

Nicole LeBoeuf, Class of 2006
Department of Medicine, Division of Dermatology

**Structure Based Drug Design: Inhibition of E6 protein in immortalized
HPV cell lines implicated in human epithelial cancers**

Nicole R. LeBoeuf MSIV, Jonathan Cherry PhD, Elliot J. Androphy MD
University of Massachusetts Medical School

Background: Human papillomavirus (HPV) has been implicated in nearly 100% of invasive cervical cancers worldwide. HPV DNA has been identified in 30-70% of sexually active women between the ages of 16 and 25 in the USA. Genital warts affect all races, genders and socioeconomic groups. According to the CDC, there are approximately 6.2 million new cases of genital warts annually. They report that there are approximately 20 million people currently infected with HPV in the USA. Concurrent immunosuppression, particularly with organ transplant or HIV, is associated with an increased occurrence of HPV and HPV-induced malignancy. HPV is a small double stranded DNA virus capable of infecting mucosal and cutaneous epithelial tissues. HPV types 16, 18, 31, 33, and 45 are considered “high-risk” as they are most frequently associated with malignant progression. DNA from the high-risk group has been isolated from over 95% of cervical cancers. Furthermore, HPV 16 DNA is found in approximately 50% of cervical cancers and is the most frequent isolate from head and neck cancers, 25-50% of which are attributable to HPV. The HPV E6 protein promotes viral replication and prevents against apoptosis by forming a ubiquitin ligase in complex with E6 associated protein (E6AP). Together, the ligase binds p53 and targets it for degradation. Normally, p53 up-regulates the expression of pro-apoptotic proteins in response to cellular stress. It is the most commonly disrupted tumor suppressor gene. The E6 protein in each of the high-risk viruses share amino acid sequences and are able to bind p53 and target it for degradation. Therefore, inhibition of E6 function is an ideal target for restoring the tumor suppressor function of p53 and allowing for apoptosis of infected cells. There is currently no specific antiviral treatment for HPV, despite its prevalence. Treatment of genital warts and cervical dysplasia involve destruction and removal of the affected tissue. While much effort has been made to develop vaccines against the E6 and E7 proteins of HPV, these options are likely several years from the market. Furthermore, they are a preventative measure and cannot treat the millions of people already infected. We aim to optimize a structure-based inhibitor of the HPV E6 protein. Given that warts tend to develop slowly and that it generally takes years for dysplasia to progress to malignancy, there is a window of opportunity for the treatment of these HPV-associated illnesses.

Objectives: This project sought to identify a reproducible and accurate method of measuring the cytotoxic effects of pharmacophores created to inhibit the E6 protein of HPV infected cells. We hope to isolate specific structure-based *in vivo* inhibitors of HPV E6 and E7 proteins from a collection of previously derived and isolated pharmacophores, identified via *in vitro* high throughput assays. We further hypothesize that killing cells infected with HPV using these specific inhibitors occurs via a p53 dependent pathway. Therefore “priming” cells with agents

known to induce p53 will allow for decreased dosing of the lead compounds such that IC_{50} values will fall into an acceptable “pre-clinical” range of low μ M or nM concentrations.

Methods: Using HPV16 immortalized SiHa cells containing E6 and E7, HPV18 immortalized HeLa cells, containing E6 and E7, and C33a cells as controls for toxicity, we tested the effect of a series of lead compounds on cell viability. SiHa, HeLa and c33a cell lines were grown in 96 well plates in the presence of varying concentrations of Doxorubicin (1.56nM-100nM), Mitomycin C (31.25nM- 2 μ M) or water. MTT cell viability assays were carried out at 24, 48 and 72 hours to determine the concentration and time point at which approximately 80% of cells continued to proliferate. Medium without phenol red was ultimately used for the MTT assay as several of the drugs tested have their own intrinsic color, which was found to affect absorbance values during analysis. The cell lines were then grown in the presence of both Mitomycin C (125nM) and varying concentrations of lead compounds. Those pharmacophores which showed killing of SiHa or HeLa cells at lower concentrations or earlier time points than c33a cells were repeated at a broader range of concentrations; MTT assay and data point collection was done at 48h, 72h, and 7 days. The medium and drug solution was changed every 48 hours to ensure adequate cellular nutrition.

Results to Date: Approximately 80% of all three cell lines were viable in Mitomycin C at 125nM concentrations until 72hours, when all cell lines showed a drop to approximately 50% viability. Doxorubicin showed less consistent killing at low concentrations, so Mitomycin C was used for presumed synergistic up-regulation of p53. Preliminary assays using both Mitomycin C and pharmacophores (G1-1 through G1-14) showed now improvement in cytotoxicity in the presence of Mitomycin C. The MTT assays were then carried out on cells grown in the presence of pharmacophores verses medium. Lead compounds G1-4, G1-9, G1-13, C-8 and F-10 exhibited greater killing of both HeLa and SiHa cells at 7days, with concentrations between 1 and 10 μ M.

Discussion: This aspect of the study is in its preliminary state. Several factors have affected the *in vivo* analysis of the isolated lead compounds. *In vivo* effectiveness can be assessed by various methods, many of which would provide more specific and objectively analyzed data. However, alternative methods also require that far fewer compounds be assessed at a time. Looking at western blots for p53 protein in the presence of the pharmacophores would provide more information about the cellular pathway in question. Additionally, it would allow us to reassess if Mitomycin C is indeed acting in the way that we presume it to be acting. Coupling the evidence from MTT assay with objective increases in p53 levels would support our hypothesis that the pharmacophores are inducing cellular killing by disrupting the E6 protein. The preliminary data allowed us to determine that the pharmacophores tend to have greater efficacy when the assays are carried out to 7days. It is most accurate and reproducible when performed using phenol red –free medium. Further, the assay is dependant on having very similar cell numbers per well on the 96 well plate. SiHa cells tend to divide more slowly than HeLa cells. Thus, finding the ideal time point and plating cells such that the cell numbers are similar and not overly confluent at that time point is a critical step in preparation. The next

step will likely be the use of this information for repeated analysis of the most promising compounds, in conjunction with western blots for p53.