Section and Cisplatin (50 µM). Each Experiment was Conducted Independently Three Times.

3.3. Flow cytometer analysis of cisplatin treated HeLa cell

The flow cytometer analysis of cisplatin and cisplatin combined with KU-55933 inhibitor in HeLa were analyzed by PI based staining. The results are presented in Figure 5 A-D. Results analysis showed that in case of cisplatin-treated HeLa sample apoptotic based PI stained cell percentage was not significant compared to untreated control. In case of cisplatin along with KU-55933, we found none noticeable presence of PI stained cell. On other hand, we compared with positive control of doxorubicin plus SCR-7 (Ajay et al., 2016) noticed with existence of PI stained HeLa. Hence, our data strongly suggest that cisplatin and cisplatin combined with KU-55933 did not result into apoptotic cell death. Our observations may rely upon cell cycle arrest and anti-proliferative mechanisms.

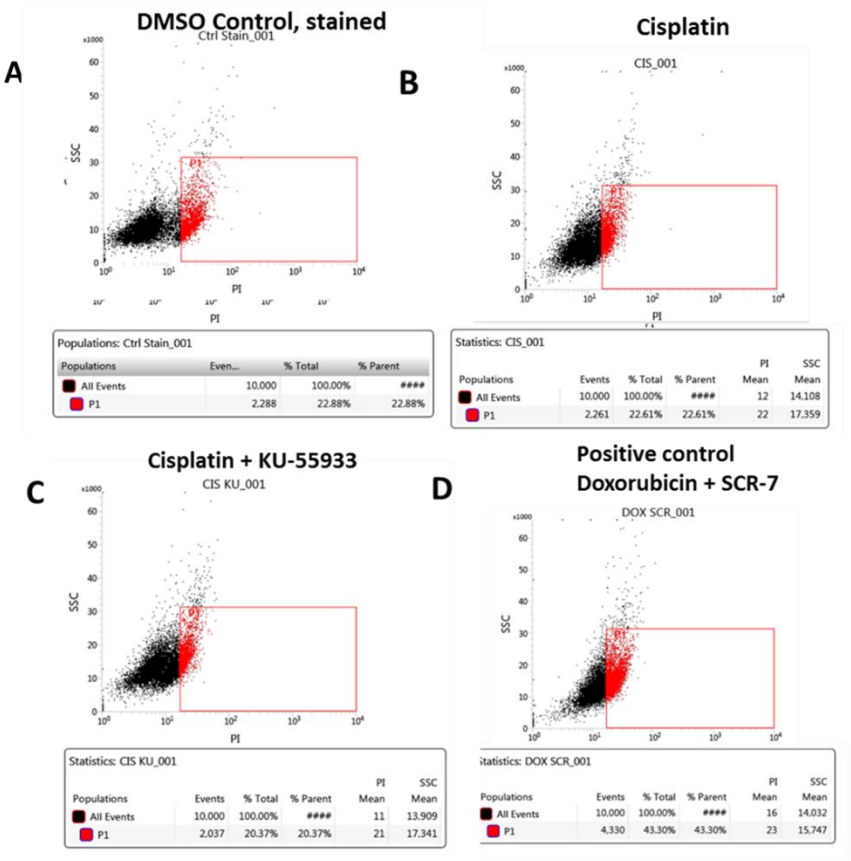


Fig. 5: This Diagram Presents the Flow Cytometer Based Cell Viability Data. (A) This Data Represents the PI Based Flow Cytometer Results for Untreated DMSO Control HeLa. (B). PI Based Cell Viability Data for Cisplatin 33 µM Treated HeLa. (C). PI Based Cell Viability Data for Cisplatin (33 µM) + KU-55933 (2.5 µM) Treated HeLa. (D). PI Based Cell Viability Data for Positive Control Doxorubicin (25 nM) + SCR-7 (29.8 µM) Treated HeLa. The Experiments were conducted in Triplicates.

It is widely accepted that cisplatin modulates ataxia-telangiectasia-mutated (ATM), a protein with clear role in double strand DNA repair, cell cycle progression and autophagy. In earlier study, evidences have suggested that KU-55933, an ATM kinase inhibitor may be able to push non-small lung cancer towards augmented radiosensitization (Toulany et al., 2015). Cisplatin is the main chemotherapeutic drug regimen for the treatment of cervical cancers. However, resistance to cisplatin is increasingly common and therefore found to have limited the efficacy and use of this drug in the clinic. Dose-dependent toxicity poses an additional challenge since patients suffer long-term and often permanent side-effects after treatment. Recently, Leisching et al (2015) have reported that cisplatin at 15 µM displayed low cytotoxicity activity due to the inherent cellular capability of HeLa. In agreement, present study using cisplatin at 33 µM demonstrated less anti-proliferative activity against HeLa.

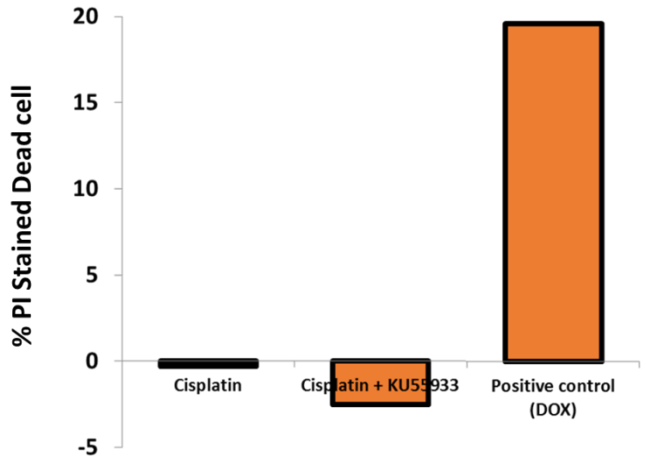


Fig. 5: (E) This Data Represents the PI Based Flow Cytometer Percentage PI Stained HeLa cell Over the DMSO Control. The Data are calculated from Flow Cytometer Cell Viability Data Presented in Figure 5 A-D.

We agree to earlier views that in case of HeLa responses against cisplatin may be modulated by the DSB pathways. When designated inhibitor as KU-55933 used along with, we found synergistic effects to show anti-proliferative potential. Therefore, DSB repair pathway ATM inhibitor KU-55933 may be a right candidate to test in combination with the cisplatin to see better responsiveness (Hickson et al., 2004; Srivastava et al., 2012; Surovtseva et al., 2016; Srivastava and Raghavan, 2015; Vecchio and Frosina , 2016; Weterings et al., 2016). Besides, there are reports on resistance towards DNA crosslinking agents such as cisplatin to be

linked with ERCC1 and FANC-BRCA1 pathway. Simultaneously, it is indicated that inactivation and deactivation such DNA repair protein may prove an option for better cisplatin based cell death response (Curtin, 2012; Puigvert et al., 2016).

There are ample evidences showing DNA double-strand breaks (DSBs) are formed during the processing of DNA inter-strand crosslinks due to treatment to cisplatin in case of proliferating HeLa. Thus, DNA inter-strand crosslinks induced by different crosslinking agents, including cisplatin may be processed and yielding to DSBs as an intermediate lesion (Inoue et al., 2014; Ha et al., 2014; Tonsing-Carter et al., 2015; Toulany et al., 2015; Ceccaldi et al., 2015). To extend and support such views, our cell cytotoxicity data point out that double strand break repair inhibitor KU-55933 demonstrated significant contribution to stop the growth of HeLa. Such findings may be due to interfering in double strand break repair signaling pathway and leading to occurrence of more lethal damage as double strand break.

4. Conclusion

In conclusion, our data provide evidences for the combinatorial option encompassing KU-55933 an ATM kinase inhibitor combined with cisplatin displaying reduction in HeLa cell proliferation. However, KU-55933 showed as anti-proliferative instead of apoptotic cell cytotoxicity to act in synergy with cisplatin. We envision that exploring detailed molecular mechanisms in HeLa and other cancer types keeping may lead to DNA repair profile based therapy.

Acknowledgements

We would like to thank the Cancer Biology Research Group for their constant support and motivation and our host institute Dr. D. Y. Patil Biotechnology & Bioinformatics Institute, Dr. D. Y Patil Vidyapeeth, Pune for providing resources. The authors acknowledge the financial support from DST-SERB, New Delhi, India and DPU, Pune, India. We acknowledge CRF, DPU, Pune facility for Flow cytometer facility

Declaration of interest

Authors declare no conflict of interest.

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