Western Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

8-19-2015 12:00 AM

High Throughput Screening For Drug Discovery In Head And Neck Squamous Cell Carcinoma

Morgan D. Black, *The University of Western Ontario*

Supervisor: Dr. Anthony Nichols, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology

© Morgan D. Black 2015

Follow this and additional works at: https://ir.lib.uwo.ca/etd

 Part of the Translational Medical Research Commons

Recommended Citation

Black, Morgan D., "High Throughput Screening For Drug Discovery In Head And Neck Squamous Cell Carcinoma" (2015). *Electronic Thesis and Dissertation Repository*. 3154.
https://ir.lib.uwo.ca/etd/3154

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

HIGH THROUGHPUT SCREENING FOR DRUG DISCOVERY IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

(Thesis format: Monograph)

by

Morgan Danielle Black

Graduate Program in Anatomy and Cell Biology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Morgan D. Black, 2015

ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and despite advancements in traditional therapies, the survival rate of ~40% remains unchanged, thus highlighting the need for novel treatments in HNSCC. High throughput robotic screening of a panel of HNSCC cell lines was carried out against 1,505 compounds, where drug activity was measured using metabolic agent alamarBlue and quantified by percent activity. Initial screening found that the majority of active compounds could be grouped based upon their cellular target(s) and/or function including: cell cycle regulation, cytoskeleton disruption, and DNA topoisomerase function. Potency was confirmed with dose response curves for 23 hit compounds and ER27319 maleate and NSC146109 hydrochloride were selected for further investigation. ER27319 maleate was observed to control tumour growth *in vivo*. This systematic high throughput screen of large panels of drugs identified a multitude of potentially effective agents for the treatment of HNSCC.

Keywords: HNSCC, HPV, high throughput, drug screening, targeted therapy, precision medicine, cell lines, spleen tyrosine kinase

CO-AUTHORSHIP STATEMENT

Dr. Alessandro Datti facilitated access to the S.M.A.R.T facility for automated, high throughput drug screening at Mount Sinai Hospital in Toronto, Ontario, Canada. Frederick Vizeacoumar, Thomas Sun, and Jenny Wang all assisted in the use of the high throughput robotic platform at the S.M.A.R.T facility as well as with processing of drug screening data through conversion of raw data to percent activities and B scores for drug activity comparisons.

All *in vivo* xenograft testing was performed under Dr. James Koropatnick's ethics approval and his lab member, Rene Figueredo, established all HNSCC cell line xenografts by performing cell line injections.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. Anthony Nichols, Dr. John Barrett, Giananthony Rizzo, Krupal Patel and Nicole Pinto for their daily guidance and encouragement. In addition, I would like to recognize Frederick Vizeacoumar, Thomas Sun, Jenny Wang and Dr. Alessandro Datti for their assistance with my high throughput drug screening at Mount Sinai Hospital in Toronto, Ontario, Canada. A special thank you to Rene Figueredo for his help in setting up my cell line xenograft models for *in vivo* drug testing. I would also like to thank Drs. James Koropatnick, David Litchfield and Alison Allan for their support as members of my supervisory committee.

Finally, I would like to thank my family for their unrelenting support throughout this entire degree. Thank you.

EPIGRAPH

"*Research is what I'm doing when I don't know what I'm doing.*"

- Wernher von Braun

TABLE OF CONTENTS

ABSTRACT ii

CO-AUTHORSHIP STATEMENT iii

ACKNOWLEDGMENTS iv

EPIGRAPH v

TABLE OF CONTENTS vi

LIST OF TABLES ix

LIST OF FIGURES x

LIST OF APPENDICES xi

LIST OF ABBREVIATIONS, SYMBOLS, NOMENCLATURE xii

1 INTRODUCTION 1

2 LITERATURE REVIEW 3

2.1 Cancer 3

2.2 Head and Neck Squamous Cell Carcinoma 5

2.3 HPV-positive HNSCC 7

2.4 Mutational Landscape of HNSCC 12

2.5 High Throughput Drug Testing in HNSCC 14

2.6 Current Treatment of HNSCC 17

2.7 Targeted Cancer Therapy 18

2.8 Precision Medicine in HNSCC 19

2.9 Project Rationale 20

3 HYPOTHESES AND OBJECTIVES 21

3.1 Hypotheses 21

3.2 Objectives 21

4 MATERIALS AND METHODS 22

4.1 HNSCC Cell Lines 22

4.2 Compound Libraries 22

4.3 Short-tandem Repeat Analysis 24

4.4 HPV Infection Status 24

4.5 High Throughput Drug Screening 25

4.6 AlamarBlue Viability Assay 26

4.7 Dose Response Curves 28

4.8 Western Blot Analysis 29

4.9 Quantitative Reverse Transcription PCR 30

4.10 Cell Line Xenografts 31

5 RESULTS 33

5.1 Large-scale, high throughput drug screening identified highly active agents in HNSCC cell lines 33

5.2 HNSCC cell lines are sensitive to known chemotherapeutics 37

5.3 HNSCC cell lines are sensitive to drugs targeting well-known pathways and targets in cancer 39

5.4 High throughput screening identified highly active agents targeting novel cellular targets in HNSCC 39

5.5 Select HNSCC cell lines were less sensitive to chemotherapeutics 41

5.6 Expanded screening identified compounds with preferential activity in either HPV-positive or HPV-negative HNSCC cell lines 42

5.7 NSC146109 hydrochloride effectively inhibited HNSCC cell line growth *in vitro* ..43

5.8 ER27319 maleate potently suppressed HNSCC cell line proliferation *in vitro* 45

5.9 ER27319 maleate suppressed tumour growth *in vivo* 47

6 DISCUSSION 50

6.1 Summary of Experimental Findings 50

6.2 Implications of Experimental Findings 51

6.2.1 High throughput drug screening identified highly active agents in HNSCC cell lines including known therapeutics and compounds targeting well-known pathways in cancer 51

6.2.2 High throughput screening identified highly active agents targeting novel cellular targets in HNSCC 54

6.2.3 Select HNSCC cell lines were less sensitive to chemotherapeutics 55

6.2.4 Expanded screening identified compounds with preferential activity in HPV-positive or HPV-negative HNSCC cell lines 56

6.2.5 NSC146109 hydrochloride effectively inhibited HNSCC cell line growth *in vitro* 59

6.2.6 ER27319 maleate suppressed HNSCC cell line proliferation *in vitro* and controlled tumour growth *in vivo* 62

6.3 Limitations to the Study 65

6.4 Future Directions 66

7 CONCLUSIONS 68

REFERENCES 69

APPENDICES 77

8 CURRICULUM VITAE 84

LIST OF TABLES

Table 1. Candidate therapeutic targets and driver oncogenic events in HNSCC. 13

Table 2. HNSCC cell lines used in this study. 23

Table 3. RT-PCR primers used in this study. 24

Table 4. Antibodies used in this study. 29

Table 5. Dosing regimens for *in vivo* cell line xenografts. 32

Table 6. Drug hits selected for drug validation studies. 34

Table 7. Hit drug compounds currently used for the treatment of cancer. 38

Table 8. Drug compounds with preferential activity in either HPV-positive (pink) or HPV-negative (white) HNSCC cell lines. 43

Table 9. Mean tumour volumes and standard errors as percent increase from untreated baseline tumour measurements. 48

LIST OF FIGURES

Figure 1. The hallmarks of cancer. 4

Figure 2. Anatomy of the head and neck. 6

Figure 3. Smoking Declines in Canada. 8

Figure 4. Frequency of HPV-positive and HPV-negative HNSCC cases. 9

Figure 5. Overall survival for HPV-positive and HPV-negative HNSCC patients. 11

Figure 6. Compounds with increased activity in HNSCC cell lines. 16

Figure 7. Protocol for high throughput drug screening of HNSCC cell lines. 27

Figure 8. Representative dose response curves from drug screening for hit confirmation. ... 35

Figure 9. Sensitivity of HNSCC cell lines to 23 compounds identified by high throughput drug screening. 36

Figure 10. Hit compounds categorized based upon their cellular target(s). 40

Figure 11. Representative amplification plots of *TP53* and *GAPDH* transcripts..... 44

Figure 12. Effects of ER27319 maleate on phosphorylation of Syk. 46

Figure 13. ER27319 maleate effectively controls tumour growth *in vivo*. 49

LIST OF APPENDICES

Appendix 1. STR profiles of HNSCC cell lines used for screening. 77

Appendix 2. Drug hits identified by high throughput drug screening in HNSCC cell lines. . 78

Appendix 3. Drug hits (in red) from expanded drug panel screening. 80

Appendix 4. Ethics approval for *in vivo* cell line xenograft model. 83

LIST OF ABBREVIATIONS, SYMBOLS, NOMENCLATURE

ALL – acute lymphocytic leukemia

AML – acute myeloid leukemia

ATCC – American Type Culture Collection

CCLE – Cancer Cell Line Encyclopaedia

cDNA – complementary DNA

CML – chronic myelogenous leukemia

CXCR2 – interleukin 8 receptor beta

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen

EAAT2/4 – excitatory amino acid transporters 2, 4

EGFR – epidermal growth factor receptor

FAAH – fatty acid amide hydrolase

FBS – fetal bovine serum

FDA – Food and Drug Administration

HER2 – human epidermal growth factor receptor 2

HNSCC – head and neck squamous cell carcinoma

HPV – human papillomavirus

HRP – horseradish peroxidase

IC_{50} – half maximal inhibitory concentration

IGF-1R – insulin-like growth factor 1 receptor

IMDM – Iscove's Modified Dulbecco's Medium

Mdm2 – E3 ubiquitin-protein ligase

mTOR – mammalian target of Rapamycin

NMDA – N-methyl-D-aspartate receptor

OPC – oropharyngeal cancer

PAR1 – protease-activated receptor

PBS – phosphate-buffered saline

PDX – patient-derived xenograft

PI3K – phosphatidylinositol-3-kinase

PKC – protein kinase C

PPAR – peroxisome proliferator-activated receptor

RARγ – retinoic acid receptor gamma

RIPA – radioimmunoprecipitation assay

RT-PCR – reverse transcription PCR

RTK – receptor tyrosine kinase

sst4 – somatostatin receptor 4

STR – short-tandem repeat

Syk – spleen tyrosine kinase

TCGA – The Cancer Genome Atlas

TRPV1 – transient receptor potential cation channel subfamily V member 1

TSG – tumour suppressor gene

VEGFR – vascular endothelial growth factor receptor

1 INTRODUCTION

Cancer is the leading cause of death worldwide, accounting for approximately 8.2 million deaths in 2012 [1]. According to the World Health Organization's International Agency for Research on Cancer, the number of new cancer cases is expected to rise about 70% over the next two decades. This predicted increase is based on both the growing elderly population worldwide as well as the continued exposure to major risk factors of cancer such as tobacco and alcohol use, unhealthy diet and physical inactivity [1]. Tobacco use continues to be the most important risk factor for cancer and is responsible for about 20% of global cancer deaths annually [1].

Head and neck cancers comprise a group of malignancies that arise in many different areas of the head and neck region including: the oral cavity, larynx, pharynx, paranasal sinuses and nasal cavity as well as the salivary glands. Head and neck cancers are currently the sixth most common cancer by incidence worldwide [2]. Tobacco and alcohol use remain the two most important risk factors for head and neck cancers, however, infection with human papillomavirus (HPV) has recently been recognized as an important risk factor for some head and neck cancers, particularly oropharyngeal cancers (OPC) involving the tonsils and base of tongue [3, 4, 5]. Over the next decade, HPV-positive OPCs are predicted to exceed the annual number of cervical cancer cases in the United States [6]. This rise in OPCs has been observed by many different countries worldwide, and presents a looming health concern for decades to come.

Currently, treatment of head and neck cancer is limited to chemotherapy, radiation therapy and/or surgery. Unfortunately, despite advances in each of these

treatment modalities, there has been no marked improvement in overall patient survival thus highlighting the need for new therapeutic options.

This thesis focuses on identifying highly active agents that can suppress the growth of head and neck cancer cell lines with the goal of identifying novel drug compounds and cellular targets for the improved treatment of head and neck cancers.

2 LITERATURE REVIEW

2.1 Cancer

Cancer is a complex disease in which cells of a specific tissue fail to respond to signals that regulate cellular survival, proliferation, differentiation and death. As a result, these non-responsive cells can accumulate and cause local disease [7]. Cancer cells have been characterized as having six hallmark traits including: resisting cell death, sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality and inducing angiogenesis (Figure 1) [7]. More recently, research has suggested that there are two additional hallmarks of cancer that are emerging, with one involving reprogramming of cellular metabolism to support cancer cell proliferation and the other which enables cells to evade destruction by the immune system. In addition, inflammation and genome instability have been found to be important factors that help enable tumour progression [7].

Cancer has long been understood to be a process of clonal evolution in which rounds of clonal selection of distinct cancer cells within a tumour give rise to extremely heterogeneous tumours [8]. This process of clonal expansion and selection leads to genetic diversification within a tumour. For example, as cancer cells rapidly divide, external pressures such as cytotoxic chemotherapy often select for clonal populations that are highly mutated and/or resistant to treatment thus resulting in treatment-resistant tumour cells with increased mutation rates and heterogeneity [8]. It has been observed

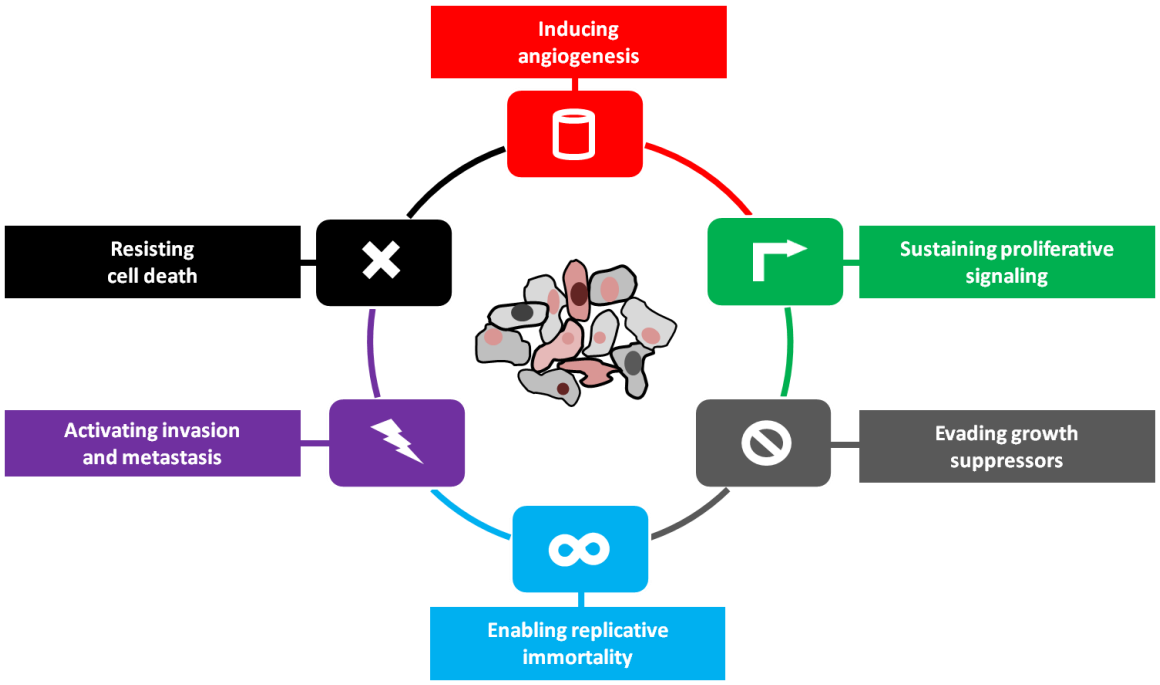


Figure 1. The hallmarks of cancer.

This schematic includes the six hallmark features that enable cancer cell growth and metastases. These hallmarks help provide a solid foundation for our current understanding of basic cancer biology. Extensive research has been performed on each of these six hallmarks to help improve our understanding of these processes as well as the overall development and progression of a tumour. Addtionally, recent research has suggested that two additional cancer hallmarks are emerging with one involving reprogramming of cellular metabolism and the other which enables cells to evade immunological destruction (adapted from Hanahan and Weinberg, 2011).

that there is extensive genetic and phenotypic diversity both between tumours (intertumour heterogeneity) and within an individual tumour (intratumour heterogeneity) [9]. One of the major causes of both intertumour and intratumour heterogeneity in cancer is genomic instability in these uncontrolled cancer cells, which results in increased mutation rates and gene expression [9]. Heterogeneity in cancer has a tremendous effect on treatment decisions and must be considered when attempting to optimize cancer therapy and improve patient outcomes [9, 10].

There are over 200 different types of cancer that have been identified to date [11]. In 2014, over 190,000 new cases of cancer were diagnosed in Canada and of these, approximately 76,000 (40%) resulted in death [12]. In order to help improve these statistics, it is essential to remember that each individual cancer is unique and tumour heterogeneity needs to be taken into consideration for improved cancer screening, diagnosis, and prognosis as well as treatment decisions [8].

2.2 Head and Neck Squamous Cell Carcinoma

Head and neck cancers are the sixth most common cancer by incidence worldwide, affecting approximately 600,000 new individuals each year [2]. Unfortunately, the mortality rate for advanced head and neck cancers remains at approximately 40% with no significant improvement observed in the last few decades [2]. Approximately 90% of head and neck cancers are squamous cell carcinoma, originating in the epithelium of the upper aerodigestive tract [13]. Head and neck squamous cell carcinoma (HNSCC) includes malignancies arising in many different sites

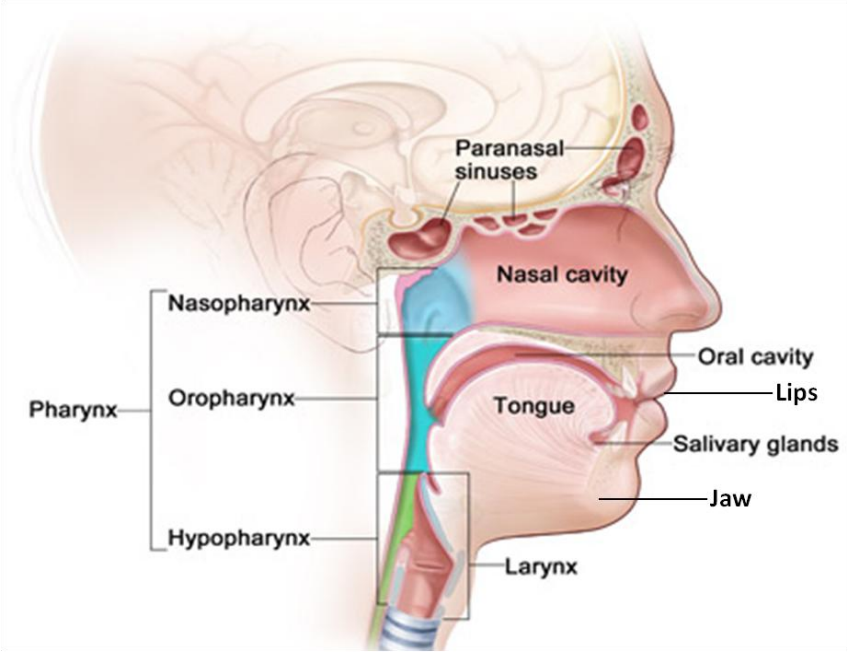


Figure 2. Anatomy of the head and neck.

Head and neck cancers have a wide variety of primary site locations including the nasal and oral cavities, larynx, paranasal sinuses, tongue, epithelium, salivary glands and the pharynx (throat) which consists of three parts: the nasopharynx, oropharynx, and hypopharynx (adapted from http://www.cancer.gov/types/head-and-neck).

within the head and neck region including: the oral cavity, paranasal sinuses, nasal cavity, tongue, lips, tonsils, salivary glands, pharynx, and larynx (Figure 2) [13].

Historically, HNSCC has long been associated with heavy tobacco use and alcohol consumption, typically presenting in elderly males [13]. In Canada, the government has invested millions of dollars into lowering the incidence of smoking over the past few decades. Fortunately, these initiatives have proven successful with the prevalence of smoking decreasing over 30% from 1965 (49.5%) to 2009 (18%) in both male and females ≥15 years of age (Figure 3) [14]. With these declines in smoking, the overall incidence of HNSCC has also been found to be decreasing. However, a subset of HNSCC cases involving the oropharynx has risen dramatically (Figure 4) [5, 15]. This rise in OPC has been shown to be caused by oral infection with HPV [5, 15].

2.3 HPV-positive HNSCC

Despite the overall reduction in HNSCC cases caused by tobacco use and alcohol consumption, primarily attributed to government efforts to decrease smoking, a subset of HNSCC cases involving the oropharynx have been found to be increasing in incidence (Figure 4) [5, 15]. The cause of this increase is due to sexually transmitted oral infection with HPV [15]. Unlike HPV-positive cervical cancers which are typically caused by infection with high-risk HPV types including HPV type 16 and 18; HPV-positive OPC are almost always caused by infection with HPV type 16 (90%+ of cases) [15]. The reasons for this increase in oral HPV infection remain unclear; however, it is

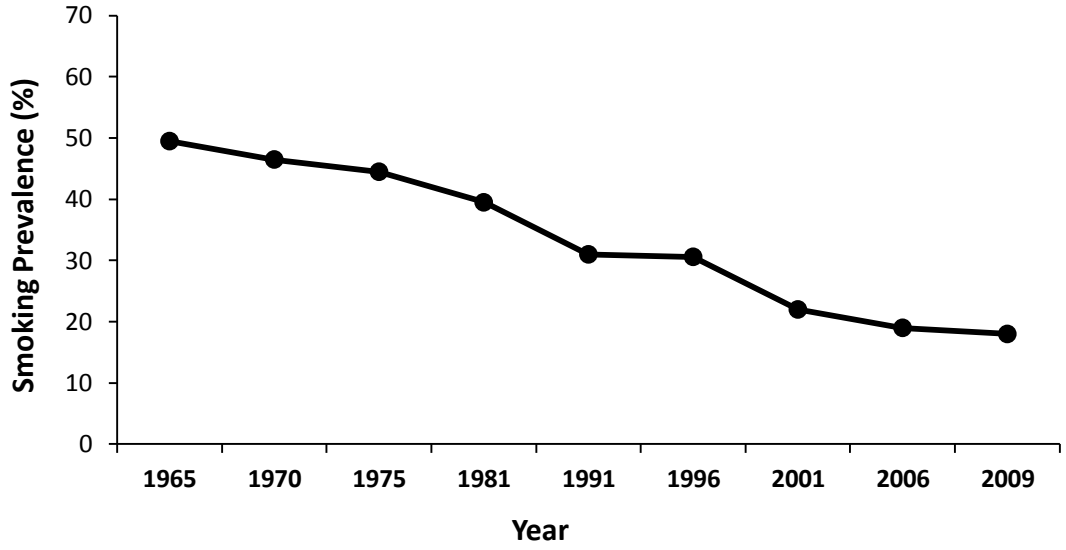


Figure 3. Smoking Declines in Canada.

This graph represents the percentage of Canadians who smoke, aged ≥15 years, on either a daily or occasional basis, based on surveys collected by the federal government from 1965 to 2009 (adapted from Physicians for a smoke-free Canada, 2012).

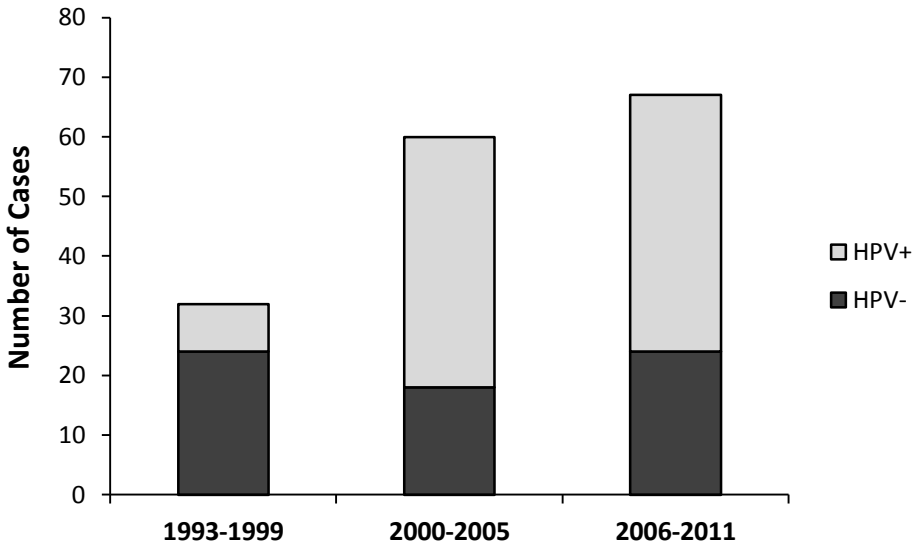


Figure 4. Frequency of HPV-positive and HPV-negative HNSCC cases.

Proportion of tonsillar carcinomas positive and negative for HPV during three sequential time intervals from patient samples obtained at the London Health Science Centre in London, Ontario, Canada, from 1993-2011. A rise in HPV-positive tonsillar HNSCC cases was observed beginning in 2000 (adapted from Nichols *et al*., 2013).

hypothesized that changes in sexual practices, specifically oral-genital contact, may be an important factor [15].

This rise in OPC has been observed in the United States [17], Australia, Japan and many other economically developed countries worldwide [18] and was also recently shown by our laboratory to be occurring in Canada [5]. Approximately 25% of tonsillar cancer cases in 1993 tested positive for HPV in a cohort of patients from Southwestern, Ontario, Canada, as compared to the 62% of cases that were found to be HPV-positive in 2011 (Figure 4) [5].

Demographically, HPV-positive patients have been found to differ quite substantially from the historical HPV-negative HNSCC patient seen in clinics who had a long history of tobacco exposure and alcohol consumption. Patients with HPV-positive tumours tend to be younger, healthier and have no history of smoking [19]. In addition, these patients have a tendency to be more highly educated, affluent, and have had a higher number of lifetime oral sex partners than HPV-negative patients [19].

Despite the increasing number of HPV-positive cases occurring worldwide, it has been observed that patients with HPV-positive HNSCC have a better five-year overall and disease-free survival prognoses than HPV-negative patients (Figure 5) [5]. Since these patients are younger and healthier than patients with HPV-negative HNSCC and are increasing in frequency, it is crucial that emerging treatments for HNSCC have maximum benefits with minimal toxicity since these patients are more likely to be living with the consequences of their treatment for years, or even decades to come.

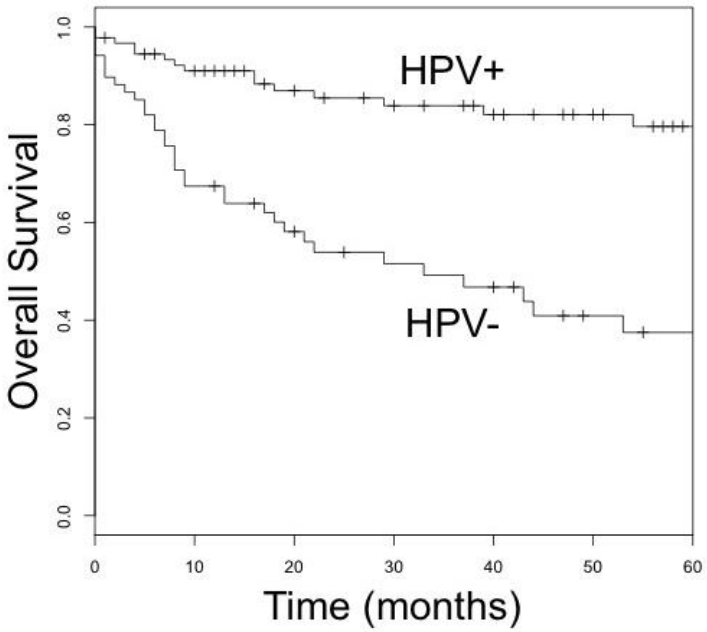


Figure 5. Overall survival for HPV-positive and HPV-negative HNSCC patients.

A survival analysis was performed using the Kaplan–Meier estimate comparing overall and disease-free survivals of 95 patients in Southwestern, Ontario, Canada with either HPV-positive versus HPV-negative oropharyngeal HNSCC tumours. HPV-positive patients were found to have significantly improved five-year overall and disease-free survivals as compared to patients with HPV-negative tumours (Nichols *et al*., 2013).

2.4 Mutational Landscape of HNSCC

In January 2015, the Cancer Genome Atlas (TCGA) Network completed a comprehensive genetic characterization of HNSCC, which included a multi-platform analysis of 279 tumour-normal pairs (HPV-negative n=243, HPV-positive n=36) including exome sequencing, copy number analysis, methylation expression arrays, and RNA sequencing [20]. Genes previously known to be altered in HNSCC were confirmed including: *TP53, CDKN2A, PTEN, PIK3CA*, and *HRAS* (Table 1) [20]. In addition, alterations in genes not previously associated with HNSCC were also identified such as loss of *TRAF3* and amplification of *E2F1* in HPV-positive tumours [20]. Finally, copy number analysis using single-nucleotide polymorphism arrays detected previously known *CCND1* amplifications, *CDKN2A* deletions and more infrequent *MYC, EGFR, ERBB2*, and *CCNE1* amplifications [20].

In addition to identifying genetic alterations that were previously associated with HNSCC as well as novel genetic variations, the mutational landscapes between HPV-positive and HPV-negative HNSCC tumours were found to differ immensely (Table 1) [20, 21, 22]. This finding is consistent with the differing etiologies of HPV-positive and HPV-negative HNSCC tumours. In HPV-negative malignancies, the disease is most often caused by long-term exposure to tobacco as well as heavy alcohol consumption. Such a cancer almost always has a mutated *TP53* gene as well as an extensive number of additional mutated genes including inactivated *CDKN2A* (Table 1) and amplification of 3q26/28 and 11q13/22 [20]. In contrast, HPV-positive HNSCC cases arise from the

Table 1. Candidate therapeutic targets and driver oncogenic events in HNSCC.

		Frequency of alteration events in HPV- (%)	Frequency of alteration events in HPV+ (%)
Receptor tyrosine kinases	*EGFR*	15	6
	FGFR1	10	0
	ERBB2	5	3
	IGF1R	4	0
	EPHA2	4	3
	DDR2	3	6
	FGFR2	2	0
	FGFR3	2	11
	MET	2	0
Oncogenes	*CCND1*	31	3
	MYC	14	3
	HRAS	5	0
PI3K	*PIK3CA*	34	56
	PTEN	12	6
	PIK3R1	1	3
TSG[1]	*TP53*	84	0
	CDKN2A	58	3
	NF1	3	0

[1]TSG, tumour suppressor gene

oncogenic action of viral oncoproteins, E6 and E7, which target essential, wild-type tumour suppressors p53 and pRb, respectively, for degradation [15]. In HPV-positive tumours, an inverse correlation has been observed between positive infection with HPV and the mutation status of *TP53* (Table 1) [20, 21]. As well, HPV-positive tumours were found to be dominated by activating mutations and focal amplifications of *PIK3CA* (56%), amplification of *E2F1* (19%) and loss of *TRAF3* (22%) [20].

Previous genetic characterizations of HNSCC have reported that the overall mutation rate of HPV-positive tumours was approximately half that of HPV-negative HNSCC malignancies (2.28 mutations/Mb and 4.83 mutations/Mb, respectively) [21], however, the most recent and arguably most comprehensive data regarding the genomic landscape of HNSCC concluded that the overall alteration rate did not differ significantly by HPV status in HNSCC tumour samples [20]. Taken together, the differences in the genetic profiles and etiologies between HPV-positive and HPV-negative HNSCC tumours, present the possibility of novel molecular targets and/or pathways being targeted for the treatment of HNSCC (Table 1).

2.5 High Throughput Drug Testing in HNSCC

Cell lines are imperfect models of cancer; however, they have proven to be invaluable tools for cancer discovery. In 2012, two large studies performed by the Broad Institute (identified forthwith as the Cancer Cell Line Encyclopedia [CCLE]) and the Sanger Institute, involved screening a large number of cancer cell lines (947 and 639, respectively) against drug panels and correlating genetic and expression data with drug

response [23, 24]. These studies confirmed known drug correlations with molecular findings, in addition to uncovering novel associations.

Unfortunately, only a small number of drugs were tested in both the CCLE and Sanger studies (24 and 121, respectively) in a relatively small subset of available HNSCC cell lines (31 and 21, respectively) [23, 24]. Using the limited information pertaining to HNSCC, our laboratory interrogated data from Garnett *et al.* (2012), in an attempt to identify drugs with increased efficacy in HNSCC cell lines [23, 25]. Four drugs, Afatinib, Bosutinib, Docetaxel and Gefitinib, were found to have significantly higher activity in HNSCC cell lines versus all other cancer cell lines tested (Figure 6) [25].

Docetaxel, an inhibitor of microtubule formation, is currently in routine clinical use for the treatment of HNSCC. In addition, Afatinib and Gefitinib, were also identified as preferentially active and both target epidermal growth factor receptor (EGFR), which is currently the only approved target for the treatment of head and neck cancers using Cetuximab [25]. The limited number of drug compounds tested in these large cancer cell line screens greatly limits the ability of novel, highly active agents being identified in HNSCC.

In addition, there were no HPV-positive HNSCC cell lines included in either of these studies. The importance of testing HPV-positive cell lines, in addition to HPV-negative HNSCC lines, is absolutely critical given the fact that these cancers are molecularly distinct (Table 1). An expanded, HNSCC-specific study including both HPV-positive and HPV-negative HNSCC cell lines is needed, as well as an *in vivo* validation of the most promising candidates, in order to improve patient outcomes.

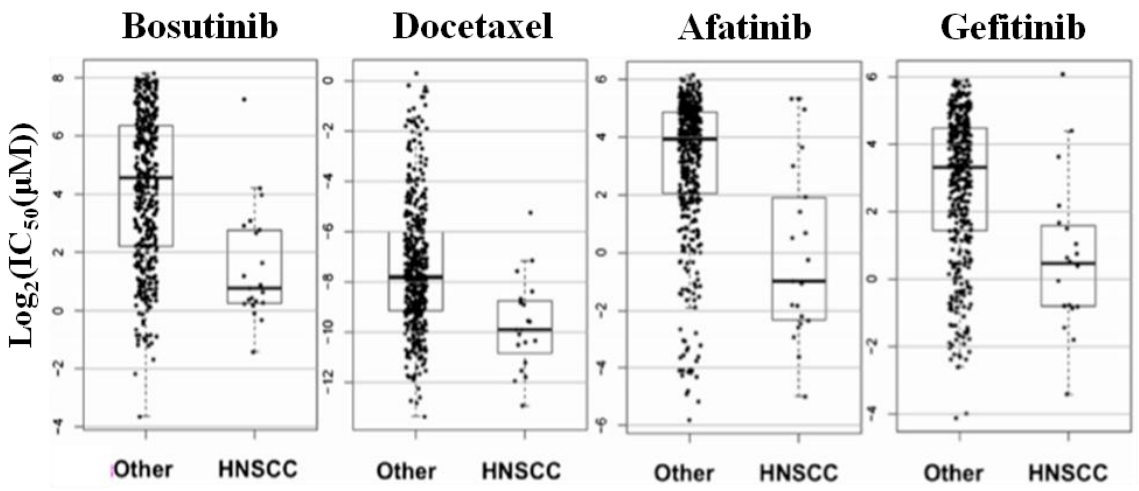


Figure 6. Compounds with increased activity in HNSCC cell lines.

Using the data published by the Sanger group in 2012, Nichols and colleagues (2014) sought to identify drug compounds that demonstrated increased activity (lower IC_{50} values) in HNSCC cell lines as compared to the remainder of the cancer cell line pool. The four drugs listed above were found to be preferentially active in HNSCC cell lines versus all other cancer cell lines screened (adapted from Nichols *et al.*, 2014).

2.6 Current Treatment of HNSCC

As mentioned previously, patients with HPV-positive tumours tend to be younger, healthier and possess a greater chance of surviving their disease (Figure 5) [5, 19]. As such, it is crucial that less harmful therapies are found for these patients, to help improve their overall quality of life. Additionally, HPV-negative patients are still at significant risk of cancer relapse and death (Figure 5) and also are at risk of severe treatment related toxicity thus highlighting the need for improved treatments for both HPV-positive and HPV-negative disease [2, 26, 27].

Contemporary treatments carry significant acute and late toxicities such as: mucositis, pain, vomiting, swallowing difficulty, hearing loss, bone necrosis, stroke, nerve injury and a risk of death of up to 3 % [27, 28]. It is evident that improved methods are needed for treating HNSCC patients including differential and ideally personalized treatment of HPV-positive versus HPV-negative tumours. Currently, only six drugs have been approved by the United States Food and Drug Administration (FDA) for the treatment of head and neck cancers including cytotoxic therapies such as cisplatin, methotrexate, 5-flurouracil, bleomycin, docetaxel as well as a single targeted agent, Cetuximab, which inhibits EGFR [26]. Our laboratory aims to develop a personalized approach to the treatment of HNSCC through high throughput drug testing and correlation of these results to the genomic alterations of the disease.

2.7 Targeted Cancer Therapy

Targeted therapies in cancer are agents that interfere with specific targets (often kinases) and/or pathways within cancer cells as opposed to non-selective traditional therapies, which simply target rapidly dividing cells and are often associated with severe toxicities. Targeted therapies are gaining traction in the development of anticancer drugs and form the basis of precision medicine. Precision medicine is an emerging approach to cancer therapy that involves the use of an individual tumour's genetic profile to specifically tailor and optimize drug treatment through utilization of targeted agents found to target their specific genetic alterations [29]. An advantage of this type of treatment is that the alterations that are suspected to be directly responsible for the growth of a tumour are specifically targeted with an appropriate targeted therapy.

Many targeted therapies have been approved by the FDA for the treatment of various cancers such as imatinib, which targets the constitutively activated BCR-Abl protein in chronic myeloid leukemia (CML), trastuzumab, which blocks the function of the estrogen receptor in breast cancers with HER2 amplifications and vemurafenib, which targets the V600E activating mutation in BRAF for the treatment of melanoma and thyroid cancer (http://www.cancer.gov/about-cancer/treatment/types/targeted-therapies /targeted-therapies-fact-sheet#q4). Many additional compounds are currently under examination in clinical trials and even more are undergoing preclinical testing.

2.8 Precision Medicine in HNSCC

To date, EGFR is the only approved target for the treatment of head and neck cancers [25, 26]. Cetuximab, a monoclonal antibody therapy that inhibits EGFR, is routinely used in the clinic for the treatment of HNSCC patients [26]. In addition to EGFR, many novel targets and pathways are currently under investigation, in both clinical trials and preclinical testing, for the treatment of HNSCC including: vascular endothelial growth factor receptor (VEGFR), phosphatidylinositol-3-kinase (PI3K), Akt, mammalian target of Rapamycin (mTOR), MET, and insulin-like growth factor receptor (IGF-1R), for example [30]. The majority of molecules being investigated in these trials are ones targeting cellular targets with previous associations to cancer with some having been implicated as being important in HNSCC specifically, by recent publications of genomic analyses of HNSCC cell lines and patient tumours.

One such target, PI3K, has been of interest to many researchers since activating mutations and focal amplification of the *PIK3CA* gene, which encodes p110α, the catalytic subunit of PI3K, are prominent in HNSCC, especially in HPV-positive tumours (Table 1) [20]. Interestingly, when attempting to identify drugs with increased efficacy in HNSCC from the large-scale high throughput drug screening performed by Garnett and colleagues (2012), Nichols *et al.* (2014) found that AZD6482, a potent inhibitor of PI3-kinase variant β, had significantly higher activity in *PIK3CA* mutant cancer cell lines as compared to *PIK3CA* wild-type cancer cell lines [23, 25]. This is an excellent example, teased out from a massive drug screening effort, of a targeted therapeutic agent having the potential to be useful against a specific genetic alteration, in this case, mutations and amplifications in the oncogene *PIK3CA*.

2.9 Project Rationale

The prospect of precision medicine in the treatment of HNSCC is looking increasingly more promising as progress is continuously being made in our understanding of HNSCC genomics as well as in molecular pharmacology, thus leading to a rapid expansion of new therapeutics being investigated [31]. Large-scale high throughput drug screens, specific to HNSCC, are necessary in order to identify new agents that exhibit increased activity in HNSCC cells and to identify novel cellular targets and/or pathways that can be interrogated in HNSCC.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

- High throughput drug screening of HPV-positive and HPV-negative HNSCC cell lines will identify drugs that effectively control cell line proliferation.

- HPV-positive and HPV-negative HNSCC are molecularly distinct and will differentially respond to a subset of therapeutic agents identified in a high throughput drug screen.

3.2 Objectives

- To identify drug compounds that are highly active in HNSCC cell lines using a high throughput drug screening platform.

- To confirm potency of top drug hits through generation of dose response curves and determination of IC_{50} values.

- To test a small subset of well characterized HNSCC cell lines against an expanded panel of drug compounds.

- To investigate the mechanism of drug action of two drug hits *in vitro* using HNSCC cell lines.

- To verify the effectiveness of Syk inhibitor, ER27319 maleate, at controlling HNSCC cell line proliferation *in vivo* using a cell line xenograft model.

4 MATERIALS AND METHODS

4.1 HNSCC Cell Lines

Twenty-eight HNSCC cell lines were used in this study, the details of which can be found in Table 2 along with their original source, all available clinical information as well as their appropriate growth media. Each individual cell line was screened on the high throughput platform in Toronto, Ontario at the same passage number for all drug screens including the generation of dose response curves (see Sections 3.5 and 3.7).

4.2 Compound Libraries

A custom-made compound library from Tocris Bioscience comprised of 1,185 compounds and a proprietary kinase inhibitor library of 320 compounds (1,505 compounds total) was used for high throughput drug screening. Two additional libraries, National Institutes of Health Clinical Collection and Prestwick compounds libraries were also used as an expanded drug panel (1,825 compounds total) to screen a subset of 6 well characterized HNSCC cell lines. All four compound libraries included compounds such as anticancer agents and kinase inhibitors that have all been previously tested in humans which helps to eliminate some regulatory hurdles for drug repurposing in HNSCC. The libraries were prepared as 1 mM stock solutions in 100% dimethyl sulfoxide (DMSO) and stored at –20°C in 384-well polypropylene plates (Sigma-Aldrich).

Table 2. HNSCC cell lines used in this study.

Cell Line	HPV Status	Tumour Site (if available)	Patient Information (if available)	Growth Medium	Source
93-VU-147T	Positive	Floor of mouth	Male, T4N2	DMEM/F12	VUMC
HMS001	Positive	Oropharynx (tonsil)	Male	DMEM/F12	Harvard Medical School
UM-SCC47	Positive	Lateral tongue	Male, T3N1M0	DMEM/F12	University of Michigan
UPCI:SCC090	Positive	Oropharynx (tongue base)	Male, T2N0	DMEM/F12	University of Pittsburgh
UPCI:SCC154	Positive	Oral cavity	Male, T4N2	DMEM/F12	University of Pittsburgh
Cal27	Negative	Tongue	Male, 56	DMEM/F12	ATCC
Detroit 562	Negative	Pharynx	Female	DMEM/F12	ATCC
FaDu	Negative	Hypopharynx	Male, 56	DMEM/F12	ATCC
SCC-4	Negative	Tongue	Male, 55	DMEM/F12	ATCC
SCC-9	Negative	Tongue	Male, 25	DMEM/F12	ATCC
SCC-15	Negative	Tongue	Male, 55	DMEM/F12	ATCC
SCC-25	Negative	Hypopharynx	Male, 56	DMEM/F12	ATCC
SCC-61	Negative	------	------	DMEM/F12	Yale
Cal33	Negative	Tongue	Male, 69	DMEM+2mM L-glu	DSMZ
JHU006	Negative	------	------	DMEM/F12	Johns Hopkins
JHU011	Negative	Larynx	Male, T3N0	DMEM/F12	Johns Hopkins
JHU029	Negative	Oropharynx	Male, T4N0	DMEM/F12	Johns Hopkins
PCI6A	Negative	------	------	DMEM/F12	University of Pittsburgh
PCI6B	Negative	Oropharynx	Male, T3N3M0	DMEM/F12	University of Pittsburgh
PCI13	Negative	Oral cavity	Male, T4N1M0	DMEM/F12	University of Pittsburgh
PCI30	Negative	------	------	DMEM/F12	University of Pittsburgh
RF15A	Negative	------	------	DMEM/F12	University of Pittsburgh
RF22A	Negative	------	------	DMEM/F12	University of Pittsburgh
RF22B	Negative	Oral cavity	Male, T4N1M0	DMEM/F12	University of Pittsburgh
RF37A	Negative	------	------	DMEM/F12	University of Pittsburgh
RF37B	Negative	------	------	DMEM/F12	University of Pittsburgh
BICR56	Negative	Tongue	Female	DMEM+2mM L-glu	Public Health England
PE/CA-PJ49	Negative	Tongue	Male, 57	IMDM+2mM L-glu	Public Health England

DMEM, Dulbecco's Modified Eagle Medium; VUMC, VU University Medical Center Amsterdam; ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; IMDM, Iscove's Modified Dulbecco's Medium

4.3 Short-tandem Repeat Analysis

Cell line identity was confirmed through short-tandem repeat (STR) profiling at The Centre of Applied Genomics in Toronto, Ontario, Canada, to ensure that there had been no contamination of the cell lines. All cell lines with published STR profiles were confirmed to be correct (Appendix 1).

4.4 HPV Infection Status

Confirmation of HPV (type 16) infection status in HPV-positive HNSCC cell lines was confirmed by quantitative reverse transcription PCR (RT-PCR) using primers designed to target HPV type 16 and *GAPDH* transcripts (Table 3). HMS-001, UM-SCC47, 93-VU-147T, UPCI:SCC090 and UPCI:SCC154 were all confirmed to be positive for HPV type 16, which is consistent with the literature.

Table 3. RT-PCR primers used in this study.

Target	Oligonucleotide Sequence	Notes
HPV-16	TTGCAGATCATCAAGAACACGTA	HPV forward primer
HPV-16	GTAGAGATCAGTTGTCTCTGG	HPV reverse primer
HPV-16	AATCATGCATGGAGATACACCTACATTGCATGA	HPV probe (JOE)
TP53	CTCAGCATCTTATCCGAGTGGAAG	TP53 forward primer
TP53	TGGTACAGTCAGAGCCAACCTCA	TP53 reverse primer
TP53	CCCTATGAGCCGCCTGA	TP53 probe (FAM)
GAPDH	GCTCATTTGCAGGGGGGAGCC	GAPDH forward primer
GAPDH	CTGATGATCTTGAGGCTGTTG	GAPDH reverse primer
GAPDH	TCTGCCCCCTCTGGTCATGCCCCCATGTTCGTCATGGGA	GADPH probe (Cy5)

4.5 High Throughput Drug Screening

All high throughput screening in this study took place at the S.M.A.R.T Facility in the Samuel Lunenfeld Research Institute at Mount Sinai Hospital in Toronto, Ontario, Canada.

Cells were seeded five days prior to screening. Cells were then transported to Mount Sinai Hospital. Each HNSCC cell line was passaged twice prior to screening in their appropriate growth medium (Table 2) (Wisent Bioproducts, St-Bruno, Quebec) supplemented with 10% fetal bovine serum (FBS) (Wisent Bioproducts), 1% hydrocortisone (Multicell) and 100mg/mL penicillin and 100U of streptomycin (Wisent Bioproducts). Cells were then transported in a T175 flask (Sarstedt, Nümbrecht, Germany) filled completely with appropriate media since the entire cell line panel is adherent. Upon arrival in Toronto, all but approximately 30 mL of media was transferred into a sterile flask and used as cell resuspension media for cell seeding.

The following day, cells were washed with phosphate-buffered saline (PBS) (Wisent Bioproducts) and trypsinized (Wisent Bioproducts), resuspended to a concentration of 12,000 cells/mL and 50μL (600 cells/well) were seeded (Beckman BioMek FX, Mississauga, Ontario) into black 384-well clear-bottom plates (Corning®, Flintshire, United Kingdom). Each 384-well plate also had cells only and media only control wells on the two outer columns of the plate, respectively. Once seeded, cells were incubated at 37°C and 5% carbon dioxide for 24 hours. Following incubation, cells were treated with drug (1 compound/well) using an automated drug pinning device (Beckman Multimek, Mississauga, Ontario). For screening of the compound libraries, all 3,330

compounds screened were tested at a single, final concentration of 4 μM. For the 23 compounds selected for hit confirmation, 10 different drug concentrations were tested ranging from 15 nM to 8 μM. Cells were incubated with the drug compounds for 48 hours at 37°C and 5% carbon dioxide (Figure 7).

4.6 AlamarBlue Viability Assay

AlamarBlue (Life Technologies, Burlington, Ontario) is a metabolic agent that is reduced by increased mitochondrial activity in viable cells [32]. AlamarBlue uses the natural reducing power of live cells to convert its inactive ingredient resazurin (blue, indicating non-viable cells) to its active form, resorufin (red, indicating viable cells), which generates a quantitative measure of cytotoxicity and viability [32]. Following addition of drug on day 1 of high throughput drug screening and the 48 hour incubation period, alamarBlue was added to each well (5%) (Beckman BioMek FX) and allowed to incubate for 4 hours (Figure 7). Fluorescence signals were then measured using a Pherastar microplate reader (BMG Labtech, Ortenburg, Germany) with peak excitation at 570 nm and peak emission at 585 nm. For determination of drug activity, the average of all media only control wells was subtracted from all test wells and the percent of drug activity, whether positive or negative, was then determined by calculating the change in fluorescence values between treated cells and untreated cells.

The top 5% (78/1,505) of compounds found to display the greatest average percent reduction in cellular growth across the entire HNSCC cell line panel were deemed hit compounds (Appendix 2).

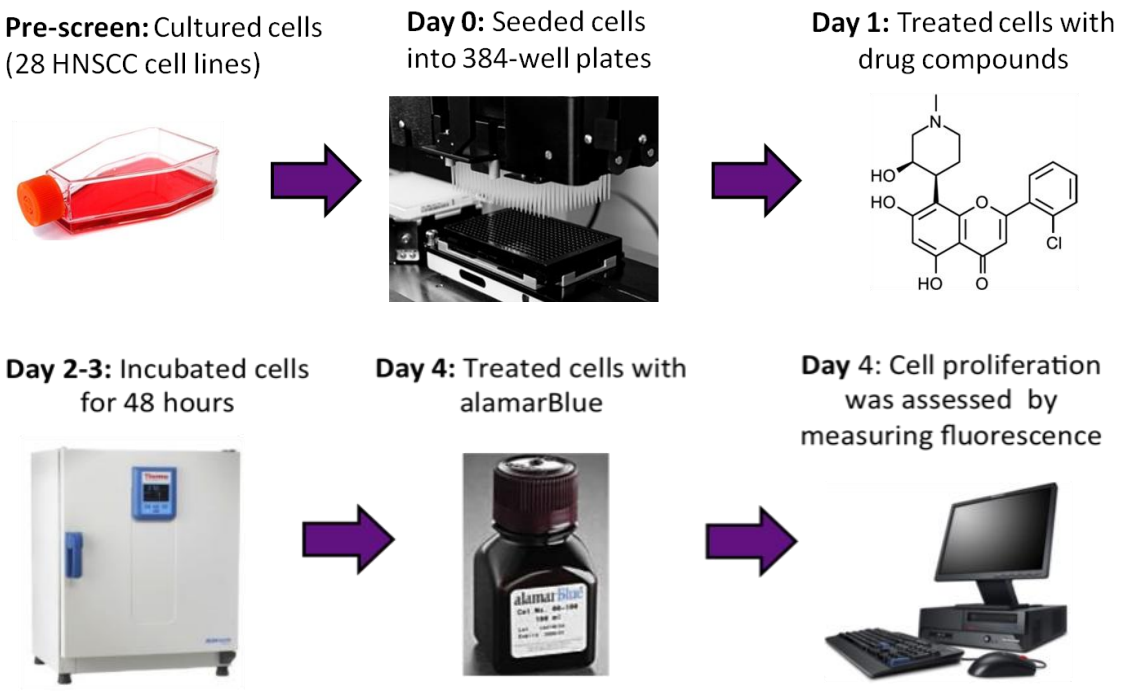


Figure 7. Protocol for high throughput drug screening of HNSCC cell lines.

Cell lines were seeded from frozen stocks and passaged twice prior to screening. Cells were seeded at 12,000 cells/mL in 384-well plates and allowed to incubate for 24h at 37°C and 5% CO_2. On day 1, drug compounds were added to each well at a final concentration of either 4 μM or 10-point concentrations ranging from 15nM to 8 μM and incubated under the same conditions. After 48h, cells were treated with alamarBlue, incubated for 4h at 37°C and 5% CO_2 and fluorescence measurements taken. Untreated cell wells were used as a baseline for cellular proliferation.

4.7 Dose Response Curves

Following the drug screening methodology described above, 23 of the 78 hit compounds (Appendix 2) were selected for drug validation based on three criteria: universal potency (compounds that were broadly effective across majority of cell lines), differential activity (compounds that were extremely potent in some cell lines while completely ineffective in others) and compounds affecting the PI3K/Akt/mTOR pathway.

HNSCC cell lines underwent the identical drug screening methodology as described above (Figure 7), however, on day 1, drugs were added at 10 doses in the range of 15 nM to 8 μM for the 23 drug compounds selected for validation. Cells were then incubated under the same conditions for 48h at which point florescence readings for cell viability were measured and data collected. Cell viability in my assays was measured with alamarBlue (Life Technologies). Absorbance measurements were taken and dose response curves were calculated for these top hits to confirm compound activity *in vitro* in HNSCC cell lines. The IC_{50} (half maximal inhibitory concentration) for each agent was then determined by sigmoidal regression using GraphPad Prism v6.0. In cases where an IC_{50} value could not be definitively extrapolated, the normalized raw data was examined to determine a drug sensitivity dose range. In rare cases, drug sensitivity data could not be converged using non-linear regression so no conclusions on drug sensitivity in these cases could be drawn.

4.8 Western Blot Analysis

Cal27 cells were seeded prior to the experiment in T25 flasks (Sarstedt). Cells were then treated with ER27319 maleate (0.62 μM) for 0.08, 0.16, 0.33, 1, 3, 6, 12, 24, or 48 hours. An additional flask was maintained as an untreated control. Cells were lysed at each time point in lysis buffer containing radioimmunoprecipitation assay (RIPA) buffer and protease inhibitor (1:250). Total protein content was quantified using a protein assay reagent (BioRad) in a Bradford assay. Equal amounts of protein (40 μg) were resolved on a 4-12% gradient SDS-polyacrylamide gel (Life Technologies). The separated proteins were then transferred to a nitrocellulose membrane (GE Healthcare, Toronto, Ontario) and blocked for 1 hour at room temperature with either 5% w/v milk (Bioshop, Burlington, Ontario) or 5% w/v bovine serum albumin (Sigma-Aldrich, Oakville, Ontario), for total and phosphorylated protein, respectively. The membrane was then incubated with primary antibody (Table 4) (1:1000) overnight at 4°C followed by incubation with the secondary, goat anti-rabbit-

Table 4. Antibodies used in this study.

Antibody Label	Molecular Weight (kDa)	Host Species (Isoform)	Commercial Source	Dilution Used
Syk (D3Z1E)	72	Rabbit	Cell Signaling	1:1000
Phospho-Syk (Tyr323)	72	Rabbit (IgG)	Cell Signaling	1:1000
Phospho-Syk (Tyr525/526)	72	Rabbit	Cell Signaling	1:1000
Anti-Rabbit IgG	---	Goat	Cell Signaling	1:5000
α-tubulin	50	Mouse	Sigma-Aldrich	1:2000
Anti-goat	--	Donkey	Jackson Laboratories	1:5000

horseradish peroxidase (HRP) (Cell Signaling, Danvers, Massachusetts) (1:5000) for 1 hour at room temperature. Finally, membranes were developed using a chemiluminescence reagent (Millipore Luminata® Crescendo, Etobicoke, Ontario). To ensure equal loading for each lane, membranes were stripped, re-blocked, probed with α–tubulin (1:2000) and incubated with goat anti-mouse-HRP (1:5000) for 1 hour at room temperature and then developed in the same manner described previously.

4.9 Quantitative Reverse Transcription PCR

Endogenous transcript levels of *TP53* were measured, using quantitative RT-PCR, to verify that cellular transcript levels were increasing upon treatment with NSC146109 hydrochloride. Cal27 cells were seeded prior to the experiment in T25 flasks (Sarstedt). The following day, cells were treated with NSC146109 hydrochloride (0.98 μM) for 0.5, 1, 3, 6, 12, 24, or 48 hours. An additional flask was maintained as an untreated control. Cells were lysed at each time point using QuickExtract RNA extraction kit (Epicentre, Madison, Wisconsin) to obtain RNA. This RNA was for construction of complementary DNA (cDNA) by reverse transcriptase PCR. This cDNA was then used as template in a multiplex quantitative PCR reaction (QuantiTect Multiplex PCR Kit, Qiagen, Toronto, Ontario) using primer and probes designed to target *TP53* (Solaris, GE Dharmacon, Ottawa, Ontario) and *GAPDH* transcripts (Table 2).

4.10 Cell Line Xenografts

Cal33 were seeded from frozen stocks in Dulbecco's Modified Eagle Medium (DMEM) (Multicell) supplemented with heat inactivated FBS (Multicell), 1% hydrocortisone (Multicell) and 100mg/mL penicillin and 100U of streptomycin (Multicell). After initial cell seeding, cells were maintained in complete media without antibiotics. Cells were collected, spun down and resuspended in PBS (Multicell). Eight week old BALB/c athymic mice (Charles River, Sherbrooke, Quebec) were obtained and 1×10^6 cells in 100 uL in PBS were injected into each flank (2 injections per mouse). Mice were monitored until tumours were palpable (8 days) at which time tumours were measured for a tumour baseline evaluation and drug treatment was initiated. There were 10 mice for each of the 3 treatment groups: vehicle control dose, 10 mg/kg dose, and 40 mg/kg (Table 5).

ER27319 maleate was administered at 10 mg/mL and 40 mg/mL doses by oral gavage along with the control group receiving PBS by oral gavage (Table 5). Mice were dosed daily (5 days/week) and tumours measured twice a week with digital calipers (Mitutoyo, Japan). ER27319 maleate treatment continued until day 26, at which time mice were sacrificed by CO_2 asphyxiation. Visible tumours, along with the liver, spleen, heart and lungs were collected and formalin fixed for preservation of tissue for potential investigations in the future.

Table 5. Dosing regimens for *in vivo* cell line xenografts.

Treatment Group	Dose (mg/kg)	# of Mice per group	Treatment Schedule	Termination
Placebo (PBS)	---	10	Daily oral gavage (5 days/week)	All mice were sacrificed at day 26 of ER27319 maleate treatment
ER27319 maleate	10			
ER27319 maleate	40			

5 RESULTS

5.1 Large-scale, high throughput drug screening identified highly active agents in HNSCC cell lines

Of the 1,505 compounds screened, 78 drugs were observed to reduce cellular growth by greater than 20% in at least 25% of HNSCC cell lines screened (top 5% of active compounds screened) (Appendix 2). These compounds were deemed 'hit' compounds and found to be active in HNSCC cell lines.

The list of compounds was then narrowed down 23 hit compounds to move forward for drug validation studies (Table 6), based on three criteria: universal potency (compounds that were extremely potent across majority of the cell line panel), differential activity (compounds that were highly active in some cell lines while completely ineffective in others) or compounds affecting the PI3K/Akt/mTOR signaling pathway (a frequently altered pathway in HNSCC cells) (Table 1). These 23 hit compounds were selected for validation based upon the above criteria as well as their potential as therapeutic agents in other malignancies as described in the literature.

Dose response curves were generated (Figure 8) for each of the 23 compounds in 28 HNSCC cell lines and IC_{50} values were determined (Figure 9). Validation of these 23 drug hits proved successful as the majority of compounds remained highly active (IC_{50} < 2 μM) in the validation step of my high throughput drug screening (Figure 9). Additionally, the trends of universal potency as well as differential activity, two of the criteria used for selection of these compounds for validation, were found to continue upon determination of drug sensitivity in these cell lines (Figure 9).

Table 6. Drug hits selected for drug validation studies.

Selection Criteria	Drug	Target	Pathway/Target Affected	Clinical Trial status (# in HNSCC)[1]
Universal Potency	Pyrrolidinedithiocarbamate ammonium	NF-kB	NF-kB	0
	NSC146109 hydrochloride	p53	p53 levels	0
	Ro 31-8220 mesylate	PKC	Signalling	6
	Ouabain	Na,K-ATPase	Ion channels	3
	BNTX maleate	δ1 opioid receptor	Opiod receptor	0
	SCS	GABAA	NTM Receptor	0
	ER27319 maleate	Syk	Immune signalling	0
	JTC 801	NOP receptor	Opiod receptor	0
	Ryuvidine	CDK4	Cell cycle	0
	BI 78D3	c-JNK	MAPK Signalling	0
Differential Activity	NSC 95397	Cdc25	Cell cycle	0
	SCH 79797 dihydrochloride	PAR1	Platelets; T cells	0
	MK-1775	Wee1	Cell cycle	21(2)
	TCS2312 dihydrochloride	Chk1,2	Cell cycle	3
	Bosutinib	Bcr-Abl, Src	Signalling	35
	AZD4547	FGFR	Signalling	10
	Foretinib	MET, VEGFR2	MAPK Signalling	11(1)
	RDEA119	MEK	MAPK Signalling	3
PI3K-pathway specific	NVP-BEZ235	PI3k/mTOR	PI3K/AKT/mTOR	22
	GDC-0941 bismesylate	PI3K	PI3K/AKT/mTOR	17
	Rapamycin	mTOR	PI3K/AKT/mTOR	1759(41)
	A-443654	AKT	PI3K/AKT/mTOR	0
	BYL719	PI3K	PI3K/AKT/mTOR	30(6)

[1]Refers to the total number of publicly and privately supported clinical studies of human participants that are currently ongoing as of June 20, 2015 with the number in parentheses referring to the number of trials intervening with head and neck cancers (www.clinicaltrials.gov).

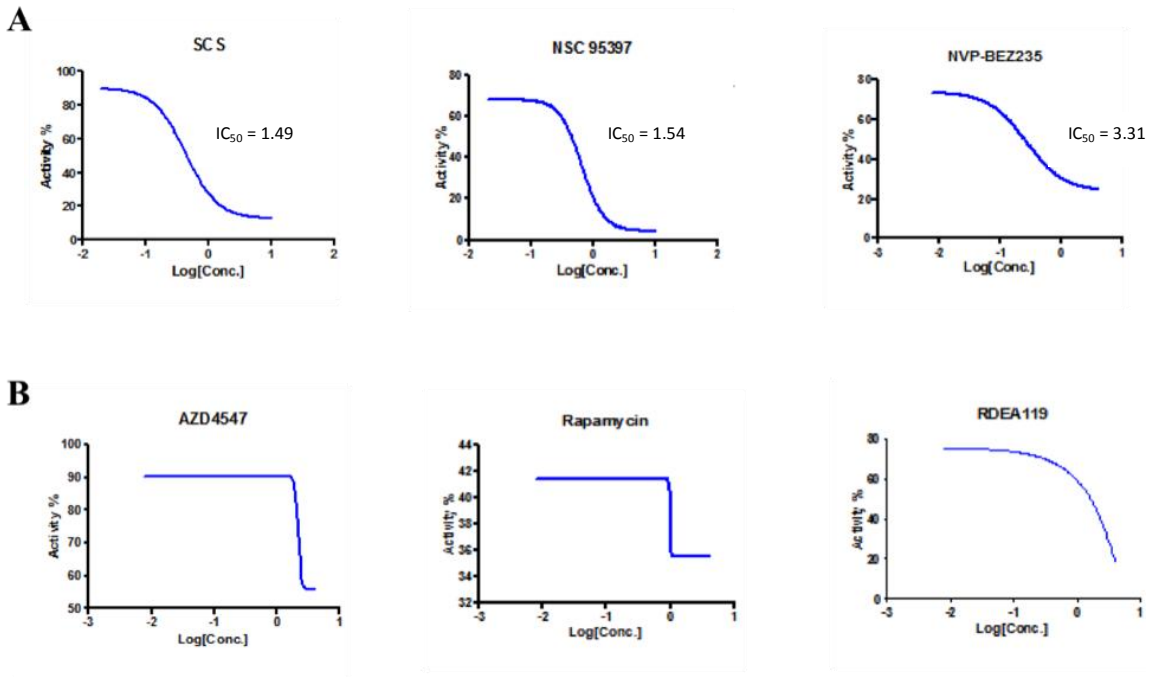


Figure 8. Representative dose response curves from drug screening for hit confirmation.

A. Examples of dose response curves with a sigmoidal decrease in percent activity of cell growth where IC_{50} values could be confidently extrapolated from the modeled data.
B. Examples of dose response curves that were found to be ambiguous meaning that specific IC_{50} values could not be calculated from this modeled data. In these situations, normalized raw data was reviewed and a drug sensitivity concentration range was determined to resolve the sensitivity of a particular cell line. In some cases the raw data simply could not be resolved and the result was 'not converged' where Prism could not converge a line of best fit to the data.

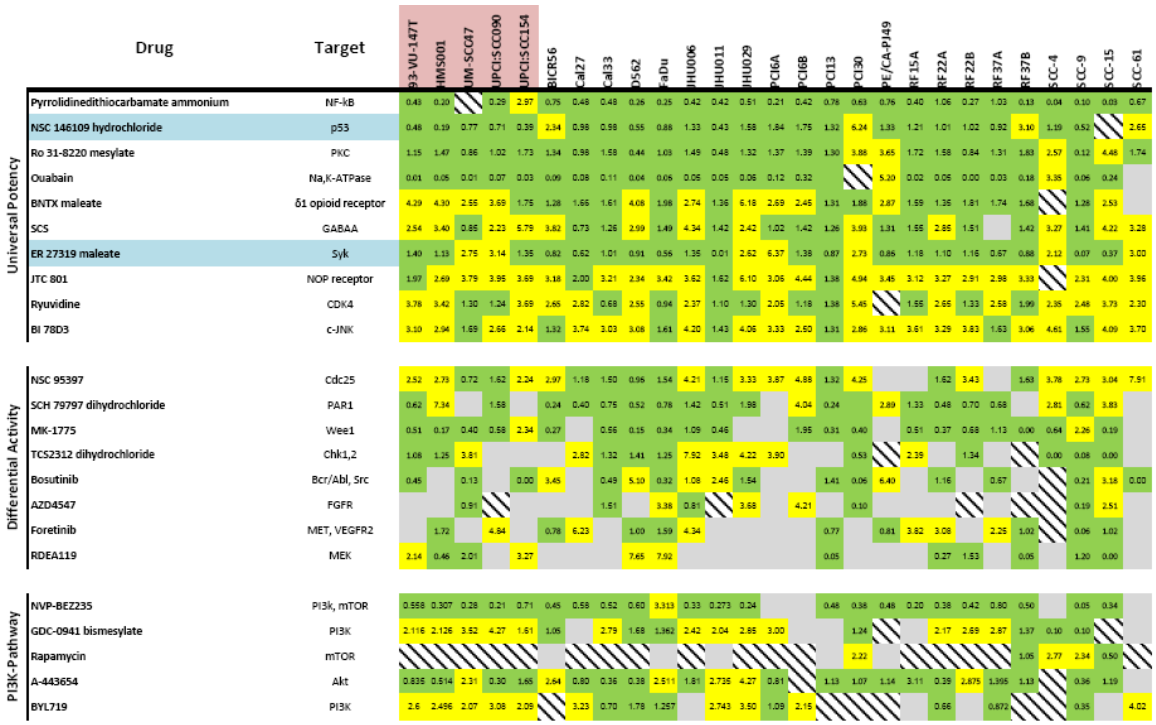


Figure 9. Sensitivity of HNSCC cell lines to 23 compounds identified by high throughput drug screening.

Ten-point dose response curves were generated for 23 drug compounds identified as being highly active in the HNSCC cell line panel where cellular viability was measured using alamarBlue. Criteria for how drugs were selected can be seen along the left. IC_{50} values, in micromolar, for each agent were determined by non-linear regression. HPV-positive HNSCC cell lines are denoted as pink and compounds selected for preclinical development in blue. Green represent exquisite sensitivity (IC_{50} < 2 μM), yellow are moderately sensitive (IC_{50} = 2-7.99 μM) and grey are resistant (IC_{50} > 8 μM, which was the highest concentration tested). Hatched squares represent data that was not converged, which occurs when non-linear regression cannot converge on a line of best fit.

5.2 HNSCC cell lines are sensitive to known chemotherapeutics

A subset of 10 compounds identified in the list of 78 hit compounds were found to be well-established chemotherapeutic agents that are currently, or have been routinely used in the treatment of various cancers, including head and neck cancers (Table 7; Appendix 2). Drugs such as daunorubicin, vincristine sulfate, etoposide, and irinotecan are used for the treatment of different malignancies and were found to be highly active in HNSCC cell lines (http://www.cancer.gov/about-cancer/treatment/drugs). In addition, doxorubicin and paclitaxel were identified as drug hits and were/are clinically used for the treatment of head and neck cancers specifically. These compounds were found to be extremely efficient at controlling HNSCC cell line proliferation *in vitro*, many of which displayed high activity across the majority of the cell line panel.

Although 10 known chemotherapeutic drug compounds were found to be active in the current screen, it was observed that some of these clinically-used compounds were not as effective as many other hit compounds (Appendix 2). For example, the drug Taxol® (paclitaxel, NSC125973) was found to be one of the 78 hit compounds; however, a multitude of other compounds that were screened displayed much higher activity across the panel of HNSCC cell lines (Appendix 2). Additionally, both cisplatin and carboplatin, which are the most frequently used chemotherapeutics in HNSCC, were included in my drug screening at 4 μM but were not identified as hits using my selection criteria.

Table 7. Hit drug compounds currently used for the treatment of cancer.

Drug Name	Cellular Pathway and/or Target	Number of Clinical Trials (head and neck specific)	Treatment Uses in Cancer	Alternate Drug Name(s)
Actinomycin D	RNA polymerase	46	Ewing's sarcoma, testicular, melanoma, soft tissue sarcoma etc	------
Camptothecan	DNA topoisomerase I	1223 (68)	Colon, rectal	Irinotecan
Clofarabine	DNA/RNA synthesis	149	ALL[1]	------
Daunorubicin	DNA/RNA synthesis	248	ALL, AML[2]	------
Doxorubicin	DNA topoisomerase II	1576 (15)	Bladder, breast, head and neck, liver, lungs, ovary, thyroid etc	------
Etoposide	DNA topoisomerase II	1135 (5)	Testicular, bladder, prostate, lung, stomach, uterine	------
Homoharringtonine	Protein synthesis	18	CML[3]	Omacetaxine mepesuccinate
Taxol	Microtubules	2482 (210)	Breast, ovarian, lung, bladder, prostate, head and neck, melanoma etc	Paclitaxel, NSC 125973
Vinblastine sulfate	Microtubules	230 (2)	Hodgkin's disease, non-Hodgkin's lymphoma, testicular, breast, lung, head and neck, bladder etc	------
Vincristine sulfate	Microtubules	861 (1)	Acute leukemia, multiple myeloma, thyroid etc	------

[1]ALL, acute lymphoblastic leukemia; [2]AML, acute myelogenous leukemia; [3]CML, chronic myelogenous leukemia.

5.3 HNSCC cell lines are sensitive to drugs targeting well-known pathways and targets in cancer

The majority of drug hits identified in the high throughput screen could be grouped together based upon the pathway(s) and/or target(s) they are known to affect within the cell (Figure 10). Many of the targets and pathways found to be affected by the top hits were ones that have been previously implicated in cancer and HNSCC in particular. Some of the most prominent targets within the top drug hits were compounds interfering with the function of various receptor tyrosine kinases (RTK) including EGFR and VEGFR. Additionally, compounds affecting cell cycle regulation, cytoskeleton assembly, NF-κβ signaling, and DNA topoisomerase function were all found to be highly active in HNSCC cell lines (Figure 10). As well, drug compounds found to inhibit protein kinase C (PKC) and interfere with protein synthesis were found to be highly active.

5.4 High throughput screening identified highly active agents targeting novel cellular targets in HNSCC

In addition to identifying targets and pathways previously associated with cancer, and HNSCC specifically, several novel targets were also identified in HNSCC cell lines (Appendix 2). Such novel HNSCC targets included: protease-activated receptor (PAR1), transient receptor potential cation channel subfamily V member 1 (TRPV1), somatostatin receptor 4 (sst4), nociceptin receptor, SETD8, $GABA_A$ receptor, interleukin 8 receptor beta (CXCR2), p38α, excitatory amino acid transporter (EAAT2/4), N-methyl-D-

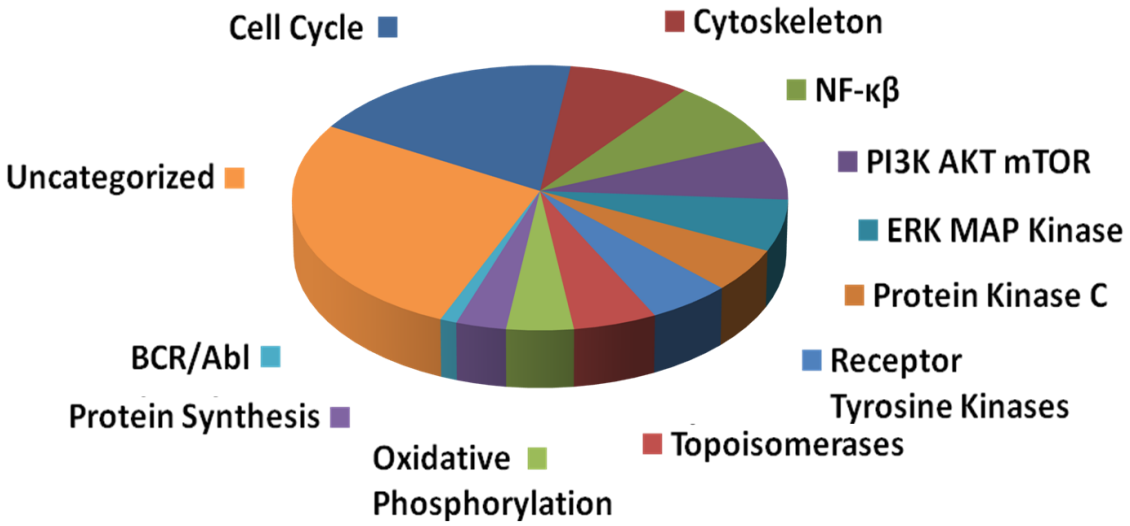


Figure 10. Hit compounds categorized based upon their cellular target(s).

HNSCC cell lines were screened, in duplicate, according to the methodology described in Figure 7. Compounds that were active in at least 25% of the HNSCC cell line panel, in both experimental replicates, were classified as 'hits'. Seventy-eight hits were identified as drug hits (Appendix 2) and could be grouped based on the pathway(s) and/or target(s) they are known to affect within the cell, as shown above.

aspartate receptor (NMDA), fatty acid amide hydrolase (FAAH), retinoic acid receptor gamma (RARγ) as well as compounds that were anthelmintics, dopamine receptor antagonists and inhibitors of DNA methylation (Appendix 2, 3). All of these compounds were found to display activity *in vitro* throughout the HNSCC cell line panel.

5.5 Select HNSCC cell lines were less sensitive to chemotherapeutics

Many of the HNSCC cell lines that were tested displayed patterns of extreme to moderate sensitivity to the top 30 of 78 compounds tested (Appendix 2). In contrast, 3 HNSCC cell lines, SCC-4, SCC-25 and PCI6A, were found to be strikingly less sensitive to many of the top 78 compounds. These cell lines were observed to have similar raw fluorescence intensity values as all other HNSCC cell lines screened when left untreated thus inferring comparable cellular growth. Additionally, these cell lines displayed this insensitivity trend in both experimental replicates. SCC-25 continued to be relatively less sensitive than the remainder of the HNSCC cell line panel when treated with the top 23 drug compounds selected for validation (Figure 9).

5.6 Expanded screening identified compounds with preferential activity in either HPV-positive or HPV-negative HNSCC cell lines

With the knowledge that HPV-positive and HPV-negative HNSCC tumours arise by distinct mechanisms and possess differing genetic landscapes (Table 1), as previously discussed, I expected to uncover compounds that were exclusively active in either HPV-positive or HPV-negative HNSCC cell lines. However, from the preliminary screening and initial hit list, I was unable to identify any compounds that displayed any significant preferential activity in either HPV-positive or HPV-negative cell lines.

To help resolve this, I performed an expanded drug screen of 1,825 additional compounds against a subset of HNSCC cell lines, including both HPV-positive and HPV-negative lines. Hits were classified as compounds that had a B score, which is a variation of a Z score that takes into account positional plate errors, that was 3 standard deviations below the experimental mean of cellular proliferation (Appendix 3). From the hit list that was generated, 5 compounds were found to be preferentially active in HPV-negative cell lines while only one compound, hycanthone, was found to be exclusively active in 2 of the 3 (93-VU-147T and HMS001) HPV-positive cell lines tested (Table 8, Appendix 3). Hycanthone exhibited negligible activity in all three HPV-negative HNSCC cell lines tested. There is some literature investigating this compound, however, it does not appear to have been explored in context of head and neck cancers thus providing a potential avenue for further research in HNSCC.

Table 8. Drug compounds with preferential activity in either HPV-positive (pink) or HPV-negative (white) HNSCC cell lines.

Drug Name	Function/Target	HNSCC cell line specificity
Puromycin dihydrochloride	Antibiotic	HPV-negative
6-azauridine	Anti-viral	HPV-negative
Primaquine diphosphate	Anti-parasitic	HPV-negative
Rosiglitazone maleate	Anti-diabetic	HPV-negative
Letrozole	Aromatase inhibitor	HPV-negative
Hycanthone	DNA/RNA synthesis	HPV-positive

5.7 NSC146109 hydrochloride effectively inhibited HNSCC cell line growth *in vitro*

NSC146109 hydrochloride was identified as highly active across the HNSCC cell line panel with an average reduction in cellular growth of approximately 84% (Appendix 2). Based on its universal potency in the cell line panel, as well as its role in reactivating p53 [33], this compound was selected as one of the 23 compounds to move forward for drug validation studies (Table 6, Appendix 2). Dose response curves were generated and IC_{50} values calculated for all HNSCC cell lines against NSC146109 hydrochloride and not unexpectedly, every cell line in the panel was found to be sensitive; with the majority of cell lines having IC_{50} values below 2 μM (Figure 9).

Quantitative RT-PCR was performed in an attempt to quantify *TP53* levels in both treated and untreated Cal27 cells. Control *GAPDH* transcripts could be efficiently detected using quantitative PCR, which indicates that the RNA extraction and cDNA production protocols were both successful, as seen in Figure 11. Unfortunately, *TP53*

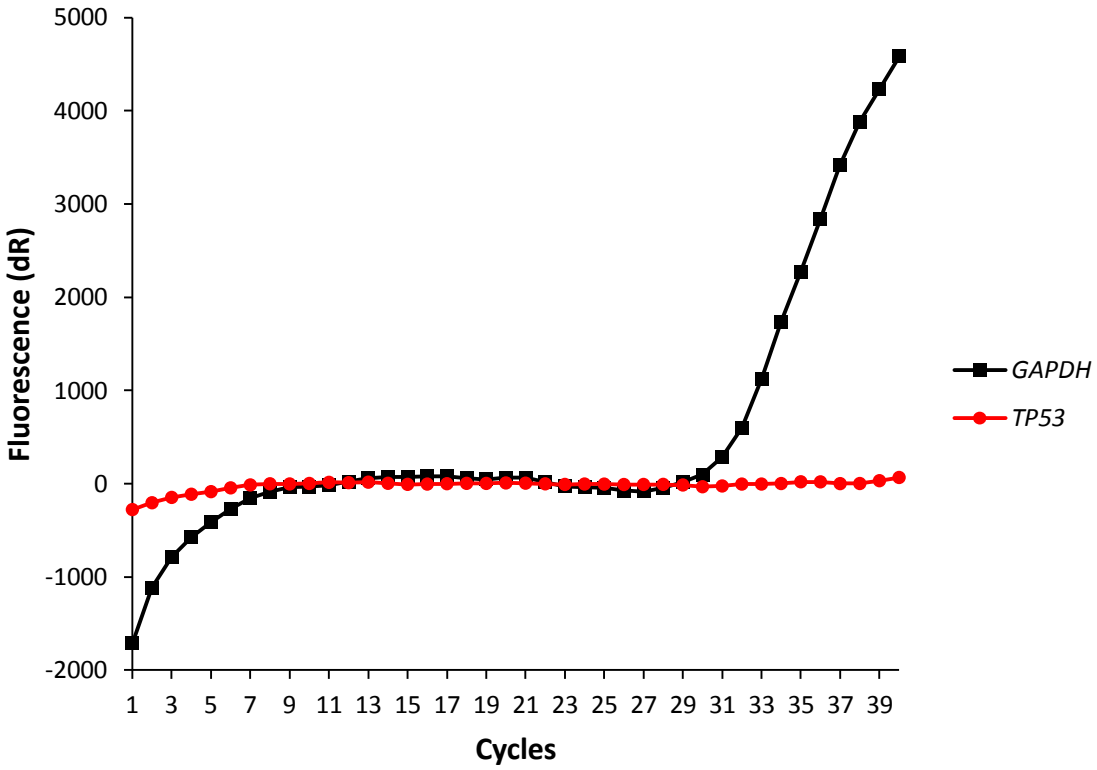


Figure 11. Representative amplification plots of *TP53* and *GAPDH* transcripts.

Cal27 cells were treated with NSC146109 hydrochloride (0.98 μM), cells were lysed and RNA collected at multiple time points. Complementary DNA was then generated by RT-PCR. This cDNA was then used as template in a multiplex quantitative PCR reaction using primer and probes designed to target *TP53* (red) and *GAPDH* (black) transcripts. Endogenous transcript levels of *TP53* were measured in an attempt to verify that cellular transcript levels increased upon treatment with NSC146109 hydrochloride. The above amplification plots are representative of all amplification curves for both *GAPDH* and *TP53* where *GAPDH* could be successfully detected, thus indicating successful RNA extraction, cDNA production and qPCR. Unfortunately, *TP53* transcripts were not efficiently detected in any sample tested.

transcript levels were too low to be detected at all time points in both treated and untreated samples, so no conclusions could be made regarding the effect of NSC146109 hydrochloride treatment on *TP53* transcript levels in Cal27 HNSCC cells *in vitro* (Figure 11).

5.8 ER27319 maleate potently suppressed HNSCC cell line proliferation *in vitro*

ER27319 maleate was identified as a highly potent compound following initial screening of my HNSCC cell line panel. On average, this compound was found to reduce cellular growth by approximately 77% of the HNSCC cell lines screened (Appendix 2). This compound was selected as one of the 23 compounds to move forward for drug validation studies (Table 6) based on the high activity observed at a single dose in preliminary screening (Appendix 2). Dose response curves were generated and IC_{50} values calculated for ER27319 maleate against all HNSCC cell lines. Almost every HNSCC cell line tested was found to be sensitive with the majority of lines having IC_{50} values below 3 μM (Figure 9).

Next, I sought to investigate the cellular mechanism of ER27319 maleate in HNSCC cell lines *in vitro* by examining spleen tyrosine kinase (Syk) phosphorylation in the presence and absence of ER27319 maleate with western blot analysis [34]. Endogenous Syk was detected at all time points in both the presence and absence of drug (Figure 12). Protein loading was observed to be sufficient and uniform based on tubulin

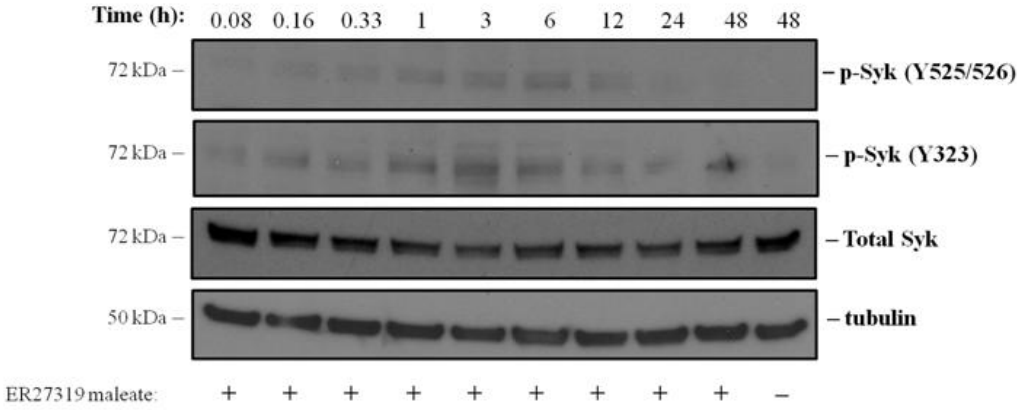


Figure 12. Effects of ER27319 maleate on phosphorylation of Syk.

Cal27 cells were treated with ER27319 maleate at 0.62 μM for 0.08, 0.16, 1, 3, 6, 12, 24, or 48 hours. An additional flask was maintained as an untreated control. Cells were lysed and equal amounts of protein were loaded into each well. Phosphorylated Syk was probed for using two antibodies; however, phosphorylated Syk in untreated samples was not detectable so no conclusions could be confidently drawn. Levels of endogenous Syk were also examined and tubulin was used as a loading control.

detection; however, I was unable to adequately visualize many of the potential phosphorylated Syk proteins. Although it initially appeared as though addition of ER27319 maleate was inhibiting Syk phosphorylation at both early and late time points (0.08, 24, 48 hours at the Y525/526 phosphorylation sites); untreated phospho-Syk was not detected, so no conclusions regarding the effect of ER27319 maleate on Syk phosphorylation could be definitively drawn (Figure 12).

5.9 ER27319 maleate suppressed tumour growth *in vivo*

Using Cal33 cell line xenografts, ER27319 maleate was orally administered at 10 mg/kg and 40 mg/kg doses. Mice were dosed daily for 26 days with tumours measured twice weekly (Table 5). ER27319 maleate was found to be extremely well tolerated, at both doses, by all mice tested. For all tumour measurements taken (days 5-26), paired Student's t-tests were performed comparing the average percent increase in tumour volume (mm^3) for both doses of ER27319 maleate-treated tumours to untreated control tumours (Table 9). Mice treated with ER27319 maleate at 40 mg/kg were found to have significantly decreased percent increase in tumour growth as compared to control tumours for every tumour measurement taken (Figure 13).

Additionally, mice treated with low dose ER27319 maleate (10 mg/kg) were observed to have significantly decreased tumour growth as compared to untreated mice at days 5, 8, 19, and 22. This inhibition in tumour growth provides excellent support for my *in vitro* drug screening and serves as a starting point where I plan to test higher doses of ER27319 maleate to see if tumour growth can be further decreased or even halted

completely. Preliminary investigation into ER27319 maleate at a dose of 70 mg/kg (n=5) found that the dose was tolerated well over 6 days of treatment.

Table 9. Mean tumour volumes and standard errors as percent increase from untreated baseline tumour measurements.

Day of ER27319 maleate Measurement	Control (% increase)	10 mg/kg (% increase)	40 mg/kg (% increase)
5	40.8 ± 12.6	-3.6 ± 7.3*	-21.2 ± 8.6***
8	67.2 ± 14.0	20.3 ± 10.0**	-0.7 ± 13.7**
12	121.7 ± 25.1	63.1 ± 15.6	10.7 ± 15.2**
15	180.0 ± 31.3	109.1 ± 16.4	30.6 ± 17.1***
19	225.6 ± 36.8	128.2 ± 21.0*	59.2 ± 21.0***
22	296.0 ± 44.9	164.8 ± 29.7*	76.9 ± 28.0***
26	341.0 ± 52.5	237.9 ± 38.1	125.0 ± 39.0**

* for $p<0.05$; ** for $p<0.01$; *** for $p<0.001$ as compared to untreated tumour growth

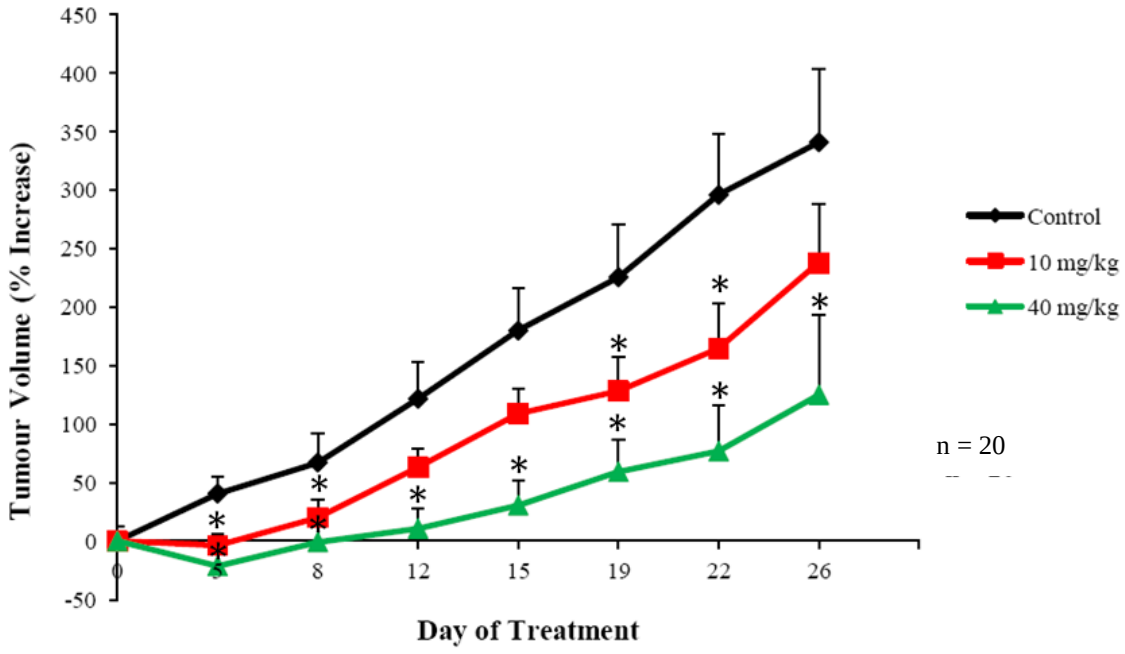


Figure 13. ER27319 maleate effectively controls tumour growth *in vivo*.

Eight-week old athymic mice were injected with Cal33 cells (1×10^6) on each flank (2 tumours/mouse). Once tumours were palpable, ER27319 maleate was administered orally at 10 mg/kg and 40 mg/kg doses daily. Tumours were measured twice weekly and tumour growth was normalized for each individual tumour to its original untreated tumour measurement for determination of the percent increase for each individual tumour. The average percent increase of tumour volume for all 20 tumours per treatment group was calculated and plotted along with the standard error. Asterisks indicate a statistically significant difference from untreated control tumour growth ($p < 0.05$).

6 DISCUSSION

HNSCC affects approximately 600,000 new individuals each year, and includes malignancies arising in the oral cavity, nasal cavity, lip, paranasal sinuses, salivary glands, pharynx, and larynx [2]. Currently, treatment is limited to chemotherapy, radiation therapy and/or surgery. High throughput drug screening has the potential to identify highly active agents for the treatment of HNSCC, a malignancy that has not seen improvement in its survival rates in decades, and is in desperate need of novel therapeutic options.

In an effort to identify novel targets and pathways implicated in HNSCC, I performed high throughput drug screening on a panel of HNSCC cell lines against 1,505 FDA-approved drug compounds. In addition, I screened a subset of HNSCC cell lines on an expanded drug panel including an additional 1,825 compounds in an effort to identify compounds with preferential activity in either HPV-positive or HPV-negative HNSCC cell lines. Following my preliminary screen and identification of hit compounds, I then selected 23 compounds to be confirmed as hits through generation of dose response curves. Finally, two compounds, ER27319 maleate and NSC146109 hydrochloride were selected as lead drug compounds to move forward into preclinical development.

6.1 Summary of Experimental Findings

- Large-scale, high throughput drug screening identified highly active agents in HNSCC cell lines.

- HNSCC cell lines are sensitive to known chemotherapeutic agents.
- HNSCC cell lines are sensitive to drugs targeting known pathways and targets in cancer.
- High throughput screening identified highly active agents targeting novel cellular targets in HNSCC.
- Select HNSCC cell lines were less sensitive to chemotherapeutics.
- Expanded screening identified compounds with preferential activity in either HPV-positive or HPV-negative HNSCC cell lines
- NSC146109 hydrochloride effectively inhibited HNSCC cell line growth *in vitro*.
- Syk inhibitor, ER27319 maleate, potently suppressed HNSCC cell line proliferation *in vitro* and *in vivo*.

6.2 Implications of Experimental Findings

6.2.1 High throughput drug screening identified highly active agents in HNSCC cell lines including known therapeutics and compounds targeting well-known pathways in cancer

Preliminary drug screening at a single concentration resulted in the identification of 78 hit compounds that demonstrated activity against HNSCC cell lines *in vitro*. Many of these highly active compounds affected cellular pathways and targets previously implicated in cancer and even HNSCC specifically. Chemotherapeutic drugs such as daunorubicin, doxorubicin, Actinomycin D, Etoposide and Taxol were found to be active against HNSCC cell lines *in vitro* and have all been extensively explored and approved

for the treatment of a variety of malignancies (Table 7). Having such chemotherapeutic agents appear within the top drug hits helps to provide internal positive controls for my high throughput screening experiments since drug compounds known and confirmed to be effective at inhibiting cancer cell growth, are proving to be highly potent on this high throughput platform.

Interestingly, although screening identified known chemotherapeutics among the hit compounds, some of these clinically established drugs were found to be relatively less potent that many other hit compounds (Appendix 2). For example, Taxol® (Paclitaxel, NSC125973) is a compound commonly used for the treatment of breast, ovarian, and non-small cell lung cancers as well as head and neck cancers (http://www.cancer.gov/about-cancer/treatment/drugs/paclitaxel). This compound has been in clinical use for over 30 years and there are currently 2,482 clinical trials ongoing, with 210 trials for its use in the treatment of head and neck cancers (https://clinicaltrials.gov/ct2/results?term= Paclitaxel&Search=Search). Despite Taxol® being identified as a hit compound; there were many additional compounds that appeared as hits in the screen that had higher activity across the HNSCC cell line panel. In addition, cisplatin and carboplatin, which are standard of care chemotherapeutics for HNSCC, were included in my high throughput drug screening. However, neither drug was found to be a hit when screened at a single dose of 4 μM. Since these drug compounds are routinely used as the workhorses for the treatment of head and neck cancers, the most likely explanation is that these drugs were not at optimal concentrations to effectively control HNSCC cell line proliferation.

Although this study was limited to *in vitro* experiments at a single drug concentration for the characterization of drug potency of these established chemotherapeutics in HNSCC cell lines, these results suggest that there are potentially a multitude of currently uncharacterized compounds in HNSCC that may be significantly more effective than current chemotherapeutics at controlling HNSCC growth and warrant further investigation by our laboratory.

Next, I wanted to confirm the activity of a subset of drug compounds through generation of dose response curves to characterize the sensitivity of each HNSCC cell lines to the top 23 drug compounds selected for hit confirmation. Compounds selected for hit confirmation were selected based on three criteria: universal potency, differential activity and PI3K-pathway specific, and IC_{50} values were calculated. IC_{50} values provide a valuable quantitative measure of the effectiveness of a drug compound at inhibiting a specific biological or biochemical function. Overall, the methodology of high throughput drug screening for drug hit identification and validation of top drug hits used in this study proved successful as majority of the top compounds were confirmed as being moderately (IC_{50} = 2 – 7.99 μM) to exquisitely sensitive (IC50 < 2 μM) across the entire HNSCC cell line panel (Figure 9). Confirmation of drug activity is essential for determination of the concentration of drug required for efficient control of HNSCC cell line proliferation *in vitro* as well as to provide sufficient preclinical data prior to testing drug activity *in vivo* using mouse xenografts.

6.2.2 High throughput screening identified highly active agents targeting novel cellular targets in HNSCC

In addition to identifying known chemotherapeutics, my large-scale screening identified many highly active compounds targeting novel cellular targets within the cell. The automated nature of the high throughput screen enabled me to screen over 3,300 compounds in total, which facilitated the identification of many novel targets that may play a significant role in HNSCC including PAR1, CXCR2, p38α, EAAT2/4 and RARγ, and others (Appendix 2, 3). Some of these targets have been previously explored in the context of other diseases or even other malignancies, however, little to no evidence of their role in HNSCC could be found in the literature. Further investigation into these compounds and novel cellular targets is required to evaluate their efficacy and confirm their activity in HNSCC.

6.2.2.1 Anthelmintics

Anthelmintics are a class of drugs that function by removing internal parasites and/or parasitic worms from the body without causing any significant damage to the host organism [35]. Many populations in developing nations suffer from such helminth infections as well as their co-morbidities such as malnutrition, reduced immunity and anemia [35]. Anthelmintic drugs have been observed to have impressive infection cure rates anywhere from 76-98%, with most of these drugs having broad-spectrum anthelmintic effects [35]. These drugs function with extremely high efficacy while being relatively inexpensive in terms of drug cost [35]. Two main types of anthelmintics were

identified as hits in my high throughput screening: benzimidazoles (including albendazole, flubendazole, mebendazole and fenbendazole) and niclosamide (Appendix 3).

In recent years, the repurposing of drugs for alternate uses than what was originally intended has become increasingly popular as investigators seek new treatment options for different human diseases. Interestingly, investigations into anthelmintics for the treatment of various cancers have proven highly promising. Flubendazole was observed to effectively enhance cytotoxicity in breast cancer cells [36], mebendazole was identified as a hit in a high throughput screen looking for compounds effective at controlling colon cancer cell line growth [37] and niclosamide has been found to effectively inhibit proliferation of human osteosarcoma cells as well as enzalutamide-resistant prostate cancer cells [38, 39]. Additionally, anti-protozoals, which include anthelmintic compounds, have been found to efficaciously kill cancer subtypes enriched for stem-like properties [40]. These recent findings along with the results from my expanded screening all support old/existing microbicides being further explored for the potential repurposing as new cancer therapeutics to eradicate HNSCC cancer cells.

6.2.3 Select HNSCC cell lines were less sensitive to chemotherapeutics

Following analysis of my preliminary drug screens, I observed that the HNSCC cell lines SCC-4, PCI6A and SCC-25 all appeared to be less sensitive overall to the majority of compounds included in my screens, with SCC-25 remaining relatively insensitive even with my confirmatory dose response curves (Appendix 2, Figure 9).

Review of the raw fluorescence data obtained for each of these cell lines found that they all had similar readings to the other HNSCC cell lines tested thus indicating comparable levels of cellular viability across all samples tested.

One possible explanation for this trend is that SCC-4 and SCC-25 were observed to have increased doubling times than most of the cell lines screened, which could account for certain compounds not appearing to be as effective while appearing highly potent in the remainder of HNSCC cell lines tested. An increased doubling time would infer that these cells are not going through as many rounds of the cell cycle, so compounds affecting regulation of the cell cycle or cytoskeleton formation during cellular division, which many of the top compounds do, may be less effective in such cell lines. Other explanations for this insensitivity could be that the cellular health of these specific HNSCC cell lines was poor and/or that these specific cell lines were contaminated, however, cell line identity was confirmed with STR profiling prior to screening and any bacterial contamination should have been obvious when screening as addition of alamarBlue would result in an extremely positive outlier for metabolic activity.

6.2.4 Expanded screening identified compounds with preferential activity in HPV-positive or HPV-negative HNSCC cell lines

Based on what was previously known about HNSCC including the differing etiologies of tobacco exposure and infection with HPV [15], I hypothesized that because HPV-positive and HPV-negative HNSCC cell lines and xenografts are molecularly distinct, they would respond differentially to a subset of therapeutic agents identified in a

high throughput drug screen. Initially, I was forced to reject my null hypothesis since no hit compounds from my preliminary screening of 1,505 compounds, were found to be preferentially active in either HPV-positive or HPV-negative HNSCC cell lines. However, when I subsequently performed a drug screen using an expanded panel of 1,825 drug compounds on a subset of HNSCC cell lines, I was able to identify compounds that affected HPV-positive or HPV-negative cells exclusively (Table 8).

6.2.4.1 Hycanthone displayed preferential activity in HPV-positive HNSCC cell lines

Hycanthone was identified in my expanded high throughput screen as being preferentially active in HPV-positive HNSCC cell lines (Appendix 3). Since HPV-positive patients have been found to have better overall survival than their HPV-negative counterparts, it is imperative that compounds targeting HPV-positive tumours specifically are identified to help treat these patients since they will likely have to live with the toxicities of their treatment. Hycanthone functions by intercalating into DNA and inhibiting RNA synthesis and it has been found to be a potential anti-neoplastic agent. Phase I clinical trials have been completed in advanced cancers [41], which are cancers that are unlikely to be cured as well as advanced metastatic cancers [42] and a phase II trial has been completed for hycanthone in advanced colorectal carcinoma [43]. One potential explanation for its preferential activity in HPV-positive HNSCC cell lines is that cells infected with HPV often depend on the expression and oncogenic function of HPV oncoproteins E6 and E7, which target p53 and pRb, respectively, for degradation [4, 15]. This dependency on expression of E6 and E7 for tumourigenesis may also depend on

increased rates of transcription, which could potentially explain why an inhibitor of RNA synthesis was found to display activity in these HPV-positive cells. To date, hycanthone has not been explored as a therapeutic option for HNSCC, thus opening up the possibility for further investigation into its cellular mechanism of action and differential activity in HPV-positive and HPV-negative HNSCC cells.

6.2.4.2 Compounds with preferential activity in HPV-negative HNSCC cell lines

Conversely, 5 compounds were found to be hits in HPV-negative HNSCC cell lines exclusively (Table 8). Upon review of the literature, I found limited evidence examining the roles of 6-azauridine, an anti-viral agent, and primaquine diphosphate, an anti-parasitic, in cancer. However, these compounds, along with many other drugs that were identified as being extremely potent across all 6 cell lines in the expanded drug screen, were found to be anti-parasitics (including anthelmintics) as well as antibiotics (Appendix 3). A recent publication by Lamb and colleagues (2015) found that puromycin, along with other antibiotics targeting protein translation, could suppress cancer stem cells in culture and control the formation of tumourspheres [44]. Furthermore, others have observed that antibiotics targeting active sites of the ribosome directly as well as indirect inhibitors of protein synthesis have an extensive cytotoxic effect on breast cancer cells *in vitro* [40]. Cuyàs and colleagues (2015) concluded that cancer subtypes that are enriched with properties of stem cells (such as the ability to divide and renew themselves) exhibit an extreme sensitivity to antibiotics and anti-protozoals targeting protein synthesis [40]. These findings could help provide an

explanation as to why many antibiotics (e.g. puromycin) and anti-protozoals (e.g. albendazole) were observed to be active in my panel of HNSCC cell lines (Table 8, Appendix 3).

6.2.5 NSC146109 hydrochloride effectively inhibited HNSCC cell line growth *in vitro*

NSC146109 hydrochloride was identified as one of the top 10 most active compounds in my high throughput drug screen (Appendix 2). NSC146109 hydrochloride is an activator of p53 that has been found to activate p53 transcription, increase levels of p53 in tumour cells and protect p53 from E3 ubiquitin-protein ligase (Mdm2)-mediated degradation [33]. To ensure that this compound was controlling HNSCC cell line proliferation *in vitro* by this cellular mechanism, I attempted to look at *TP53* transcript levels using quantitative RT-PCR. Unfortunately, *TP53* transcript levels were too low to be accurately detected and compared (Figure 11). In addition, a suitable positive control was not obtained and I was therefore unable to normalize the untreated samples in order to gain an understanding of baseline *TP53* transcript levels within HNSCC tumour cells.

Currently, I am attempting to use normal, non-cancerous human cell lines as a positive control to redo the RT-PCR experiment to see if I can get a better measure of *TP53* levels in untreated cell lines and subsequently, using the now normalized untreated *TP53* transcript levels to compare to NSC146109 hydrochloride-treated cells with the expectation of *TP53* transcript levels increasing.

Next, I plan to investigate p53 levels at the protein level using Western blot analysis where I will compare levels of p53 protein in treated cells versus untreated cells.

Examination of p53 protein enables conclusions to be drawn regarding levels of p53 at the functional level since this is the molecule that acts as an essential tumour suppressor within the cell. Looking exclusively at *TP53* transcript levels is not a direct measurement of levels of p53 protein, which is why investigation into p53 protein levels upon treatment with NSC146109 hydrochloride is essential to confirm its mechanism of action within the cell.

Finally, I will examine cellular levels of p21 in response to reactivation of p53 as p21 is a well-documented downstream functional target of p53 activation [45, 46]. P21 is a potent and universal inhibitor of cyclin-dependent kinases and functions as a regulator of cell cycle progression [47]. Increases in p53 have been found to result in increased levels of p21, which inhibits the proliferation of mammalian cells [45, 47]. Since NSC146109 hydrochloride functions to reactivate p53 within the cell by protecting it from Mdm2-mediated degradation, I expect increased levels of p53 protein within the cell along with increased levels of its downstream target p21, upon treatment of HNSCC cells with this compound.

Testing of NSC146109 hydrochloride using *in vivo* cell line xenografts was intended, however, there were issues with the solubility of the compound when attempting to resuspend it for intraperitoneal dose delivery in mice. This issue is currently being examined where I am pursuing different reagents (e.g. DMSO, ethanol) in varying concentrations in an attempt to solubilize NSC146109 hydrochloride at concentrations suitable for *in vivo* mouse xenograft experiments.

6.2.5.1 TP53 and Cancer

TP53, which encodes p53 protein, is one of the most well known tumour suppressor genes in cancer and is mutated or lost in approximately 50% of all human malignancies [48]. As the "guardian of the genome", p53 regulates cell cycle progression by initiating G1-arrest in response to DNA damage and loss of functional p53 results in unregulated cellular division, or cancer cell formation [48]. A recent genomic characterization of HNSCC reported the frequency of p53 mutations and/or deletions at 84% in HPV-negative HNSCC tumours and 3% HPV-positive tumours [20]. Interestingly, disruptive mutant p53 has been found to be correlated with decreased recurrence-free survival as well as a valuable predictive biomarker in the development of local recurrences compared to patients with non-disruptive mutations or wild-type *TP53* [49, 50, 51].

Investigations into small molecule activators of p53 have found that these compounds can induce apoptosis and enhance cytotoxicity in HNSCC cell lines [52]. Compounds such as nutlin-3, RITA, CP-31398, PRIMA-1 and Minnelide™ have all been shown to successfully control HNSCC cell line proliferation *in vitro* against HNSCC cell lines through reactivation of p53 [52, 53]. Although these compounds have proven successful *in vitro* and in select preclinical models of HNSCC [52], there are currently no clinical trials investigating such compounds in head and neck cancer, thus providing a potential opportunity to explore this target as a treatment for patients suffering from HNSCC.

6.2.6 ER27319 maleate suppressed HNSCC cell line proliferation *in vitro* and controlled tumour growth *in vivo*

In addition to NSC146109 hydrochloride, the compound ER27319 maleate was also selected for preclinical development due to its high activity in HNSCC cell lines. ER27319 maleate has been found to inhibit phosphorylation of spleen tyrosine kinase (Syk) within the cell [34, 54]. In order to verify that ER27319 maleate is controlling HNSCC cell line proliferation through inhibition of Syk phosphorylation, both endogenous and phosphorylated Syk were examined in both treated and untreated HNSCC cells. Syk has 3 different sites that can undergo phosphorylation (Tyr323; Tyr525/526), all of which may be affected upon treatment with ER27319 maleate, so it was important to attempt to look at all of these sites in my studies. Although observation of endogenous Syk using Western blot analysis proved successful, I was unable to effectively visualize phosphorylated Syk, particularly in the context of untreated HNSCC cells and thus was unable to make any concrete conclusions regarding ER27319 maleate inhibiting Syk phosphorylation *in vitro* (Figure 12).

Currently, I am attempting to boost universal cellular phosphorylation by growing cells in serum-free media, which induces a stressed state and increases global phosphorylation within the cell. By increasing cellular phosphorylation, I hope to elevate phosphorylated Syk levels to levels that can be effectively visualized by Western Blow analysis. Once phosphorylated Syk can be efficiently visualized through universal amplification of cellular phosphorylation, comparisons between ER27319 maleate-treated cells and untreated cells can be made to ensure that ER27319 maleate is in fact controlling HNSCC cell line growth by inhibiting phosphorylation of Syk.

Additionally, future studies with this compound will involve examination of both endogenous and phosphorylated Syk levels across my entire panel of HNSCC cell lines. Gaining a better understanding of total Syk and phosphorylated Syk levels in all 28 HNSCC cell lines will enable appropriate cell line selection for future experiments when attempting to examine changes in Syk phosphorylation (i.e. examination of an HNSCC cell line with increased levels of phosphorylated Syk in untreated cells may allow for an visible and ideally indisputable inhibitory effect on Syk phosphorylation to be observed upon addition of ER27319 maleate).

With the knowledge that ER27319 maleate could effectively control HNSCC cell line proliferation *in vitro*, I wanted to examine the effect this compound had in an *in vivo* cancer model using cell line xenografts. Mice treated with ER27319 maleate (40 mg/kg) had significantly reduced tumour growth as compared to untreated control tumours (Table 9, Figure 13). In addition, the compound was extremely well tolerated amongst all animals tested as evidenced by subjective observation. Future experiments will involve treating cell line xenografts with an increased dose of ER27319 maleate (70 mg/kg), which was also found to be well tolerated, to see if I can further reduce tumour growth or even stop tumour growth completely. This preliminary data helps support my findings of ER27319 maleate demonstrating high activity both *in vitro* and *in vivo* in HNSCC cell lines and opens up the possibility of further preclinical modeling of this compound towards its development for the treatment of HNSCC.

6.2.6.1 Spleen Tyrosine Kinase

Spleen tyrosine kinase (commonly referred to as Syk) is a member of the Syk family of tyrosine kinases that has historically been associated with transmitting cellular immune signals within B cells in the initiation of the immune response [55]. More recently, Syk has been recognized as functioning as a promoter of cell survival in a variety of different cell types [55]. Interestingly, Syk has also been found to promote cell motility and progression *in vitro* in HNSCC cell lines [56]. Furthermore, an investigation by Du and colleagues (2012) observed that Syk levels are elevated in nasopharyngeal cancer and this was found to be correlated with poor patient prognosis thus presenting the opportunity for a new prognostic biomarker [57].

ER27319 maleate was one of the top drugs uncovered in my high throughput drug screening that displayed extremely high activity across all HNSCC cell lines tested. This evidence of its ability to efficiently control HNSCC cell line proliferation both *in vitro* and *in vivo*, combined with its newly associated role in epithelial cell motility and cell cycle progression, presents the possibility of a new and promising therapeutic target in HNSCC.

6.3 Limitations to the Study

One of the major limitations of this study was the use of cell lines as a model of HNSCC. Cell lines grow in a two dimensional monolayer in culture and are thus not the best representation of a three dimensional patient tumour. In addition, due to their two dimensional growth, cell lines are unable to replicate the tumour microenvironment, a series of complex interactions that facilitate tumorigenesis including stromal interactions, tumour vascularisation, various signaling molecules as well as the interactions with the extracellular matrix. This lack of accurate tumour replication, including the tumour microenvironment, does not enable us to assess drug delivery as well as drug metabolism in the context of an actual tumour environment.

Another major limitation of my study was the number of drugs tested. In my high throughput drug screen, libraries of 1,505 and 1,825 drug compounds were screened against HNSCC cell lines. Although this screen is the largest for the number of drug compounds performed to date, for HNSCC specifically, there is still the possibility that many active compounds may have been missed due to their lack of presence in my screen. Although not completely comprehensive, my drug screens were extremely extensive when it comes to drug testing in HNSCC. Additionally, I was able to successfully identify many novel agents that warrant further investigation for the treatment of HNSCC.

An additional limitation was the readout assay used for my high throughput drug screening. The alamarBlue viability assay, which provides a readout of cellular metabolic activity, enabled me to perform high throughput drug screening on a large-scale at a relatively low cost. Unfortunately, this assay does not give us any specific information on

whether or not cells stop proliferating due to apoptosis or senescence, for example. Additional assays could have been used as readouts for my screening such as caspase assays, which look at apoptosis as well as β-galactosidase assays, which examine cellular senescence, however, the reagents for these assays tend to be quite expensive. Examination of the effects of the 23 drugs selected for hit confirmation using additional readouts such as cellular senescence and apoptosis, as mentioned above, may provide further insight into the mechanisms of actions of these compounds as well as be more cost effective as compared to large-scale screening with these specific assays.

Finally, a major limitation of this study was that my large-scale drug screening was only performed at a single drug concentration. Screening at a single dose was necessary in order to test as many cell lines against the number of drugs that I screened; however, it is very possible that some highly active compounds were not identified. Some drugs may require increased doses for an anti-proliferative effect to be observed so it is likely that some potentially potent compounds were missed due to the selected screening dose not being in the optimal activity range for such compounds.

6.4 Future Directions

Further investigation is required for elucidation of the mechanisms of actions for the top drug compounds identified in my high throughput screening. In addition, combinational drug therapies will be explored using both data from my drug screening as well as relevant background information regarding drug and cellular targets from the literature. Combinational drug screening is important to overcome potential issues of

drug resistance as well as to identify possible synergistic drug effects. In addition, I plan on testing top drug hits in patient-derived xenografts (PDX) models. PDX will enable me to validate my *in vitro* screening and *in vivo* cell line xenograft model using actual patient tumours. PDX have been found to maintain the genomic alterations of the original patient tumour and have been found to be accurate in predicting patient response [58, 59].

The long-term goal of this project is to pair my drug sensitivity data to HNSCC cell line genomic data in order to identify biomarkers of drug sensitivity and/or resistance. The automated nature of this project has enabled a large number of HNSCC cell lines to be screened against extensive chemical panels, which is crucial for providing the statistical power required to make correlations between drug response and genetics. There is currently a lack of reliable predictive biomarkers for treatment response in HNSCC. One reason for this is due to the heterogeneity of HNSCC tumours, which makes it difficult to identify patient cohorts that can predict responses to particular therapeutics. In an effort to overcome this issue, it is essential that a large number of drug compounds are screened so active compounds can be identified. As well, it is vital that an extensive number of HNSCC cell lines are screened against these large chemical libraries so conclusions regarding markers of drug sensitivity and/or resistance can be accurately made.

7 CONCLUSIONS

High throughput drug screening identified therapeutic agents that are highly active in a panel of HNSCC cell lines. My screen of 1,505 compounds, along with an expanded screen of 1,825 drugs against a subset of HNSCC cell lines, identified both known and novel targets in HNSCC. Additionally, 23 compounds were confirmed as being highly active in HNSCC cell lines *in vitro* through the generation of 10-point dose response curves. One of these compounds, ER27319 maleate, an inhibitor of Syk phosphorylation, was found to reduce tumour growth *in vivo* in a cell line xenograft model.

Further investigations need to be performed to systematically and comprehensively characterize each compound as well as its ability to control HNSCC cell line and tumour growth *in vitro* and *in vivo*, respectively. In addition, my drug sensitivity data will eventually be paired with cell line genomic analyses to facilitate the identification of genetic biomarkers of drug response. Once combined, this strategy of high throughput drug screening paired with genomic analysis has the potential to not only identify novel agents to improve outcomes in HNSCC, but also identify ideal patient populations to offer each drug to, thus delivering the goal of precision medicine to HNSCC.

REFERENCES

1. Stewart B and BP Wild (2014) World Cancer Report. *IARC Non Serial Publication*.
2. Parkin DM, Bray F, Ferlay J and P Pisani (2005) Global cancer statistics, 2002. *CA Cancer J Clin* **55**(2):74–108.
3. Gandini S, Botteri E, Iodice S, Boniol M, Lowenfels AB, Masionnauve P and P Boyle (2008). Tobacco smoking and cancer: A meta-analysis. *Int J Cancer* **122**(1):155–169.
4. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E *et al.* (2011) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *J Clin Oncol* **29**(32): 4294–4301.
5. Nichols AC, Palma DA, Dhaliwal SS, Tan S, Theuer J, Chow W *et al.* (2013) The epidemic of human papillomavirus and oropharyngeal cancer in a Canadian population. *Curr Oncol* **20**(4):212–219.
6. Kreimer AR and AK Chaturvedi (2011) HPV–associated Oropharyngeal cancers-Are they preventable? *Cancer Prev Res* **4**(9):1346–1349.
7. Hanahan D and RA Weinberg (2011) Hallmarks of Cancer: The Next Generation. *Cell* **144**:646–674.
8. Marte B (2013) Tumour heterogeneity. *Nature* **501**:327.
9. Burrell RA, McGranahan N, Bartek J and C Swanton (2013) The causes and consequences of genetic heterogentiy in cancer evolution. *Nature* **501**:338–345.

10. Errico A (2015) Clonal and subclonal events in cancer evolution–optimizing cancer therapy. *Nat Rev Clin Onc* Published online.
11. Cancer Research UK (2015) What is cancer? Published online: http://www. cancerresearchuk.org/about-cancer/what-is-cancer.
12. Canadian Cancer Society, Canadian Cancer Statistics 2014, May 2014 0835-2976.
13. Leemans CR, Braakhuis BJM and RH Brakenhoff (2011) The molecular biology of head and neck cancer. *Nat Rev Cancer* **11**(1):9–22.
14. Canadian Cancer Society Advisory Committee on Cancer Statistics (2013) Canadian Cancer Statistics 2013. Toronto, ON: Canadian Cancer Society.
15. Gillison ML (2004) Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. *Sem Oncol* **31**(6):744 –754.
16. Physicians for a smoke-free Canada (2012) Fact sheets. Published online: http://www.smoke-free.ca/factsheets/pdf/prevalence.pdf.
17. Gillison ML, Broutian T, Pickard RK, Tong ZY, Xiao W, Kahle L *et al*. (2012) Prevalence of oral HPV infection in the United States, 2009-2010. *JAMA* **307**(7):693–703.
18. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S *et al*. (2013) Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. *J Clin Oncol* **31**(36):4550–4559.
19. D'Souza G, Zhang HH, D'Souza WD, Meyer RR and ML Gillison (2010) Moderate predictive value of demographic and behavioural characteristics for a diagnosis of HPV16-positive and HPV-negative head and neck cancer. *Oral Oncol* **46**(2):100–104.

20. The Cancer Genome Atlas Network (2015) Comprehensive genomic characterization of head and neck squamous cell carcinoma. *Nature* **517**:576–582.
21. Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A *et al.* (2011) The mutational landscape of head and neck squamous cell carcinoma. *Science* **333**(6046):1157–1160.
22. Agrawal N, Frederick MJ, Pickering CR, Bettegowda C, Chang K, Li RJ *et al.* (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* **333**(6046):1154–1157.
23. Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW *et al.* (2012) Systematic identification of genomic markers of drug sensitivity in cancer cells. *Nature* **483**(7391):570–575.
24. Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim SS *et al.* (2012) The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**(7391):603–607.
25. Nichols AC, Black M, Yoo J, Pinto N, Fernandes A, Haibe-Kains B *et al.* (2014) Exploiting high-throughput cell line drug screening studies to identify candidate therapeutic agents in head and neck cancer. *BMC Pharmacol Toxicol* **15**:66.
26. Wen Y and JR Grandis (2015) Emerging Drugs for head and neck cancer. *Expert Opin Emerging Drugs* **11**(3):461-7.
27. Machtay M, Moughan J, Trotti A, Garden AS, Weber RS, Cooper JS *et al.* (2008) Factors associated with severe late toxicity after concurrent chemoradiation for locally advanced head and neck cancer: an RTOG analysis. *J Clin Oncol* **26**(21):3582–3589.

28. Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tân PF *et al.* (2010) Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med* **363**(1):24–35.
29. Mendelsohn J (2013) Personalizing Oncology: Perspectives and Prospects. *J Clin Onc* **31**(15):1904–1911.
30. Dorsey K and M Agulnik (2013) Promising new molecular targeted therapies in head and neck cancer. *Drugs* **73**(4):315–325.
31. Razzouk S (2014) Translational genomics and head and neck cancer: toward precision medicine. *Clin Genet* **86**:412–421.
32. Page B, Page M and C Noel (1993) A new fluorometric assay for cytotoxicity measurement in-vitro. *Int J Oncol* **3**(3):473–476.
33. Berkson RG, Hollick JJ, Westwood NJ, Woods JA, Lane DP and S Lain (2005) Pilot screening programme for small molecule activators of p53. *Int J Cancer* **115**:701–710.
34. Moriya K, Rivera J, Odom S, Sakuma Y, Muramato K, Yoshiuchi T *et al.* (1997) ER-27319, an acridone-related compound, inhibits release of antigen-induced allergic mediators from mast cells by selective inhibition of fcepsilon receptor I-mediated activation of Syk. *Proc Natl Acad Sci USA* **94** (23):12539–44.
35. Grover JK, Vats V, Uppal G, and S Yadav (2001) Anthelmintics: a review. *Trop Gastroenterol* **22**(4):180-9.
36. Hou ZJ, Luo X, Zhang W, Peng F, Cui B, Wu SJ *et al.* (2015) Flubendazole, FDA-approved anthelmintic, targets breast cancer stem-like cells. *Oncotarget* **6**(8):6326-40.

37. Nygren P, Fryknäs M, Agerup B, and R Larsson (2013) Repositioning of the anthelmintic drug mebendazole for the treatment for colon cancer. *J Cancer Res Clin Oncol* 1**39**(12):2133-40.

38. Liu C, Lou W, Zhu Y, Nadiminty N, Schwartz CT, Evans CP, and AC Gao (2014) Niclosamide inhibits androgen receptor variants expression and overcomes enzalutamide resistance in castration-resistant prostate cancer. *Clin Cancer Res* **20**(12):3198-210.

39. Liu C, Lou W, Armstrong C, Zhu Y, Evans CP, and AC Gao (2015) Niclosamide suppresses cell migration and invasion in enzalutamide resistant prostate cancer cells via Stat3-AR axis inhibition. *Prostate* doi: 10.1002/pros.23015.

40. Cuyàs E, Martin-Castillo B, Corominas-Faja B, Massaguer A, Bosch-Barrera J and JA Menendez (2015) Anti-protozoal and anti-bacterial Antibiotics that inhibit protein synthesis kill Cancer cubtypes enriched for stem cell-like properties. *Cell Cycle* **13**:0.

41. Kovach JS, Moertel CG, Schutt AJ and ET Eagan (1979) Phase I study of hycanthone. *Cancer Treat Rep* **63**(11-12):1956–9.

42. Legha SS, Grose WE and GP Bodey (1978) Phase I study of hycanthone. *Cancer Treat Rep* **62**(8):1173–6.

43. Schutt AJ, Dalton RJ, Kovach JS, Moertel CG and MJ O'Connell (1983) Phase II study of hycanthone in patients with advanced colorectal carcinoma. *Cancer Treat Rep* **67**(6):593–4.

44. Lamb R, Harrison H, Smith DL, Townsend PA, Jackson T, Ozsvari B *et al.* (2015) Targeting tumor-initiating cells: eliminating anabolic cancer stem cells

with inhibitors of protein synthesis or by mimicking caloric restriction. *Oncotarget* **6**(7):4585–4601.

45. Krummel KA, Lee CJ, Toledo F and GM Wahl (2005) The C-terminal lysines fine-tume P53 stress response in a mouse mdoel but are not required for stability control or transactivation. *Proc Natl Acad Sci USA* **102**(29):10188–93.
46. Rozan LM and WS El-Deiry (2007) p53 downstream target genes and tumor suppression: a classical view in evolution. *Cell Death Differ* **14**:3–9.
47. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R and D Beach (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* **366**:701–704.
48. Muller PAJ, Vousden KH, and JC Norman (2011) p53 and its mutants in tumor cell migration and invasion. *J Cell Biol* **192**(2):209–218.
49. Ganci F, Sacconi A, Bossel Ben-Moshe N, Manciocco V, Sperduti I, Strigari L *et al*. (2013) Expression of TP53 mutation-associated microRNAs predicts clinical outcome in head and neck squamous cell carcinoma patients. *Annals Oncol* **24**(12):3082–3088.
50. Osman AA, Neskey DM, Katsonis P, Patel AA, Ward AM, Hsu TK *et al*. (2015) Evolutionary Action Score of TP53 Coding Variants Is Predictive of Platinum Response in Head and Neck Cancer Patients. *Cancer Res* **75**(7):1205-1215.
51. Neskey DM, Osman AA, Ow TJ, Katsonis P, McDonald T, Hicks SC *et al*. (2015) Evolutionary Action Score of TP53 Identifies High-Risk Mutations Associated with Decreased Survival and Increased Distant Metastases in Head and Neck Cancer. *Cancer Res* **75**(7):1527-36.

52. Roh J, Kang SK, Minn IL, Califano JA, Sidransky A and WM Kocha (2012) p53-Reactivating small molecules induce apoptosis and enhance chemotherapeutic cytotoxicity in head and neck squamous cell carcinoma. *Oral Oncol* **47**(1):8–15.

53. Caicedo-Granados E, Lin R, Fujisawa C, Yueh B, Sangwan V, and A Saluj (2013) Wild-type p53 reactivation by small-molecule Minnelide™ in human papillomavirus (HPV)-positive head and neck squamous cell carcinoma. *Oral Oncol* **50**(2):1149–1156.

54. Lusková P and P Dráber (2004) Modulation of Fcepsilon receptor I signling by tyrosine kinase inhibitors: search for therapeutic targets of inflammatory and allergy diseases. *Curr Pharm Des* **10**(15):1727–37.

55. Geahlen RL (2014) Getting Syk: spleen tyrosine kinase as a therapeutic target. *Trends Pharmacol Sci* **5**(8):414-22.

56. Luangdilok S, Box C, Patterson L, Court W, Harrington K, Pitkin L *et al.* (2007) Syk tyrosine kinase is linked to cell motility and progression in squamous cell carcinomas of the head and neck. *Cancer Res* **15**;67(16):7907-16.

57. Du ZM, Kou CW, Wang HY, Huang MY, Liao DZ, Hu CF *et al.* (2012) Clinical significance of elevated spleen tyrosine kinase expression in nasopharyngeal carcinoma. *Head Neck* **34**(10):1456-64.

58. Hennessey PT, Ochs MF, Mydlarz WW, Hsueh W, Cope L, Yu M *et al.* (2011) Promoter methylation in head and neck squamous cell carcinoma cell lines is significantly different than methylation in primary tumors and xenografts. *PLoS One* **6**(5):e20584.

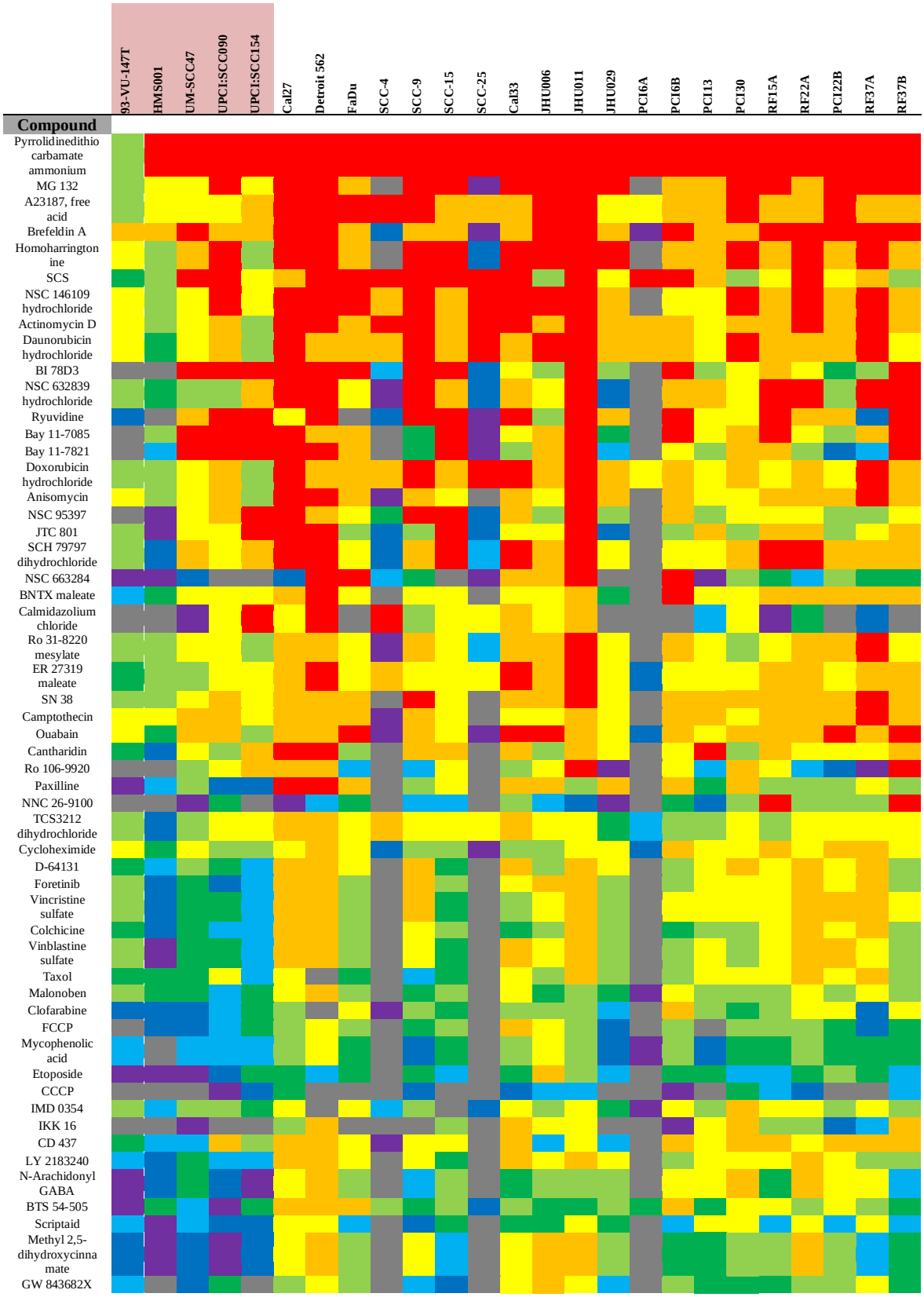
59. Siolas D and GJ Hannon (2013) Patient-derived tumor xenografts: transforming clinical samples into mouse models. *Cancer Res* **73**(17):5315–5319.

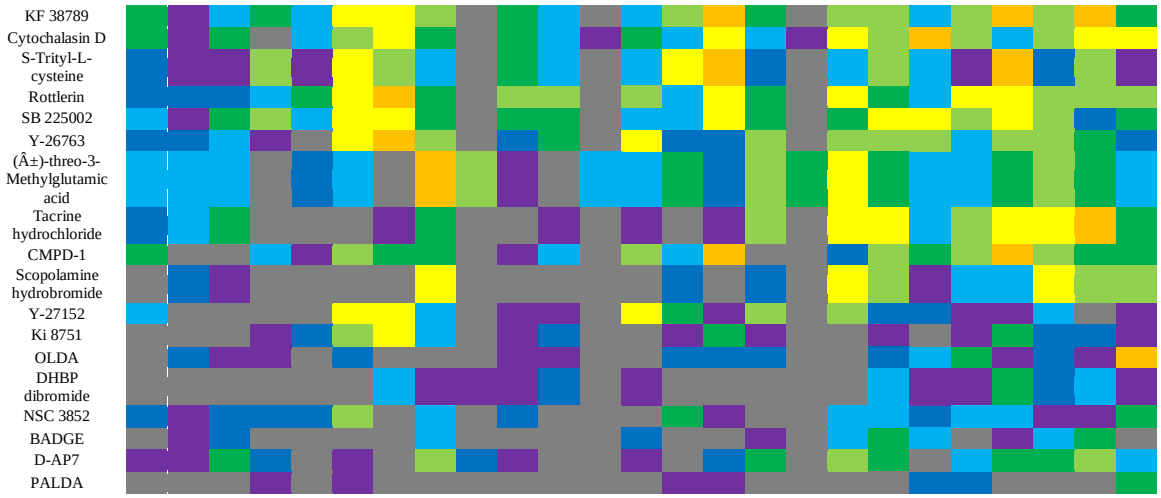
APPENDICES

Appendix 1. STR profiles of HNSCC cell lines used for screening.

Cell Line	Amelogenin	CSF1PO	D13S317	D16S539	D5S818	D7S820	TH01	TPOX	vWA	D18S51	D19S433	D21S11	D2S1338	D3S1358	D8S1179	FGA
93-VU-147T	X,Y	11,11	8,11	9,12	11,12	12,12	6,6	8,8	17,17	15,20	12,13	28,29	21,21	15,15	12,15	24,24
HMS001	X,Y	11,11	8,8	9,12	11,12	12,12	6,6	8,8	17,17	15,20	13,14	28,29	21,21	15,15	13,15	24,24
UM-SCC47	X,Y	11,13	8,11	8,13	11,12	11,11	7,9.3	10,11	18,18	18,18	14,15	29,30	25,25	15,15	15,15	25,25
UPCI:SCC090	X,Y	11,12	11,11	12,13	11,12	9,10	7,7	8,8	17,17	14,18	12,13	39,31	22,22	14,14	12,12	20,20
UPCI:SCC154	X,Y	10,12	9,12	13,13	11,12	9,10	7,7	8,9	17,17	15,15	15.2,16	28,29	25,25	16,16	12,12	20,24
Cal27	X,X	10,12	10,11	11,12	11,12	10,10	6,9.3	8,8	14,17	13,13	14,15.2	28,29	23,24	16,16	13,15	25,25
Detroit 562	X,X	11,13	12,12	11,11	11,12	8,10	8,9	8,10	16,16	15,15	14,14	28,30	25,25	15,16	13,13	21,21
FaDu	----	12,12	8,9	11,11	12,12	11,12	8,8	11,11	15,17,18	16,16	14,16	31.2,31.2	19,19	17,18	13,13	25,25
SCC-4	X,Y	11,11	11,13	12,12	13,13	9,11	9.3	8,8	15,17	15,15	12,14	32.2,32.2	16,24	18,18	14,14	21,22
SCC-9	X,Y	11,11	9,9	10,11	12,12	8,8	8,9	9,11	17,17	12,14	12,14	28,28	19,21	15,15	13,13	20,25
SCC-15	X,Y	10,13	9,14	12,15	12,12	10,11	9,9.3	8,8	15,17	16,16	12,15	30,30	16,23	16,16	10,13	24,24
SCC-25	X,X	10,10	13,13	11,12	12,12	12,12	8,8	8,12	17,19	16,16	13,14	30,30	17,19	17,17	13,13	20,24
Cal33	X,Y	11,12	8,13	11,11	11,12	8,10	9,9.3	8,8	17,17	14,14	14,15.2	29,30	20,25	17,17	13,13	21,22
JHU006	X,Y	10,10	11,11	12,12	12,12	9,10	6,6	8,8	14,16	16,16	14,14.2	31,31	17,25	13,13	13,14	19,19
JHU011	X,X	10,12	12,12	9,14	9,12	11,11	6,9	8,9	16,17	13,15	13,14	31,31	17,26	18,18	13,13	23,23
JHU029	X,Y	8,12	12,13	10,13	13,15	10,11	8,8	9,11	15,15	15,15	13,13	33.2,33.2	19,26	16,16	14,14	22,25
PCI6A	X,X	12,12	9,11	11,11	11,12	8,12	6,6	11,11	15,18	12,17	15,16.2	31.2,31.2	17,24	18,18	14,14	23,23
PCI6B	X,Y	10,11	10,11	12,13	12,12	12,13	6,7	8,9	16,18	16,16	12,14	32.2,32.2	23,24	18,18	13,14	20,20
PCI13	X,X	10,14	10,11	11,11	11,14	11,12	9,9.3	6,8	13,17	16,16	13,15.2	29,30	24,24	14,14	12,13	22,22
PCI30	X,Y	10,10	13,13	12,13	11,11	10,12	6,6	10,11	14,17	12,15	12,12	30,32.2	18,18	15,15	13,15	21,21
RF15A	X,X	10,12	12,12	9,9	10,12	10,11	6,6	8,11	17,17	16,18	13,14	29,29	20,23	16,16	14,14	22,22
RF15B	X,Y	11,11	11,11	11,11	11,11	11,11	6,6	8,8	18,18	14,14	14,15.2	28,28	20,25	16,16	14,14	23,23
RF22A	X,X	10,10	8,12	9,11	12,12	8,9	6,6	8,11	15,18	18,18	14,15	28,28	17,20	16,16	11,13	22,24
RF22B	X,X	10,12	8,11,12	9,12,13	10,12,13	9-12	6,6	8,11	15-18	16,18	12,13,14	27,29	20,22,23	16,18	13-16	18,22
RF37A	X,X	11,11	10,13,14	11,14	11,11	7,10	7,9.3	8,11	16,17	13,13	15,16	30,32.2	24,24	17,17	11,13	21,21
RF37B	X,X	10,12	12,12	9,9	10,12	10,11	6,6	8,11	17,17	16,18	13,14	29,29	20,23	16,16	14,14	22,22
BICR56	X,X	12,12	12,12	11,11	11,12	8,12	9,9.3	8,9	15,16	14,17	14,15	28,29	24,26	14,14	11,13	21,21
PE/CA-PJ49	X,X	11,12	8,11	11,11	8,12	8,9	6,10	8,8	16,19	24,24	14,14	32,32	17,18	16,16	10,11	20,20

Appendix 2. Drug hits identified by high throughput drug screening in HNSCC cell lines.

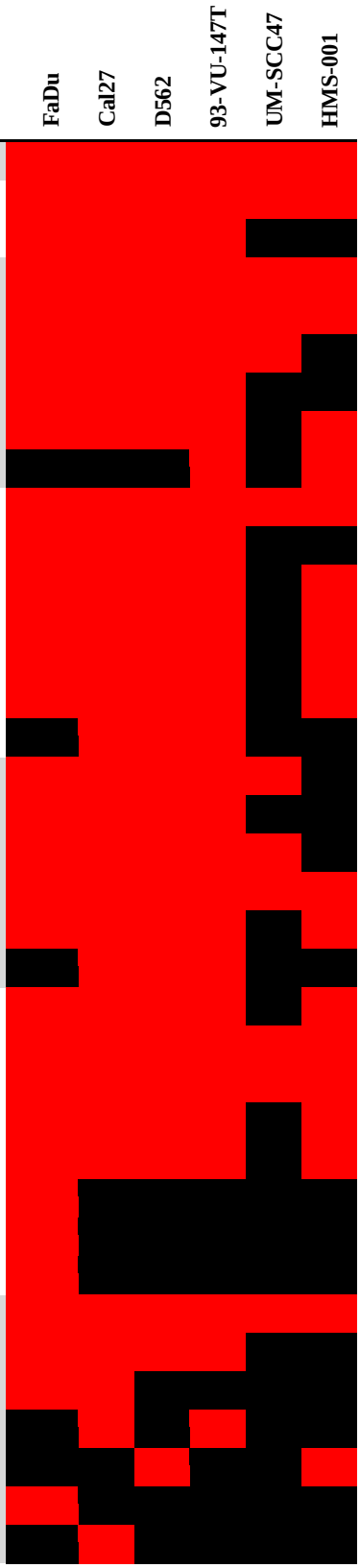




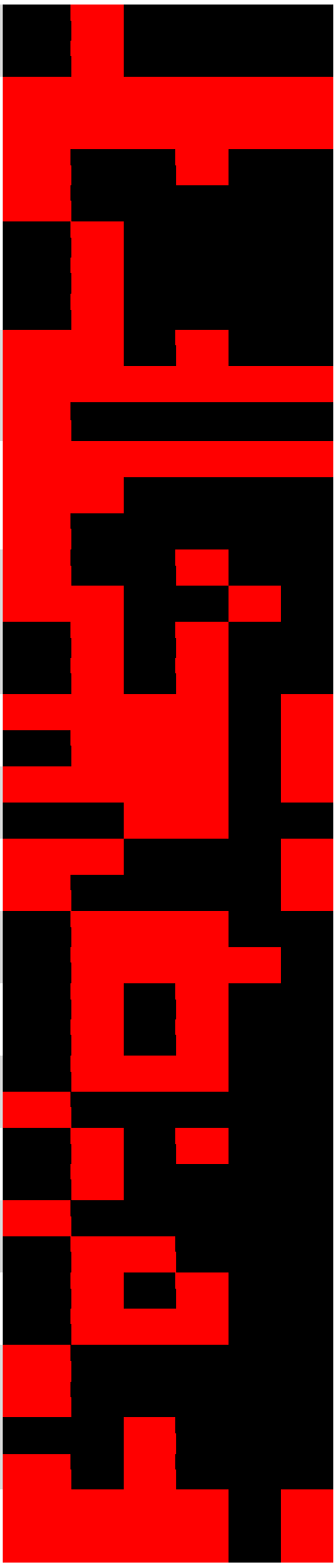
Reduction in cellular proliferation: red, 90-100%; orange, 80-90%; yellow, 70-80%; light green, 60-70%; dark green, 50-60%; light blue, 40-50%; dark blue, 30-40%, purple, 20-30%; grey, <20%.

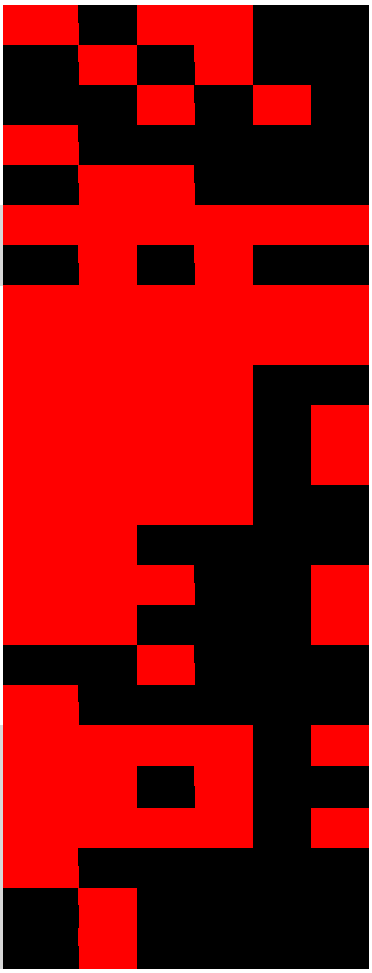
Appendix 3. Drug hits (in red) from expanded drug panel screening.

Library	Drug	Target	FaDu	Cal27	D562	93-VU-147T	UM-SCC47	HMS-001
NIH	Homoharringtonine	Protein synthesis						
Prestwick	Piperlongumine	Anti-cancer						
Prestwick	Mycophenolic acid	Anti-cancer						
NIH/Prestwick	Daunorubicin Hydrochloride	DNA/RNA synthesis						
NIH	Mitoxantrone	DNA synthesis						
NIH	Epirubicin Hydrochloride	DNA/RNA synthesis						
NIH	2-chloro-2'-deoxyadenosine	DNA synthesis						
NIH	Podofilox	Antimitotic						
Prestwick	Hycanthone	RNA/DNA synthesis						
NIH/Prestwick	Albendazole	Anthelmintic						
NIH	Flubendazole	Anthelmintic						
NIH/Prestwick	Mebendazole	Anthelmintic						
Prestwick	Parbendazole	Anthelmintic						
Prestwick	Niclosamide	Anthelmintic						
Prestwick	Fenbendazole	Anthelmintic						
Prestwick	Oxantel pamoate	Anthelmintic						
NIH	Vincristine Sulfate	Microtubules						
NIH	Vindesine Sulfate	Microtubules						
NIH	Vinorelbine Bitatrate	Microtubules						
Prestwick	Colchicine	Microtubules						
Prestwick	Nocodazole	Microtubules						
NIH	Docetaxel	Microtubules						
NIH	Irinotecan HCl (trihydrate)	Topoisomerase I						
NIH	Idarubicin HCl	Topoisomerase II						
NIH	Doxorubicin Hydrochloride	Topoisomerase II						
NIH	Topotecan HCL	Topoisomerase						
Prestwick	Podophyllotoxin	Topoisomerase						
NIH	Etoposide	Topoisomerase						
Prestwick	Ellipticine	Topoisomerase						
Prestwick	Etoposide	Topoisomerase						
NIH	Dactinomycin	Antibiotic						
NIH	Telithromycin	Antibiotic						
Prestwick	Puromycin dihydrochloride	Antibiotic						
Prestwick	Metampicillin sodium salt	Antibiotic						
NIH/Prestwick	Metronidazole	Antibiotic						
Prestwick	Thonzonium bromide	Antibiotic						
NIH	Cephalexin monohydrate	Antibiotic						



Prestwick	Thiostrepton	Antibiotic
Prestwick	Benzylpenicillin sodium	Antibiotic
NIH	Triptolide	NF-κB
Prestwick	Parthenolide	NF-κB
NIH	Clofazimine	Anti-inflammatory
NIH	Valdecoxib	Anti-inflammatory
NIH	Diphenylcyclopropenone	Topical Immunotherapy
NIH	Hydrocortisone	Immunosuppressant
NIH	Dipyridamole	Thrombus formation
NIH	Raclopride	Dopamine D2/D3 receptor
Prestwick	Thiethylperazine malate	Dopamine antagonist
Prestwick	Apomorphine hydrochloride hemihydrate	Dopamine agonist
NIH	Rythmol	Antiarrhythmic
Prestwick	Ajmaline	Antiarrhythmic
Prestwick	Clofilium tosylate	Antiarrhythmic
NIH	Mosapride Citrate	5-HT4 agonist
NIH	5-Nonyloxytryptamine	5-HT1 eceptor
NIH	Tegaserod Maleate	5-HT4 agonist
Prestwick	Scoulerine	5-HT receptor
Prestwick	Chloropyramine hydrochloride	Antihistamine
Prestwick	Brompheniramine maleate	Antihistamine
Prestwick	Tranylcypromine hydrochloride	Histone demethylation
NIH	Azacitidine	DNA methylation
NIH	Cogentin Mesylate	Acetylcholine levels
Prestwick	Gallamine Triethiodide	Acetylcholine levels
NIH	Cerivastatin Na	Statin
NIH	Itavastatin Ca	Statin
NIH	Methyltestosterone	Oral steroid
Prestwick	Fluorometholone	Corticosteroid
Prestwick	Methyldopate hydrochloride	Anti-hypertensive
NIH	Fenoldopam Mesylate	Anti-hypertensive
NIH	LY 171883	PPARα/PPARγ
NIH	Rosigltazone HCl	PPARγ
NIH	Valaciclovir Hydrochloride	Anti-viral
NIH	6-Azauridine	Anti-viral
NIH	Fluphenazine HYDROCHLORIDE	Anti-psychotic
Prestwick	Thioridazine hydrochloride	Anti-psychotic
Prestwick	Benzethonium chloride	Anti-microbial
Prestwick	Ciclopirox ethanolamine	Anti-fungal
NIH/Prestwick	Itraconazole	Anti-fungal
Prestwick	Primaquine diphosphate	Anti-parasitic
Prestwick	Methimazole	Anti-thyroid
Prestwick	Fenoterol hydrobromide	Adrenergic agonist



Prestwick	Proxyphylline	Xanthine derivative	
Prestwick	Strophantine octahydrate	Cardiac glycosides	
NIH/Prestwick	Tomelukast	Antiasthmatic	
NIH	Tiagabine HCl	Anti-convulsive	
NIH	Rosiglitazone Maleate	Anti-diabetic	
NIH	Ranitidine hydrochloride	Histamine H2 receptor	
NIH	Toremifene Citrate	Estrogen receptor	
NIH	Tetraethylthiuram Disulfide	Alcohol dehydrogenase	
Prestwick	Disulfiram	Acetaldehyde dehydrogenase	
NIH	Procaine hydrochloride	Na+ channel	
NIH	Pyrimethamine	MATE transporters	
Prestwick	Omeprazole	Proton Pump Inhibitor	
Prestwick	Quinacrine dihydrochloride dihydrate	MAO-A/B inhibitor	
Prestwick	Letrozole	Aromatase Inhibitor	
Prestwick	Bucladesine sodium salt	Phosphodiesterase inhibitor	
Prestwick	Proscillaridin A	Na+/K+ ATPase	
NIH/Prestwick	Moxisylyte hydrochloride	α-blocker	
NIH	Bifemelane	Antidepressant (MAO)	
NIH	Hydrochlorothiazide	Diuretic	
NIH	3-hydroxy-1,2-dimethyl-4(1H)-pyridone	Iron chelator	
Prestwick	Chelidonine monohydrate (+)	Unknown	
NIH	1H-CAS	Unknown	
Prestwick	Methotrimeprazine maleate salt	Unknown	
Prestwick	Chrysene-1,4-quinone	Unknown	

Appendix 4. Ethics approval for *in vivo* cell line xenograft model.

PI Info

PI_FULL_NAME	Koropatnick, J.
CAMPUS_NAME	Victoria Research Laboratory
ORGANIZATION	Schulich School Of Medicine & Dentistry
DEPARTMENT	Oncology
PRIMARY EMAIL	
WORK PHONE	

Protocol Info

AUP_NUMBER	2008-069
DATE_APPROVED	08/26/2013
AUP_TITLE	Testing Novel Synthesized Compounds for Antitumor Activity in Nude Mice
WORKFLOW LOCATION	AUS

Funding Sources

FUND_TYPE	FUND_SOURCE	FUND_TITLE
Agency	Ontario Ministry Research & Innovation	Ontario-Palestine Collaborative Research Program: "New Directions in Cancer Research"
Agency	Ontario Centre of Excellence	In vivo testing of novel therapeutics
Agency	Canadian Institutes of Health Research	The role of IDO1 vs IDO2 on tumour immune evasion and immune therapy.
Agency	Ontario Research Fund	Ontario Preclinical Imaging Consortium ORF-RE-03-051
Agency	Trillium Therapeutics	Villers, Natasja | To Develop Immunotherapeutic Agents f
Agency	Uwo-schulich Joint Subsidy	Peptides Specific for Glypican-3 as Next-Generation Imaging

8 CURRICULUM VITAE

Name: Morgan Black

Post-secondary Education and Degrees:

University of Western Ontario
London, Ontario, Canada
2008-2012 B.M.Sc

The University of Western Ontario
London, Ontario, Canada
2013-2015 M.Sc.

Honours and Awards:

Seiz Travel Award for Most Innovative Research
Oncology Research and Education Day
2015

ACB Travel Prize
2015

Gabriel G. Altman Research Award – Masters Biological Research
Anatomy and Cell Biology Research Day
2014

Canadian Institutes of Health Research Strategic Training Program in Cancer Research and Technology Transfer
2013-2015

Western Graduate Research Scholarship
2013-2015

Dean's Honour List
2011-2012

Biochemistry Undergraduate Summer Research Program Studentship Recipient
2011

Related Work Experience

Private Tutor
Mississauga, Ontario, Canada
2009-2012

Publications:

Theurer JA, Stecho W, Yoo J, Kwan K, Wehrli B, Harry V, **Black M**, Pinto N, Winquist E, Palma DA, Richter S, Barrett JW, MacNeil D, Fung K, Howlett C and AC Nichols (2014) Feasibility of targeting PIK3CA mutations in head and neck squamous cell carcinoma. Pathol Onc Res (accepted)

Ross JA, Trussler RS, **Black MD**, McLellan CR and DB Haniford (2014) Tn5 transposition in Escherichia coli is repressed by Hfq and activated by over-expression of the small non-coding RNA SgrS. Mobile DNA 5(1):27.

Nichols AC, **Black M**, Pinto N, Fernandes A, Haibe-Kains B, Boutros PC and JW Barrett (2014) Exploiting high-throughput cell line drug screening studies to identify candidate therapeutic agents in head and neck cancer. BMC Pharmacol Toxicol 15(1):66.

Pinto N, **Black M**, Patel K, Yoo J, Mymryk JS, Barrett JW, and AC Nichols (2014) Genomically driven precision medicine to improve outcomes in anaplastic thyroid cancer. J Oncol doi: 10.1155/2014/936285.

Rizzo G, **Black M**, Mymryk J, Barrett J, and AC Nichols (2014) Defining the genomic landscape of head and neck cancers through next-generation sequencing. Oral Dis doi: 10.1111.

Mundi N, Um S, Yoo J, Rizzo G, **Black M**, Pinto N, Palma DA, Fung K, MacNeil D, Mymryk JS, Barrett JW and AC Nichols (2014) The control of anaplastic thyroid carcinoma cell lines by oncolytic poxviruses. Virus Res 190C:53–59.