PRECLINICAL EVALUATION OF IMMUNOMODULATORY EFFECTS OF AURORA KINASE INHIBITION IN HUMAN PAPILLOMAVIRUS POSITIVE CANCERS

Pragya Sinha

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations

Part of the Cancer Biology Commons, and the Immunotherapy Commons

Recommended Citation
Sinha, Pragya, "PRECLINICAL EVALUATION OF IMMUNOMODULATORY EFFECTS OF AURORA KINASE INHIBITION IN HUMAN PAPILLOMAVIRUS POSITIVE CANCERS" (2023). The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access). 1281.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/1281

This Thesis (MS) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digcommons@library.tmc.edu.
PRECLINICAL EVALUATION OF IMMUNOMODULATORY EFFECTS OF AURORA KINASE INHIBITION IN HUMAN PAPILLOMAVIRUS POSITIVE CANCERS

By

Pragya Sinha, B.S.

APPROVED:

______________________________
Jagannadha Sastry, Ph.D.
Advisory Professor

______________________________
Faye M. Johnson, M.D., Ph.D.

______________________________
Roza I. Nurieva, Ph.D.

______________________________
Subrata Sen, Ph.D.

______________________________
Pamela Wenzel, Ph.D.

APPROVED:

______________________________
Dean, the University of Texas

MD Anderson Cancer Center UTHHealth Graduate School of Biomedical Sciences
PRECLINICAL EVALUATION OF IMMUNOMODULATORY EFFECTS OF AURORA KINASE INHIBITION IN HUMAN PAPILLOMAVIRUS POSITIVE CANCERS

A

DISSERTATION

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Pragya Sinha, B.S.

Houston, Texas

May, 2023
DEDICATION

To Mumma, Papa and Phantom,
who always picked me up on time
and encouraged me to go on every adventure,
especially this one.
ACKNOWLEDGEMENTS

I would first like to acknowledge my supervisor, Dr. Jagan Sastry, for giving me the opportunity to be an MS student in his lab. He has been an inspiring, patient and supportive mentor and I consider myself very fortunate to have continued my scientific career in his lab. Thank you for all the cheer-leading and motivation to do better and show passion for my work.

I wish to thank the members of my graduate supervisory committee, Drs. Faye M. Johnson, Roza I. Nurieva, Subrata Sen, and Pamela Wenzel for their guidance and encouragement throughout the course of my studies. I am grateful to have been surrounded by all the brilliant and helpful scientists and staff at MD Anderson and GSBS that have helped me get to where I am at today, it is truly an honor to be surrounded by this incredible network of people.

I would like to thank past and present members of the Sastry Lab, Madison O’ Hara and Ananta Yanamandra. Our lab space is shared with the Johnson Lab (also our collaborators) and I particularly must thank Drs. Soma Ghosh, Tuhina Mazumdar, and Pooja Shah for being kind and exceptional people.

Finally, I would like to thank my parents for their constant love and support and for instilling in me the importance of hard work and education. Last, but not least, I am very fortunate to have kind and wonderful best friends who always listen to my frustrations and celebrate my achievements, no matter how small they are. I know that this would not have been possible without the support of my family and friends.

Lastly, I would like to thank the GSBS for providing such an inviting and caring environment for its students. I’m grateful for the friends I’ve made at GSBS along the way, and the opportunities
I have had to grow my leadership and teambuilding skills, particularly as a member and leader of the International Student Association (ISA).
PRECLINICAL EVALUATION OF IMMUNOMODULATORY EFFECTS OF AURORA KINASE INHIBITION IN HUMAN PAPILLOMAVIRUS POSITIVE CANCERS

Pragya Sinha, B.S.

Advisory Professor: Jagannadha K. Sastry, Ph.D.

ABSTRACT:

Human papillomavirus (HPV) is the causative agent of cervical cancer and some cancers of the penis, vulva, vagina, anus, and oropharynx. Current therapies for these cancers include a combination of surgery, radiotherapy, and chemotherapy that often results in permanent, life altering adverse effects. Immunotherapy is partially effective, but with significant recurrence and lower long-term survival. Importantly, there are no few biomarker-selective targeted therapies for these cancers. To address this unmet need, our collaborators conducted a large-scale drug screen and identified Aurora Kinase (AK) inhibitors as a unique class of reagents to induce selective apoptosis in HPV+, but not HPV- human tumor cells in vitro and in vivo in HPV+ patient derived xenografts (PDX) mouse models. We hypothesized that Aurora kinase inhibition mediated HPV+ cancer cell apoptosis would lead to immunogenic cell death (ICD) that would promote antitumor efficacy of immune checkpoint therapy. The current investigation focused on the effectiveness of alisertib, an Aurora Kinase A inhibitor using preclinical mouse tumor models of HPV+ cancers (mEER, TC-1, and C3.43). In vitro, alisertib treatment when compared to the vehicle control, reduced the level of phospho-Aurora Kinase A confirming the targeted activity. We observed morphological changes to cells suggesting cell death that was confirmed via annexin V 7-AAD staining as apoptosis. Furthermore, western blot analyses revealed DNA damage, in terms of
increased levels of γH2AX levels, and pyroptosis, in terms of increased levels of cleaved gasdermin E. Importantly, in both mouse and human HPV+ cancer cell lines treatment with alisertib, relative to vehicle control, resulted in significantly higher cell surface expression of calreticulin (CRT), and high mobility group box 1 protein (HMGB1) in the culture supernatants, both of which are markers for (ICD). Studies with the mEER tumor cells implanted in immunocompetent syngeneic mice showed partial in vivo efficacy of Aurora Kinase inhibition that when combined with immune checkpoint blockade using anti-CTLA-4 antibody, resulted in significant tumor growth reduction and a survival advantage. Thus, data from this investigation support the suitability of targeted Aurora kinase inhibition in combination immune therapeutic approaches for the clinical management of HPV+ cancers.
# TABLE OF CONTENTS

- Approval page .............................................................................................................i
- Title page ..................................................................................................................ii
- Dedication page ..........................................................................................................iii
- Acknowledgements .....................................................................................................iv-v
- Abstract .......................................................................................................................vi-vii
- Table of Contents .......................................................................................................viii
- List of Illustrations .....................................................................................................ix
- List of Tables ................................................................................................................x
- Chapter 1: Introduction ..............................................................................................1-12
- Chapter 2: Materials and Methods ............................................................................13-20
- Chapter 3: Results ......................................................................................................21-35
- Chapter 4: Discussion ...............................................................................................36-42
- References ..................................................................................................................43-51
- VITA .............................................................................................................................52
List of Illustrations

Figure 1.................................................................3
Figure 2.................................................................5
Figure 3...............................................................11
Figure 4...............................................................21
Figure 5...............................................................22
Figure 6...............................................................24
Figure 7...............................................................25
Figure 8...............................................................26
Figure 9...............................................................28
Figure 10............................................................30
Figure 11............................................................31
Figure 12............................................................33
List of Tables

Table 1.........................................................................................................................6
1. Introduction

1.1 Human Papillomavirus (HPV)

HPV is a non-enveloped double-stranded DNA virus with eight protein-coding genes. The virus contains an icosahedral capsid and a roughly 8000 base pair circular double-stranded DNA genome [1].

Most HPV types tend to infect cutaneous tissues, yet a few of them can also infect mucosal tissues. Each of the nearly 200 HPV types that have been identified so far shares less than 10% homology with the other strains [2]. Based on the genes it encodes and their functions, the HPV DNA can be divided into three sections: the upstream regulatory region (URR), the early protein coding area (E1, E2, E4, E5, E6, and E7), and the late-protein encoding region (L1 and L2), capsid proteins [3].

HPVs are classified into low risk and high-risk types based on their ability to cause cancer. The low-risk types, such as HPV 6 and 11, have been linked to non-cancerous disorders including skin and genital warts, pulmonary papillomatosis, and other non-cancerous lesions. On the other hand, the high-risk types, for example HPV16 and 18, can cause cancers of the cervix, and other anogenital, as well as the oropharynx [4].

The E6 oncoprotein binds to and degrades the tumor suppressor protein p53, which is involved in regulating cell growth and division and promoting cell death when DNA damage occurs. By inactivating p53, E6 can allow cells with DNA damage or other genetic abnormalities to
continue dividing and proliferating, increasing the risk of cancer development. The E7 oncoprotein, on the other hand, binds to and inactivates another tumor suppressor protein, known as retinoblastoma (Rb). Rb normally acts to inhibit cell cycle progression and promote cell differentiation. Inactivation of Rb by E7 can lead to uncontrolled cell proliferation and reduced cell differentiation, further promoting cancer development. Together, the E6 and E7 oncoproteins of HPV work to disrupt the normal regulatory mechanisms of the cell cycle, leading to abnormal cell growth and division, reduced cell differentiation, and increased risk of cancer development. Additionally, the E6 and E7 oncoproteins can also induce chromosomal instability, genomic mutations, and alterations in gene expression, further promoting cancer development and progression [5].
Fig 1: Mechanisms of high-risk HPV encoded E6 and E7 oncogenes towards cellular transformation and malignancy. The tumor suppressor protein p53 is targeted by the E6 protein for rapid ubiquitin-mediated degradation. When the E7 protein attaches to the retinoblastoma (Rb) family of tumor suppressor proteins, it causes their instability and the loss of Rb/E2F complexes, which restrict the transcription of genes necessary for cell cycle progression. Figure created with BioRender.com.
1.2 HPV+ Cancers

HPV causes about 5% of all human cancers. HPV infection can result in head and neck squamous cell carcinoma (HNSCC) and cervical cancers. HNSCC associated with high-risk HPV infection are the most common malignancy in head and neck and make up to 3.5% of all malignancies in the United States and Europe [6].

The HNSCC represent the 6th most prevalent cancer worldwide, with an annual incidence of 600,000 cases. The two additional key common risk factors for HNSCC are tobacco use and alcohol intake [7]. According to the anatomical site, there are different associations between HPV and head and neck malignancies, with the oropharynx accounting for majority of the associations (90%) [8]. Furthermore, HPV16 incidence in HPV+ oropharyngeal squamous cell carcinoma (OPSCC) is approximately 90%, with HPV18 prevalence being 3% [9].

Almost all occurrences of cervical cancer are caused by high-risk type HPV [10]. Due to weaker surveillance and higher incidence in the developing and low-income countries, it ranks as the second most common cancer in women globally despite the decreasing incidence in developed nations [11]. The annual mortality surpasses 250,000 and the annual incidence is above 500,000 worldwide [12].
1.3 Prevention of HPV+ Cancers

The currently available HPV vaccines (Table 1) contain virus-like particles (VLPs) with the viral L1 proteins but no viral DNA, and they can elicit an immune response comprised of strong virus
neutralizing activity to prevent HPV infection, and thus are termed as “prophylactic vaccines”. Most prevalent high-risk HPV infections are effectively prevented by these current vaccines; however, they do not treat existing HPV lesions [13].

The first vaccine approved for human use was Gardasil, a quadrivalent vaccine from Merck, and it targets not just HPV-16 and 18 but also genital wart-causing HPV subtypes HPV-6 and 11. The second approved vaccine was Cervarix from GSK, which protects against high-risk HPV types 16 and 18. The third and latest approved HPV vaccine is a nonvalent Gardasil-9 from Merck, targeting nine different HPV types (6, 11, 16, 18, 31, 33, 45, 52, and 58) [14].

<table>
<thead>
<tr>
<th>What is the name of the vaccine?</th>
<th>Who is the manufacturer?</th>
<th>What is the vaccination schedule?</th>
<th>Who is it for?</th>
<th>What HPV infection it prevents?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervarix</td>
<td>GlaxoSmithKline</td>
<td>3 doses: day 1, month 1, and month 6</td>
<td>Girls 9 to 26 years old</td>
<td>HPV types 16 and 18</td>
</tr>
<tr>
<td>Gardasil</td>
<td>Merck</td>
<td>3 doses: day 1, month 2, and month 6</td>
<td>Girls and boys 9 to 26 years old</td>
<td>HPV types 6, 11, 16 and 18</td>
</tr>
<tr>
<td>Gardasil 9</td>
<td>Merck</td>
<td>3 doses: day 1, month 2, and month 6</td>
<td>Girls: 9 to 26 years old Boys: 9 to 15 years old</td>
<td>HPV types 16, 18, 31, 33, 45, 52, 58, 6 and 11</td>
</tr>
</tbody>
</table>

Table 1: Comparison of the 3 prophylactic HPV vaccines: Cervarix, Gardasil and Gardasil 9
1.4 Treatment of HPV+ Cancers

Despite the availability of these preventative vaccines for HPV+ cancers, there is a high morbidity rate related to HPV+ cancers as there are millions of people who were already infected with the virus prior to the vaccine availability or who are unable or unwilling to receive the vaccine [15]. The treatment options for HPV+ cancers generally include surgical interventions, chemotherapy and/or radiotherapy either alone or in combinations, all of which exhibit toxic side effects and quality-of-life issues [16, 17].

The development of a less toxic therapy is a research goal because HPV+ HNSCC primarily affects younger (<55yr) individuals who have lifelong side effects from therapy and poor quality of life [18].

A large-scale drug screen conducted by our collaborator Dr. Faye Johnson’s group revealed that Aurora kinase inhibitors (AKI) specifically cause cell death in HPV+, but not HPV-, cancer cell lines and reduce the size of HPV+ HNSCC patient-derived xenograft (PDX) tumors in vivo in immunodeficient mice [19]. These results were supported by the discovery of Aurora kinase A in a kinase screen of HPV+ cervical cancer cells, and the induction of apoptosis in these cells by the AKI alisertib [20]. In recent joint investigations, we found that drugs that inhibit Aurora kinases, essential cell cycle regulators, specifically led to cell death in HPV+ cancer cell lines but not in HPV- cancer cell lines [19].

1.5 Aurora Kinases
Aurora Kinases are a family of serine/threonine kinases that are known to be involved in cell cycle regulation and are vital players during mitosis. There are three members of the Aurora kinase family referred to as Aurora A, Aurora B, and Aurora C. Functioning and activity of these Aurora kinases are closely regulated, and their disruption/dysregulation can cause genomic instability due to defects caused in the centrosome function, spindle assembly, and chromosomal alignment. Aurora kinase A plays a crucial role in mitotic spindle assembly by facilitating the maturation of centrosomes. Aurora kinase A (AURKA) is a protein that plays a critical role in mitosis, the process of cell division. Specifically, it is involved in regulating the separation and segregation of chromosomes during mitosis. During mitosis, AURKA is activated and accumulates at the centrosomes, which are the microtubule organizing centers in the cell. AURKA then phosphorylates various substrates, including proteins involved in microtubule organization, chromosome alignment, and cytokinesis. This activity helps to ensure proper spindle formation and chromosome segregation during mitosis. In summary, AURKA is an essential regulator of mitotic progression and is critical for proper chromosome segregation during cell division. Inhibition of AURKA has been proposed as a potential strategy for cancer therapy, as cancer cells often have increased AURKA activity and rely heavily on it for their rapid cell division [21] [22, 23] [24].

An N-terminal domain (39–139 aa), a kinase domain (250–300 aa), and a C-terminal domain (15–20 aa) are present in all Aurora kinases. The kinase domain is highly conserved among Aurora kinases, with AurA/B, AurA/C, and AurB/C sharing 71%, 60%, and 75% of homology, respectively [25].
The Aurora kinase B is involved in the regulation of chromatid proteins as well as separation, including cytokinesis [26].

Aurora kinase C (AURKC) is a protein that also plays a critical role in cell division, specifically in meiosis, which is the process of cell division that leads to the formation of gametes (i.e., sperm and egg cells) [27].

In various types of human cancers, the expression, as well as activity of Aurora kinases, are highly upregulated suggesting a role in tumorigenesis and therefore constitute an important target and focus of many novel anti-cancer drug studies [28].

1.6 Aurora Kinases in Cancer

Dysregulation of Aurora Kinases may disrupt cell cycle checkpoint operation as well as spindle assembly, resulting in centrosome amplification and chromosome missegregation, which eventually hampers cell division. These mitotic irregularities contribute to carcinogenesis [29].

Many human cancers, including malignant squamous cervical carcinoma and cervical intraepithelial neoplasm 3 (CIN 3), were shown to have elevated levels of the Aurora A and B kinases [30]. It has also been found that inhibiting Aurora kinase A is synthetically lethal on the background of HPV E7 expression, according to a screen of the human kinome [31]. This result indicates that these kinases might be regarded as novel targets for the development of anticancer treatments.
The goal of this study is to test alisertib, an Aurora kinase inhibitor, particularly to investigate the cellular and immunological effects of inhibiting Aurora A kinase in preclinical models for HPV+ cancers.

1.7 Alisertib

The drug alisertib (MLN-8237) is being developed for the treatment of solid cancers, such as triple negative breast cancer, recurrent or metastatic HNSCC, recurrent and refractory central nervous system atypical teratoid rhabdoid tumors, and stage IV EGFR-mutated non-small cell lung cancer. It is a second-generation, orally accessible, selective small chemical inhibitor of Aurora A kinase with potential anticancer properties. The mitotic spindle apparatus construction, chromosomal segregation, and cell proliferation may all be disrupted by alisertib's binding to and inhibition of Aurora A kinase [33].

Alisertib inhibits Aurora kinase A by binding to the ATP-binding pocket of the kinase domain, which prevents the transfer of phosphate groups to target proteins and thereby disrupts the normal functions of Aurora kinase A in cell division and spindle assembly. Specifically, alisertib binds to the hinge region of the kinase domain, which is adjacent to the ATP-binding pocket, and induces a conformational change that prevents ATP from binding to the pocket. This inhibits the phosphorylation of downstream targets by Aurora kinase A, leading to cell cycle arrest and apoptosis in cancer cells [33].
Alisertib (MLN8237) exhibited potent anti-cancer effects in preclinical trials conducted before clinical testing, thereby encouraging its advancement into clinical trials [33]. A broad range of cell cultures, including those derived from human tongue squamous cell carcinoma [33], malignant bladder cancer [37], and cervical cancer cell lines [38] showed growth inhibition following alisertib treatment.

Our research revealed that by inhibiting Aurora kinase, HPV+ cancer cells underwent apoptosis. Our hypothesis was that this inhibition would result in ICD, DNA damage, and improve the effectiveness of immune checkpoint inhibition *in vivo*. To examine this hypothesis, we conducted
a study using preclinical mouse tumor models of HPV+ cancers (mEER, TC-1, and C3.43) and evaluated the efficacy of alisertib, an inhibitor of Aurora Kinase A.

Therefore, the goal of our lab and my thesis project is to employ preclinical models of HPV cancers for *in vitro* and *in vivo* studies to test the anti-tumor effects of alisertib towards potential application in designing clinically applicable testing strategies.
2. Materials and Methods

2.1 Cell Cultures

We use three HPV+ murine cancer cell lines TC-1, mEER, and C3.43 and three HPV+ human cancer cell lines SiHA, CaSki, and UM-SCC-47.

The TC-1 cells were derived from primary lung epithelial cells of C57BL/6 mice. These cells express the HPV-16 E6 and E7 oncogenes and the H-Ras oncogene [39] [40]. The TC-1 cells were cultured in RPMI supplemented with heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, and gentamycin and incubated at 37°C in 5% CO2.

The mEER cell line is an established preclinical model of HPV-positive oropharyngeal squamous cell carcinoma. The mEER cell line is derived from murine tonsil epithelial cells and expresses the HPV-16 E6 and E7 and HRas oncogenes [40, 41]. This cell line was cultured in DMEM supplemented with Ham’s F12 nutrient mixture, heat inactivated FBS, hydrocortisone, transferrin, insulin, triiodothyronine, epidermal growth factor, cholera toxin, penicillin, streptomycin and incubated at 37°C in 5% CO2.

The C3.43 cell line was established by transfecting the full-length HPV 16 genome as well as activated H-Ras into B6 MEC C57L6 mouse embryo cells. The C3.43 cells were cultured in RPMI supplemented with heat inactivated FBS, penicillin, streptomycin, and gentamycin and incubated at 37°C in 5% CO2.
The UM-SCC-47 cell line is a unique HNSCC that was isolated from the primary tumor of the lateral tongue of a male patient. The UM-SCC-47 cell line contains 18 copies of the integrated HPV-16 [41, 42]. The UM-SCC-47 cells were cultured in RPMI supplemented with heat-inactivated FBS, penicillin, streptomycin, and gentamycin and incubated at 37°C in 5% CO2.

The SiHa cell line is isolated from fragments of a primary uterine tissue sample from a 55-year-old, female, HPV-16+ Japanese patient with squamous cell carcinoma. The cell line expresses the p53 and pRB genes. The SiHa cells were cultured in RPMI supplemented with heat inactivated FBS, penicillin, streptomycin, and gentamycin and incubated at 37°C in 5% CO2.

The CaSki cell line is an epithelial cell line that was established from the cervix of a 40-year-old female. It is an HPV type 16-positive cell line that serves as a model for the study of advanced cervical carcinoma. The CaSki cells were cultured in RPMI supplemented with heat inactivated FBS, penicillin, streptomycin, and gentamycin and incubated at 37°C in 5% CO2.

These HPV+ murine and human cancer cell lines were cultured either in 10cm or 6 well plates with their respective media described above. The confluency for these cells was checked daily and they were passaged every 2 days.

For harvesting the cells, the media was decanted using a pipette and 2 mL of trypsin was added for a 10cm plate or 1 mL/well for a 6-well plate. After adding the trypsin, the cells
were incubated at 37°C in 5% CO2 until the cells were visually seen to be detached from
the surface of the plates/wells. The plates were then washed with media to deactivate the
trypsin and cells were centrifuged at 1,500 rotations per minute (rpm) and 4°C for 5
minutes. After the centrifugation, the supernatant of the cell suspension was decanted, and
the cells in the pellet were resuspended in their respective media. Thereafter, the cells were
plated according to the desired confluency required with their respective media. All HPV+
murine and human cell lines were Mycoplasma-free at the time of testing as determined
using a Mycoplasma Detection Kit. (ATCC Universal Mycoplasma Detection Kit 30-
1012K)

2.2 SDS-PAGE and Western Blotting

For preparing the samples for SDS-PAGE, the cells of interest were lysed using RIPA
lysing buffer that contains protease inhibitor, and phosphatase inhibitor (Thermo Scientific
Halt™ Protease Inhibitor Cocktail, EDTA-free (100X) Catalog number: 78425) to prevent
protein and phosphoprotein degradation.

For preparing samples for conditioned media, the culture medium was centrifuged in
protein concentrators at 1,500 rpm to remove small polypeptides and debris.

After this, a bicinchoninic acid assay (BCA) was carried out to determine the protein
concentration as well as volume to be used in the samples for SDS-PAGE. After the BCA
analysis, the concentration of protein was calculated and the amounts of each sample to be
used along with Laemmli buffer, and β-mercaptoethanol were calculated and prepared. The
samples were then boiled for 5 minutes at 95°C to ensure that proteins denatured to their primary structures. After cooling, samples and a molecular weight size marker ladder mix were each loaded into individual wells of the SDS-PAGE gels in a tris-glycine buffer. The electrophoresis chamber was set to 120 V for 60-70 minutes.

The gel was removed carefully using apparatus and placed in a chilled transfer buffer. A nitrocellulose membrane along with 2 transfer sponges was also soaked in the chilled transfer buffer. For the transfer stack, one transfer sponge was placed on the cassette of the western blot apparatus, on top of this the nitrocellulose membrane, the gel with our proteins, and finally, the other transfer sponge was placed on top. After the assembly of this sandwich, the bubbles were removed using a roller before closing the cassette. The locked cassette was then put into the western blot transfer machine and the process was started.

After the transfer was complete, the membrane was washed with tris-buffered saline tween 20 (TBST). Then, the membrane was incubated in a blocking buffer (5% nonfat dry milk in TBST) for 1 hour (h) on a rocker to prevent antibodies from non-specifically binding to the membrane. The membrane was then washed with TBST 3 times for 5 minutes each, and the primary antibodies were added to the membrane in a blocking buffer.

For the cell lysates, the primary antibodies used were specific to Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198) (Cell Signaling Technology #2914), Total Aurora A (Thermo Fisher Scientific #45-8900), GAPDH (Cell Signaling Technology #97166), Cleaved PARP (Cell Signaling Technology #9541), Cleaved Caspase-3 (Cell Signaling Technology #9661), Actin (Cell Signaling Technology #3700), GSDME (Abcam
#ab215191), H2A.X (Thermo Fisher Scientific #PA5-28778), cGAS (Cell Signaling Technology #31659). Antibodies specific to actin and GAPDH were used as a loading control.

For the conditioned media, or cell supernatant, HMGB1 (Cell Signaling Technology #3935S), Cytochrome c (Cell Signaling Technology #4272) were used as primary antibodies.

The membranes were incubated with their respective primary antibodies for 24 h at 4° C on a gentle rocker. Following incubation, the membranes were washed 3 times for 10 minutes with TBST with gentle rocking to remove excess antibodies, then incubated at room temperature with gentle rocking with the appropriate secondary antibody (anti-rabbit or anti-mouse) in blocking buffer. After one h of incubation, the membranes were washed with TBST 3 times for 10 minutes each to remove unbound secondary antibodies. Throughout this process, the membrane was not allowed to dry in order to reduce the background and improve signal clarity.

The chemiluminescence substrate solution was prepared, and the membrane was incubated with the solution for 5 minutes. Subsequently, the membrane was imaged with a chemiluminescence imaging system. Following imaging, the membranes were washed with and stored in TBS and the procedure was repeated for the remaining primary antibodies.

2.3 Apoptosis Flow Cytometry
One million cells of interest were plated per well into 6 well plates and allowed to adhere. The next day, the cells were treated with 300nM alisertib for 48 h along with vehicle (DMSO) control. Floating, dead cells and trypsinized cells were washed in Corning 1X Phosphate-buffered salt (PBS) solution that did not contain calcium or magnesium and collected in FACS tubes. After being spun in a centrifuge for 5 minutes at 1500rpm, cell pellets were obtained, and the supernatant was decanted out of each sample.

The samples were then placed on ice and the pellets were resuspended in 50uL of 1X Annexin V buffer (BD Biosciences) containing 5 µl of FITC Annexin V (BD Biosciences) and 10 µl 7-AAD. The samples’ tubes were flicked to mix, covered with aluminum foil, and stored in the dark for 30 minutes. Subsequently, the cells were washed with 1X Annexin V binding buffer and then 250uL of 1X Annexin V binding buffer was added and the samples were analyzed for flow cytometry.

The samples were analyzed in a Beckman Coulter Gallios 561 flow cytometer at MD Anderson Cancer Center North Campus Flow Cytometry and Cellular Imaging Facility. Preliminary results were gathered from this core facility and further results were analyzed using FlowJo (v10.8.1).

2.4 CRT Flow Cytometry

1 million cells of interest were plated per well into 6 well plates and allowed to adhere. The next day, the cells were treated with alisertib at a concentration of 300nM for 48 h along with vehicle (DMSO) control. Trypsinized cells were washed in Corning 1X Phosphate-
buffered salt (PBS) solution that did not contain calcium or magnesium and collected in FACS tubes. After being spun in a centrifuge for 5 minutes at 1500rpm, cell pellets were obtained, and the supernatant was decanted out of each sample.

The samples were then placed on ice and the pellets were resuspended in 100 µl of FACS buffer containing 1 µl of CRT antibody (Cell signaling technology #77344) and 0.3 µl of LIVE/DEAD™ Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific; L34966). The samples’ tubes were flicked to mix, covered with aluminum, and stored in the dark for 30 minutes. After thirty minutes, the cells were washed with FACS buffer and then the cells were fixed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) for 30 minutes, then washed with FACS buffer and finally resuspended in 250 µl of FACS buffer and the samples were analyzed for flow cytometry [42].

The samples were analyzed in a Beckman Coulter Gallios 561 flow cytometer at MD Anderson Cancer Center North Campus Flow Cytometry and Cellular Imaging Facility. Preliminary results were gathered from this core facility and further results were analyzed using FlowJo (v10.8.1).

2.5 Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 8 for Windows. Statistical significance was determined using ordinary one-way ANOVA plus multiple comparisons.

2.6 In vivo Tumor Experiments
The *in vivo* tumor experiment involved male C57BL/6J mice, which were implanted with 1 million mEER cells to form flank tumors. A total of 30 mice were used, with 7 mice each in the control and alisertib-alone group, and 8 mice each in the anti-CTLA-4-alone and combination of alisertib and anti-CTLA-4 group. Alisertib (30mg/kg) treatment began on day 2 and was given for 7 days, followed by a 3-day break, and continued in this cycle. Alisertib was dissolved in phosphate-buffered saline (PBS) with 20% β-cyclodextrin (Sigma) and administered orally to mice at a daily dose of 30mg/kg in a final volume of 0.2 ml. For anti-CTLA-4 treatment (InVivoMAb anti-mouse CTLA-4 (CD152) Catalog #BE0131), 200μg antibody in 200μl of PBS was administered intraperitoneally on days 5, 8, and 11, and this cycle was continued for a total of 5 cycles. The mice were monitored for tumor growth and body weight twice a week. The combined treatment of alisertib and anti-CTLA-4 resulted in a significant improvement in survival and a marked reduction in tumor size in mice bearing HPV+ mEER tumors, indicating the potential of this combination therapy in treating such tumors.
3. Results

3.1 Alisertib induces changes in HPV+ cancer cell morphology

We determined the effects Aurora A kinase inhibition in mEER cells by incubating them with 500nM or 1500nM alisertib for 48 h. We observed morphological changes through confocal microscopy in the drug-treated cultures compared to cells incubated with the vehicle control (DMSO). The morphological changes in alisertib-treated cultures included enlarged, rounded, and swollen cells as well as detached floating cells (Fig. 4).

Fig. 4: Alisertib treatment results in morphological changes related to cell death in HPV+ murine tumor cells. Confocal microscopic images in the murine tumor cell line mEER showing morphological features of cell death between vehicle (DMSO) and alisertib (500nM and 1500nM) treatment for 48h. The experiment was conducted three times and yielded consistent results across all experiments.
3.2 Alisertib inhibits the phosphorylation of Aurora kinase A in HPV+ murine tumor cells

To determine whether alisertib effectively targeted the Aurora kinases, we examined by immunoblotting the phosphorylation levels of AURKA, AURKB, and AURKC in TC-1, C3.43, and mEER cells treated \textit{in vitro} with 300nM alisertib for 48 h. We observed that alisertib completely inhibited the expression levels of phosphorylated AURKA in mEER and C3.43 cells, while in TC-1 cells there was only partial inhibition. Similarly, phosphorylation of AURKB and AURKC was reduced with alisertib treatment, when compared to the vehicle control as seen in Fig 5. Even though alisertib is an Aurora kinase A specific inhibitor, there is decrease in the levels of phospho-Aurora B and C, which may be because of the dose of alisertib we used despite being a physiologically relevant dose. It was also observed that the there was a slight increase in the total Aurora A level after alisertib treatment. This is due to the phosphorylated version of Aurora kinases being inhibited by alisertib which in turn accumulates and is observed in the total Aurora A protein levels.
3.3 Alisertib induces apoptosis in HPV+ murine and human cancer cell lines

Flow cytometry analysis was used to examine whether the growth inhibition observed with alisertib using confocal microscopy is due to apoptosis in the three HPV+ murine tumor cell lines, mEER, C3.43, and TC-1 along with two HPV+ human tumor cell lines, SiHa and UMSCC-47. The frequencies of apoptotic cells in the different cell lines were quantified by using an Annexin V 7-AAD kit after treatment with 300nM alisertib for 48h. The frequency of apoptotic cells was significantly enhanced in HPV+ murine cell lines tested other than TC-1 tested (Fig. 7). We also observed a significant percentage of apoptotic cells in the HPV+ human cell lines SiHa and UMSCC-47 treated with alisertib when compared to the vehicle treated cultures, confirming earlier [43, 44]. These results strongly suggest that alisertib treatment results in apoptosis in both the murine and human HPV tumor cell lines.
To further probe the effects of alisertib treatment, we examined the levels of cleaved-PARP (Poly (ADP-ribose) polymerases) and cleaved caspase 3, two of the standard markers of cells undergoing apoptosis. Immunoblotting analyses clearly showed increased levels of cleaved PARP in mEER and C3.43 cells treated with alisertib (300nM for 48h) (Fig. 8). Furthermore, increased levels of cleaved caspase 3 were observed in all three HPV+ murine tumor lines treated with alisertib.

Together, this data clearly showed that AURKA inhibition with alisertib lead to apoptosis in HPV+ murine cancer cell lines as well as HPV+ human cancer cell lines.
Fig. 6: Example of gating strategy used for flow cytometry determination in mEER cells for apoptosis using the Annexin V/7-AAD assay after treating cells with vehicle (DMSO) or alisertib (300nM) for 48 h.
Fig. 7: Aurora kinase inhibition induces apoptosis in HPV-positive mouse and human cancer cell lines in vitro. HPV+ murine (mEER, TC-1, and C3.43) and human (UMSCC-47 and SiHa) cells were treated with 300nM alisertib for 48 h before being subjected to Annexin V 7-AAD staining to measure apoptosis. The experiment was conducted two times with 3 replicates for each group and yielded consistent results across all experiments. Statistical significance was calculated using a one-way ANOVA, p<0.05 (*), p<0.005 (**), p<0.0005 (***).
Fig. 8: Aurora kinase inhibition induces apoptosis in HPV-positive mouse cancer cell lines *in vitro*. Murine cells (mEER, TC-1, and C3.43) were treated with 300nM alisertib for 48 h before being subjected to (A) lysis and immunoblotting with the indicated antibodies to full-length and cleaved PARP, and cleaved caspase 3. The loading control used for the immunoblotting was
actin. Levels of cleaved PARP and cleaved caspase 3 were normalized to actin (bottom panel). The experiment was conducted twice and yielded consistent results across all experiments. Statistical significance was calculated using a one-way ANOVA, $p<0.05$ (*), $p<0.005$ (**), $p<0.0005$ (***)

3.4 Alisertib treatment induces DNA damage and pyroptosis in the HPV+ murine tumor cells

In addition to apoptosis, anti-cancer agents may induce DNA damage and a specific type of cell death called pyroptosis. [44] Among the well-established markers of DNA damage are $\gamma$-H2AX and cytochrome c. [45] Using immunoblotting analyses, we tested for DNA damage after treatment with 300nM alisertib for 48h in the three HPV+ murine cell lines (C3.43, mEER, and TC-1). We observed significantly increased levels of $\gamma$-H2AX in all three HPV+ murine cell lines after alisertib treatment relative to DMSO control. Furthermore, we observed increased levels of cytochrome c in alisertib-treated mEER and C3.43 cells when compared to vehicle control (Fig. 9).

Gasdermin E (GSDME) is a marker for pyroptosis, and cells undergoing pyroptosis exhibit activated and/or cleaved GSDME [46]. Using immunoblotting, we tested for GSDME levels in the three HPV+ murine cancer cell lines after treatment with 300nM alisertib for 48 h. As seen in Fig. 9, cleaved GSDME levels were increased in C3.43 cells after alisertib treatment when compared to the vehicle control. The cleavage of GSDME was not seen in mEER and TC-1 cells, which may be due to the expression of other types of gasdermin (e.g. GSDMA/B/C/D and PJVK) in these cells that are not detected with the antibody used.
Fig. 9: Aurora kinase inhibition induces DNA damage and pyroptosis in HPV-positive murine cancer cell lines. The three murine cell lines (mEER, TC-1, and C3.43) were treated with 300nM alisertib for 48 h before being subjected to lysis and immunoblotting with the indicated antibodies to detect the different markers of DNA damage and pyroptosis and ICD. For the determination of released HMGB1 and cytochrome-C, equal volumes of conditioned media were subjected to western blot analysis; Ponceau S staining was used as a loading control. Levels of γ-H2AX, c-GAS, HMGB-1 and cytochrome-c were normalized to actin and ponceau s respectively as mentioned in the figure (bottom panel). The labels GSDME-FL and GSDME-CL refer to full length and cleaved forms of gasdermin E. The experiment was conducted twice and yielded consistent results across all experiments. Statistical significance was calculated using a one-way ANOVA, p<0.05 (*), p<0.005 (**), p<0.0005 (***)

3.5 Alisertib treatment induces immunogenic cell death in HPV+ murine and human cancer cells

Immunogenic cell death (ICD) is a process in which tumor cells dying from natural or treatment-related events promote innate and adaptive immune responses via the release of soluble factors and/or tumor-associated antigen [47].

First, we assessed the cell surface expression of calreticulin (CRT) (Fig. 10), because CRT exposure is the result of the relocation of ER-resident CRT to the plasma membrane [48]. Surface CRT expressions act as an “eat me” signal from dying cells that in turn triggers antigen-presenting cell (APC)-mediated dead-cell antigen uptake, a crucial event for promoting the adaptive immune system. Therefore, we used flow cytometry to assess the membrane
expression of CRT on the HPV+ murine and human cancer cells. The results in Fig. 11 showed that CRT surface expression was significantly increased after 48h of alisertib treatment when compared to the vehicle control.

In addition to CRT, we also assessed by immunoblotting the supernatants from alisertib-treated HPV+ murine tumor cells for high mobility group box 1 (HMGB1), another well-established marker of ICD [49]. We observed, increased levels of HMGB1 in mEER and C3.43 cells that were treated with alisertib when compared to the vehicle control (Fig. 9).
Fig. 10: Example of gating strategy used for detecting ICD in mEER cells using a CRT antibody after treating cells with vehicle (DMSO) for control and alisertib (300nM) for 48 h. The cell surface calreticulin expression was analyzed by flow cytometry.

Fig. 11: Aurora kinase inhibition induces increased cell surface calreticulin in HPV-positive mouse and human cancer cell lines. The three different HPV+ murine (C3.43, mEER, and TC-1) and two HPV+ human cells (SiHA, CaSki, and UM-47) were treated with 300nM alisertib for
48 h and the cell surface calreticulin expression was analyzed by flow cytometry. Average values from triplicate cultures of alisertib treatment in each cell line were compared to DMSO control treatment. The experiment was conducted two times with 3 replicates for each group and yielded consistent results across all experiments. Statistical significance was calculated using a one-way ANOVA, p<0.05 (*), p<0.005 (**), p<0.0005 (***).

3.6 Combination of CTLA-4 blockade with Aurora kinase inhibition conferred significantly enhanced survival and tumor reduction in HPV+ mEER tumor bearing mice

Based on our data showing apoptosis and ICD in mEER tumor cells after Alisertib treatment, we investigated the in vivo effectiveness of Aurora kinase inhibition alone and in combination with immune checkpoint blockade (ICB) therapy targeting CTLA-4, an inhibitory co-stimulatory marker expressed on T cells (refs) HPV+ mEER tumor-bearing C57BL/6 mice. Separate groups of mice with flank-injected mEER tumors were administered PBS (control), alisertib (30mg/kg), anti-CTLA-4 (200μg/dose/mouse), and a combination of alisertib and anti-CTLA-4. Alisertib was administered via oral gavage, with a schedule of 7 days on and 3 days off, while anti-CTLA-4 (200μg) was delivered intraperitoneal in 200μl of PBS on days 5, 8, 11, and so on. The treatment protocol was conducted for a total of 5 cycles. A total of 30 mice were used, with 7 mice each in the control and alisertib-alone group, and 8 mice each in the anti-CTLA-4-alone and combination of alisertib and anti-CTLA-4 group.

Our findings demonstrated that the combination treatment approach resulted in a significant survival benefit for the mice compared to the control and monotherapy groups. Specifically, we observed (in Fig. 12) a marked reduction in tumor size in the combination group, as well as an
extended survival time for the mice receiving the combination treatment (47 days), compared to the control group (38 days). These results indicate that the combination of alisertib and anti-CTLA-4 may represent a promising therapeutic approach for the treatment of HPV+ mEER tumors in mice.

Fig. 12: Combination treatment with alisertib and anti-CTLA-4 results in improved survival and reduced tumor size in HPV+ mEER tumor-bearing C57BL/6 mice. Separate groups of mice (n=7 to 8) were injected with mEER tumor cells on the right flank and untreated or treated with Alisertib and/or a-CTLA-4 antibody and monitored for survival (A) along with measuring tumor sizes at several intervals (B). The combination group showed a marked increase in survival compared to the control and monotherapy groups (A) and the tumor growth (shown as fold change in tumor volume for each treatment group) (B). The combination group showed a significant reduction in tumor volume compared to the control and monotherapy groups.
Statistical significance was calculated using a one-way ANOVA, $p<0.05$ (*), $p<0.005$ (**), $p<0.0005$ (***).
4. Discussion

Compared to HPV-negative HNSCC, HPV+ HNSCC tends to have a better prognosis and is more responsive to treatment with chemotherapy and radiation. However, despite the favorable prognosis of HPV+ HNSCC, it still presents significant challenges for treatment. For example, patients with HPV+ HNSCC may experience treatment-related toxicities, and some may not respond well to standard treatments. Additionally, some patients may experience disease recurrence after initial treatment, which can be difficult to manage [50].

Therefore, there is a need for targeted therapies that can improve outcomes and reduce treatment-related toxicities for patients with HPV+ HNSCC. Targeted therapies are designed to selectively target cancer cells based on specific molecular or genetic characteristics and may be more effective and less toxic than traditional chemotherapy and radiation.

There has been an elevated interest in discovering new effective drugs for HPV+ cancers. AURKA is a kinase that is expressed in the cell cycles at late S phase, with a peak at the G2/M phase. It is involved in centrosome maturation and separation, bipolar spindle assembly, and mitotic entry. AURKA has become an important aspect when it comes to cancer therapies [51]. The overexpression of AURKA has been seen in human tumor cells and has been shown to be related to tumor proliferation. Research in different cancers has shown that AURKA can induce chemotherapeutic resistance and regulate several key signaling pathways related to the cell cycle, apoptosis, autophagy, and EMT in cancer cells, suggesting AURKA’s role as a central node in cancer cell signaling [52].
Human papillomavirus has been identified as a major risk factor for head and neck squamous cell carcinoma. Studies have shown that HPV+ HNSCC has a better prognosis and is more responsive to chemotherapy and radiation compared to HPV-negative (HPV-) HNSCC. However, despite the favorable prognosis, HPV+ HNSCC still presents significant challenges for treatment [53]. Therefore, there is a need for targeted therapies that can improve outcomes and reduce treatment-related toxicities for patients with HPV+ HNSCC.

Targeted therapies are designed to selectively target cancer cells based on specific molecular or genetic characteristics and may be more effective and less toxic than traditional chemotherapy and radiation. AURKA is a kinase that is expressed in the cell cycle at late S phase, with a peak at the G2/M phase. It is involved in centrosome maturation and separation, bipolar spindle assembly, and mitotic entry. AURKA has become an important aspect when it comes to cancer therapies. The overexpression of AURKA has been seen in human tumor cells and has been shown to be related to tumor proliferation. Research in different cancers has shown that AURKA can induce chemotherapeutic resistance and regulate several key signaling pathways related to the cell cycle, apoptosis, autophagy, and EMT in cancer cells, suggesting AURKA’s role as a central node in cancer cell signaling [54].

To utilize the Aurora A kinase pathway for anticancer therapy, we assessed the effects of the Aurora kinase A inhibitor: alisertib on HPV+ human and murine cell lines. Our findings indicated that alisertib treatment resulted in inhibition of phosphorylated Aurora A kinase in various HPV+ human and murine cancer cell lines.
Subsequently, we investigated the impact of alisertib on apoptosis in HPV+ human and murine cancer cell lines. Apoptosis is identified by distinct morphological and biochemical changes in dying cells. Our observations revealed enlarged, rounded, and swollen cells as well as detached floating cells in both HPV+ murine and human cancer cells treated with alisertib. Prior studies have demonstrated that alisertib induces apoptosis in multiple cancer cell types, such as human epithelial ovarian cancer cells, human hepatoblastoma cells, and leukemic cells [55, 56].

The Annexin V-7-AAD flow cytometry analysis for apoptosis (fig. 7) revealed that alisertib induced apoptotic cell death in mEER and C3.43 (HPV+ murine cancer cells) and in UMSCC-47 and SiHa (HPV+ human cancer cells), but not significantly in TC-1. This lack of response in TC-1 may be attributed to various mechanisms by which cancer cells develop resistance to cancer drugs, such as increased drug efflux. In this process, cancer cells use efflux pumps to expel drugs, decreasing the drug concentration inside the cell and rendering it less effective. Cancer cells can also use ATP-binding cassette (ABC) transporters to develop resistance to cancer drugs. ABC transporters are membrane proteins that pump drugs out of cancer cells, decreasing their concentration inside the cell and making them less effective [57, 58].

Caspase family members execute apoptotic cell death. There are two routes that can activate caspase-mediated execution: the intrinsic pathway, involving mitochondria and cytochrome c, and the extrinsic pathway, involving death receptors. Both pathways are interconnected and can activate caspases 3, 6, and 7 [59].

After mitochondrial rupture, cytochrome c is known to be released and initiate the apoptotic process [60]. In our study, we observed a significant increase in the cytosolic level of cytochrome
c in the conditioned supernatant of mEER, TC-1, and C3.43 after treatment with 300nM alisertib for 48 hours. This increase in cytochrome c subsequently activated caspase 9 and caspase 3, which ultimately induced apoptosis [61]. Our findings suggest that alisertib induces mitochondria-dependent apoptosis in HPV+ murine cancer cells.

Our findings (Fig. 8) indicate that treating HPV+ murine cancer cell lines (mEER, TC-1 and C3.43) with 300nM alisertib for 48 h resulted in a significant increase in cleaved caspase 3 expression when compared to vehicle control. Additionally, we observed elevated levels of PARP cleavage, suggesting the induction of apoptosis. Therefore, our data suggest that alisertib-mediated inhibition of Aurora kinase A induces apoptosis in HPV+ murine cancer cell lines.

Based on our previous findings that alisertib induces apoptosis in HPV+ human and murine cell lines, we further investigated its ability to induce immunogenic cell death in these tumor cells. Immunogenic cell death is characterized by the expression of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT), which can activate immune cells and stimulate an immune response against the tumor [62]. Our results suggest that alisertib treatment increases the expression of DAMPs in these tumor cells, particularly CRT, indicating its potential to promote immunogenic cell death and enhance its antitumor activity.

Our findings indicate that alisertib treatment significantly upregulated CRT expression in HPV+ murine cancer cells compared to the vehicle control. This aligns with recent research that suggests chemotherapeutic drugs can induce the release of ICD markers such as HMGB1 and CRT, which can activate the immune system to attack cancer cells [48]. We observed an increase in both CRT and HMGB1 expression upon treatment, suggesting that alisertib has the potential to induce
immunogenic tumor cell death. Nevertheless, further validation is necessary to determine whether it can activate tumor immune function *in vivo*.

We investigated whether alisertib can induce pyroptosis in HPV+ murine cancer cell lines, as recent research has shown that pyroptosis can play a significant role in the treatment of tumors with chemotherapy drugs [63]. Cells undergoing pyroptosis exhibit membrane perforation on the cell surface and cell swelling, caused by the cleaved gasdermin family members [45]. Our study results showed that alisertib treatment induced gasdermin E cleavage in HPV+ murine cell line C3.43 and resulted in membrane perforation and gasdermin E cleavage in these cells, indicating that alisertib can induce pyroptosis in HPV+ murine cancer cells.

Alisertib's effect on the mitochondrial apoptotic pathway was examined in HPV+ murine cancer cell lines (mEER, TC-1, C3.43). The release of cytochrome c from the mitochondria to the cytosol initiates the caspase-dependent apoptotic cascade. This release is an early event in apoptosis, and cytochrome-c associates with procaspase 9 to activate caspase 3 and trigger apoptosis [64]. These findings align with a study showing that alisertib induces apoptosis in peripheral T-cell lymphomas, highlighting its potential therapeutic value [65].

To test the efficacy of the Aurora kinase A inhibitor (alisertib) against HPV+ cancers, we measured γH2AX, a DNA damage marker. Alisertib treatment significantly increased γH2AX expression in HPV+ murine cancer cells (Fig. 9), indicating DNA damage. Previous studies suggest that DNA damage can trigger pyroptosis and apoptosis in various cancers [66], providing further relevance to our study.
Alisertib induces immunogenic cell death in cancer cells by activating the DNA damage response pathway, which leads to the release of danger signals or damage-associated molecular patterns (DAMPs), including HMGB1 and calreticulin. This triggers an immune response by dendritic cells, which present tumor antigens to T cells, leading to their activation and proliferation [67]. Overall, alisertib-induced ICD may contribute to the therapeutic efficacy of alisertib in cancer treatment, by activating the immune system against the tumor and potentially leading to long-lasting antitumor immunity [68].

CTLA-4 is a receptor on activated T cells that negatively regulates T cell activation and immune tolerance by binding to B7 ligands on APCs. Blocking CTLA-4 can enhance the antitumor immune response by releasing T cell suppression. Combining alisertib with anti-CTLA-4 antibodies can further improve efficacy by enhancing the antitumor immune response and blocking inhibitory signals on T cells. This combination may help overcome limitations imposed by Tregs and MDSCs, leading to long-lasting antitumor effects [69].

In this study, we found that alisertib has anti-cancer effects against HPV+ murine and human cancer cells through several mechanisms, including immunogenic cell death, DNA damage, pyroptosis, and apoptosis. Our findings are consistent with a review of alisertib's potential clinical effects in various types of cancers. These results support the use of preclinical HPV+ murine models for testing the efficacy of alisertib alone or in combination with other treatments, such as checkpoint inhibition, therapeutic vaccines, and adoptive cell therapies. Our study shows that alisertib, an Aurora kinase inhibitor, can effectively target HPV+ cancer cells, and may be used alone or in combination with other treatments. These findings provide a basis for further research
to evaluate the potential of alisertib and related approaches in treating HPV+ cancers more effectively and in a targeted way, potentially reducing mortality and minimizing toxic side effects. In summary, alisertib holds promise as a new and targeted therapeutic drug for improving the outcomes and quality of life of HPV+ cancer patients.
References:


32. *Alisertib by Puma Biotechnology for Recurrent Head And Neck Cancer Squamous Cell Carcinoma: Likelihood of Approval*. February 24, 2023; Available from:


VITA

Pragya Sinha completed her bachelors in 2020 at Shiv Nadar University in India with a major in Biotechnology and a minor in Design. After her bachelor’s degree completion, she enrolled at the UT MD Anderson Cancer Center (MDACC) UTHealth Graduate School of Biomedical Sciences in August 2020 and in October 2020 began as a graduate research assistant at MDACC Department of Thoracic Head and Neck Medical Oncology. At University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, she has served as the treasures for the International Student Association (ISA), and GSBS representative member of the Student Inter Council (SIC) as well as member of the Immunology Media Committee.