An investigation into mechanisms of action of colchicine, zinc acetate and paracetemol - potential candidates for drug repurposing in head and neck cancer therapy.

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Abstract

The incidence of head and neck cancers (HNC) is on the rise. The common therapies used, are associated with long-term toxicities that hamper the quality of life of HNC patients. Novel therapeutic strategies are required to reduce these. As drug discovery is expensive, time consuming and unreliable, in this project we have considered drug repurposing. Paracetemol, zinc acetate and colchicine are used for pain relief, common cold lozenges and gout treatment, respectively. We initially assessed their efficacy on the proliferation of a HNC cell line, SCC040, using an Alamar blue colometric assay. Results indicate zinc acetate significantly inhibited proliferation (62.68 $\pm$ 3.75% reduction, p≤0.001) compared to vehicle control. Secondly cell cycle analysis was carried out using propidium iodide staining and flow cytometry analysis. Colchicine was found to be a potent G2/M cell cycle blocker (p≤0.001) compared to its untreated control. Finally the effect of the drugs on the migration of SCC040 cells was assessed using a wound-healing assay. Colchicine was found to significantly inhibit migration compared to its vehicle control (79.55 ± 4.02% reduction p≤0.001). These results suggest that zinc acetate and colchicine are potential candidates for less toxic therapies in HNC patients.

Acknowledgements

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1. Introduction

1.1 Head & Neck cancer (HNC)

Cancer is defined as the uncontrolled division and proliferation of abnormal cells (National Cancer Institute, 2013), to produce a malignant tumour (Weinberg, 2013). Carcinogenesis, the transformation from normal cells to cancer cells, is a complex pathway, which is characterized by a group of genetic mutations (Osborne *et al,* 2004). These mutations change the normal function of cellular pathways, which control regulatory mechanisms such as growth; and this leads to the development of cancer (Osborne *et al,* 2004).

HNCs are a group of related neoplasms (Hashibe *et al,* 2007), which are carcinomas of the upper aero-digestive tract (Dobrossy, 2005). Almost all are squamous cell carcinomas (Argiris *et al,* 2008), meaning that they arise from the mucosal surface (Rezende *et al,* 2010). There are over 30 affected areas, which fall into the HNC group (NHS, 2012). HNC is therefore a term that groups epithelial malignancies, which arise from the nasal cavity, oral cavity, pharynx, larynx and paranasal sinuses (Rezende *et al,* 2010).
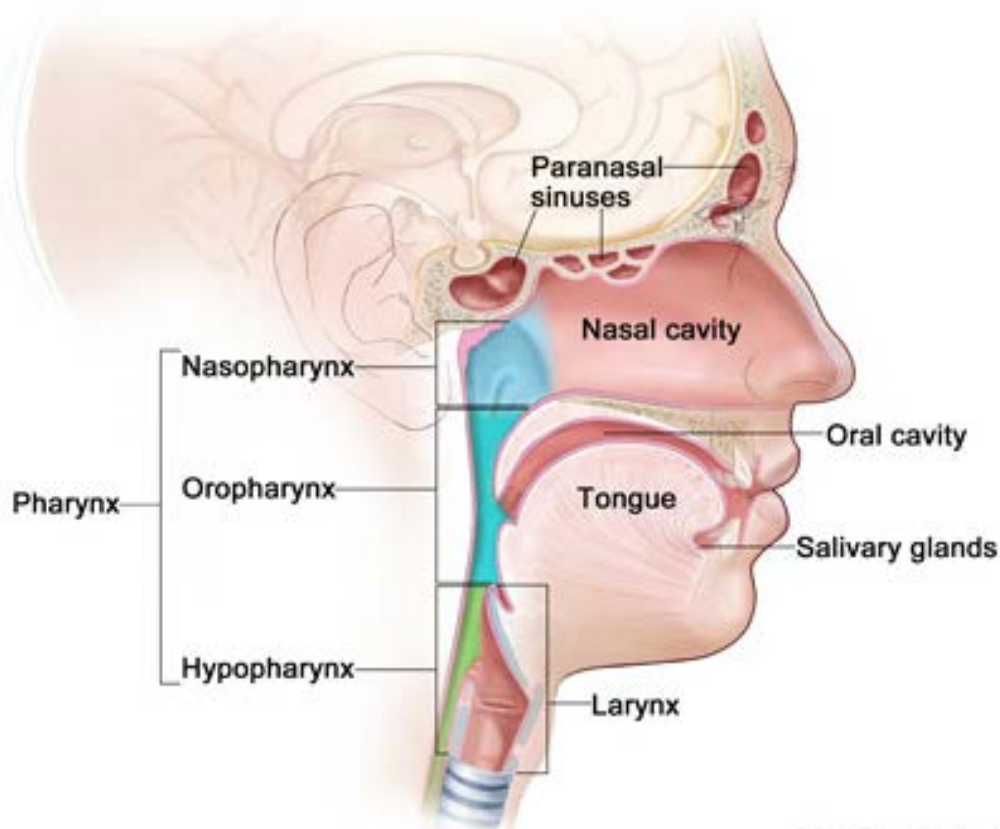
Figure 1: Illustrates the regions in the HNC group affected by carcinomas (National Cancer Institute, 2013).

Symptoms associated with HNCs vary according to the site, but can include sore throat, hoarseness, difficulty swallowing and dysphagia and upon examination patients present with an identifiable primary site with a palpable neck mass (Haddad & Shin, 2008). From this, HNCs can therefore impact basic daily functions such as speaking, swallowing and breathing and from this have physical and emotional effects on the patient (Hanna *et al,* 2013).

1.1.2 HNC epidemiology

The incidence of HNCs is on the rise within the industrialized world (Schoder, 2013). There are approximately 600,000 new cases per year and 300,000 deaths per year occurring worldwide from HNCs (Hashibe *et al,* 2007). The World Health Organization estimates that the oral cavity was the most commonly affected site with 389,000 new cases yearly (Boyle & Levin, 2008).

	Africa	The Americas[†]	Eastern Mediterranean	Europe	South East Asia	Western Pacific
Population	737 536	874 380	519 688	883 311	1 671 904	1 738 457
Incidence	30	38	29	72	195	74
Mortality	22	25	22	51	158	56

*Numbers should be multiplied by 1000.
[†]North America, South America, and Canada.

Figure 2: Demonstrates the population, incidence and mortality of HNCs around the world (Mehanna et al, 2010)

The highest incidences of HNCs are specifically seen in France, Brazil, Australia, South Africa and the Indian subcontinent (Mehanna *et al,* 2010). The lower rates seen in the Eastern Mediterranean, Americas and Europe may be due to a decline in exposure to carcinogens for example smoking (*Figure 2;* Mehanna *et al,* 2010). Other risk factors may explain high rates in south East Asia, where tobacco chewing is prominent (Her, 2001). Secondly nasopharyngeal cancer is largely restricted to China, which is linked to dietary risk factors such as the consumption of salted and preserved foods (Her, 2001). This includes salted fish, which contains the known risk factor *N*-nitro-sodimethylamine (Her, 2001).

Tobacco and alcohol alone are accountable for at least 75% of diagnosed HNCs in Europe and USA (Hashibe *et al,* 2007). Non-smokers who have 3 alcoholic beverages a day increase their risk of developing the disease by a factor of two. As well as first hand smoking, passive smokers are also at risk of developing the disease (Rezende *et al,* 2010). When studied, 1114 patients from 4 separate areas of the USA, who drank alcohol and smoked, were found to have increased their risk of the development of HNC by 35 fold (Blot, 1998). Secondly the type of drink taken was a variable factor, where hard liquor was a higher risk factor than beer (Blot, 1998). Genetics may also add to this factor as polymorphisms that occur in genes that encode for the metabolism of alcohol and tobacco can be mutated which contributes to HNC development (Rezende *et al,* 2010).

As well as tobacco and alcohol, human papillomavirus (HPV) plays a strong role as a causative agent in the development of HNC (Argiris *et al,* 2008). HPV is a family of over 100 virus types and is a viral infection that spreads through contact with infected skin, body fluids and mucous membranes (Keogh, 2013). HPV 16 and 18 are the most significantly associated with the development of HNCs, whereas HPV 6 and 11 are less likely to be causative factors (Chen *et al,* 2008). There is a strong indication that oral HPV is likely to be sexually acquired (Goon *et al,* 2009). The risk factors of HPV related HNCs are similar to that of cervical cancers and include the number of sexual partners, practice of oral sex and history of genital warts (Syrjanen, 2010). It is also seen that 6+ sexual partners increases the risk from 1.25 to 1.54 for

men, and early age of first sexual intercourse increases the risk from 2.36 to 5.05 (Heck *et al,* 2010).

1.2 HNC management

HNC has various management techniques, which have gradually evolved from surgery being the main option (Bonner *et al,* 2006). Combination therapy is now more emphasized such as surgery followed by radiation, which is highly successful in those patients with early stage cancer (Garden *et al,* 2004). Secondly a combination of radiotherapy and chemotherapy is a standard of care for the treatment of more advanced squamous cell carcinoma of the head and neck and has demonstrated a better overall 5 year survival rate over radiotherapy alone (Riaz *et al,* 2013).

Cisplatin is a chemotherapy agent and when used alone or in combination with other chemotherapy agents such as fluorouracil, which produces clear improvements in locoregional control and is associated with higher longer survival rates in 60-90% of patients (Forastiere, 2013). This however, is still not as effective as chemoradiation, which when studied in multiple phase III clinical trails showed better overall survival rates (Forastiere, 2013).

A downfall to the use of chemotherapy is the associated toxicities. With cisplatin there is an increase of short-term adverse effects, such as tiredness, fatigue and nausea (Cancer Research UK, 2013)**.** Treatment can therefore, be majorly hampered by the long term major toxicicties, and adds difficulty when trying to intensify the treatment (Merlano *et al,* 2010). Patients that have

other medical conditions or are unable to withstand the effects of chemotherapy need alternative therapies that produce long survival rates without adverse effects (Merlano *et al,* 2010).

EGFR is abnormally overexpressed in epithelial cancers including HNCs, and this high expression is associated with poor clinical outcome (Bonner *et al,* 2006). In recent years the management of HNC has been advanced by the development of therapies using monoclonal antibodies (Kabolizadeh *et al,* 2012). Cetuximab is directed against the epidermal growth factor receptor (EGFR) and shows clinical activity in HNC (Kabolizadeh *et al,* 2012). Cetuximab works by inhibiting the ligand-binding domain of EGFR and sensitizes cells to the effects of radiation (Bonner *et al,* 2006). When studied by Bonner *et al* (2006) it was found the there was prolonged survival in patients treated with cetuximab and radiotherapy over those treated with radiotherapy alone in advanced stage disease. This offers a less toxic option for therapy (Riaz *et al* 2013).

HNCs have a huge impact on the quality of life of a patient and they often require long term care, which can place financial pressure on the institution providing the treatment (Hunter *et al,* 2005). This shows that it is highly necessary to develop advanced treatment methods to not only reduce mortality but to reduce complications from treatment by developing more targeted therapies such as monoclonal antibodies, which may be able to improve patient quality of life (Hunter *et al,* 2005).

1.3 Drug repurposing

At present pharmaceutical drug discovery is a very expensive process with the cost of developing one therapeutic soaring as high as $2billion and taking 10 – 15 years (Boguski *et al,* 2009). Another downfall to the current drug development programmes is that the number of new chemical and biological entities that are being approved by the Federal Drug Agency (FDA) has been declining since the late 1990's (Boguski *et al,* 2009). However drugs that have already been approved by the FDA, are eligible for approval for a second purpose that it's seen to be fit for (Boguski *et al,* 2009). This makes the process of drug development much quicker (Boguski *et al,* 2009).

From this it is very apparent that there is a need for a different approach to drug discovery, which is where drug repurposing becomes of huge interest. Drug repurposing is the development of existing licensed drugs for new indications (Sleigh & Barton, 2010). Repurposing therefore requires finding novel therapeutic indications of drugs compared to the initial use, for which, they were designed and approved for (Oprea & Mestres, 2012).

Repurposing programmes can make research and development less costly, more reliable and more predictable (Boguski *et al,* 2009). This predictability comes from existing clinical and pharmacokinetic data on drugs, which can be implemented into new repurposing, programmes and creates and accelerated route for drug discovery (Li & Jones, 2012).

Drug repurposing has already had a presence in the treatment of cancer, for example, the commonly used diabetes type II drug, Metformin (Gotlieb *et al,* 2008). Metformin has been evaluated for its efficacy in ovarian cancer cell lines it has been found to induce dose and time dependent inhibition of growth (Gotlieb *et al,* 2008). Secondly in patients, metformin decreases cancer incidence (Sahra *et al,* 2010). Anti proliferative effects of metformin have been observed in cancers of the breast, ovary, glioma, prostate and colon, showing its wide variation in possible cancer therapies from a repurposing programme (Rattan *et al,* 2009).

Another example of a repurposed drug is a sedative, called thalidomide, which was used for morning sickness in the late 1950's, however patients who were taking the drug were found to have children born with severe birth defects and the drug was withdrawn from the market (Boguski *et al,* 2009). However at present the drug has been found to have a great use in leprosy and also showed anti-angiogenesis properties, which led to its use in the treatment of multiple myeloma (Shaughnessy, 2011).

Examples of repurposed medicines		
Drug	**Original use**	**Repurposed use**
Allopurinol	Antineoplastic	Gout
Amantadine	Antiviral	Essential tremor
Atomoxetine	Antidepressant	Attention deficit hyperactivity disorder
Bimatoprost	Glaucoma	Eyelash growth
Bromocriptine	Parkinson's disease	Type 2 diabetes
Colchicine	Gout	Post-pericardiotomy syndrome
Colesevelam	Cholesterol lowering	Type 2 diabetes
Doxepin	Antidepressant	Sleep
Metoclopramide	Gastro-oesophageal reflux	Migraine
Thalidomide	Sedation	Multiple myeloma

Figure 3: Shows repurposed drugs, with their initial use and repurposed use (Shaughnessy, 2011)

Repurposing of drugs is a multistep pathway that begins with the initial assessment of a potential drug candidate. Cell lines are screened to evaluate potential of possible drug targets, and these candidates can be taken forward for further investigation and clinical trials to validate and prove findings (Bogsuki *et al,* 2009). Repurposing programmes are a highly productive as although they involve vast amounts of specific research and clinical trials, the lessened cost and time taken to get a drug on the market compared to developing one from scratch, is of great interest. (Padhy & Gupta, 2011).

1.4 Mechanisms and uses of the target drugs

1.4.1 Colchicine

Colchicine is an alkaloid, derived from a plant source known as *Colchicium autumnale* (Neil & Schermann, 2006). Colchicine possesses anti-inflammatory and anti-mitotic properties (Neil & Schermann, 2006). Its initial purpose is in gout and Mediterranean fever as well as more recently cystic fibrosis and cirrhosis (Neil & Schermann, 2006). Gout is a type of arthritis, where crystals of sodium urate forms inside of the joints (NHS, 2012a) and Mediterranean fever is an auto-inflammatory disorder, which causes unprovoked inflammation (Rolden *et al* 2008). Colchicine is used due to its anti inflammatory properties (Neil & Schermann, 2006). The precise mechanism of action of colchicine is unknown (Ly *et al* 2007). However it is thought to act by blocking the inflammatory response, which prevents uric acid crystal formation in gout (Ly *et al* 2007). Secondly it is thought to act by inhibiting cellular pathways in the inflammatory response to stop the secretion of cytokines (involved in promotion of cell signaling) and chemokines (involved in promoting migration) (Neil & Schermann, 2006).

Colchicine also possesses anti mitotic properties, which works by preventing tubulin incorporation into microtubules, which in turns prevents the elongation of microtubules and disrupts the mitotic spindle during cell division (Neil & Schermann, 2006). This halts cell division and induces cell cycle arrest leading to apoptosis of the cell (Zhou, 2005).

Microtubule drugs are important in cancer chemotherapy, as their specific ability to arrest the cell cycle and initiate programmed cell death in many tumour types can be highly beneficial, as it can inhibit aggressive tumour growth (Checchi *et al,* 2003). Microtubule targeting agents are already successfully being used clinically to treat cancers and open a huge interest in pursuing new options for therapy in other cancers as well as improving the efficacy and toxicity profiles of the existing drugs (Migralese & Carlson, 2006).

This mechanism can therefore be used to target cancer cells and induce apoptosis, which in a previous study by Chen *et al* (2012) where in normal liver cells, colchicine was able to induce a loss of mitochondrial membrane potential, which is an early apoptosis event, which in turn activated caspase 3 and cause apoptosis. This may explain other studies where colchicine has had a positive effect in treatment of breast, cervical, lung and gastric cancers (Chen *et al,* 2012).

1.4.2 Zinc Acetate

Zinc acetate is a salt of its pure form, zinc (NCBI, 2013) and its main use is in Wilson's disease, which is an inherited disorder caused by the accumulation of copper in the brain/liver (NCBI, 2013). The accumulation of copped is toxic and in worst-case scenarios leads to liver failure (Wilsons Disease Support Group, 2012). Zinc acetate works by inducing metallothionein, which when bound to zinc, prevents the absorption of copper from the intestinal tract and endogenously secreted copper to reduce copper levels (EuroWilson, 2012).

Zinc acetate can also be found in lozenges for the common cold where it gives a reduction in nasal discharge and coughing (Fitzgerald & Bao, 2000), which is thought to be by inhibition of viral replication (such as the rhinovirus (Hulisz, 2004) by interfering with protein cleavage (Jackson *et al,* 2000).

In studies carried out in prostate cancer, zinc acetate has shown to have a positive effect on tumour volume in mice, however the results obtained in human studies are highly inconsistent (Gonzales *et al,* 2009).
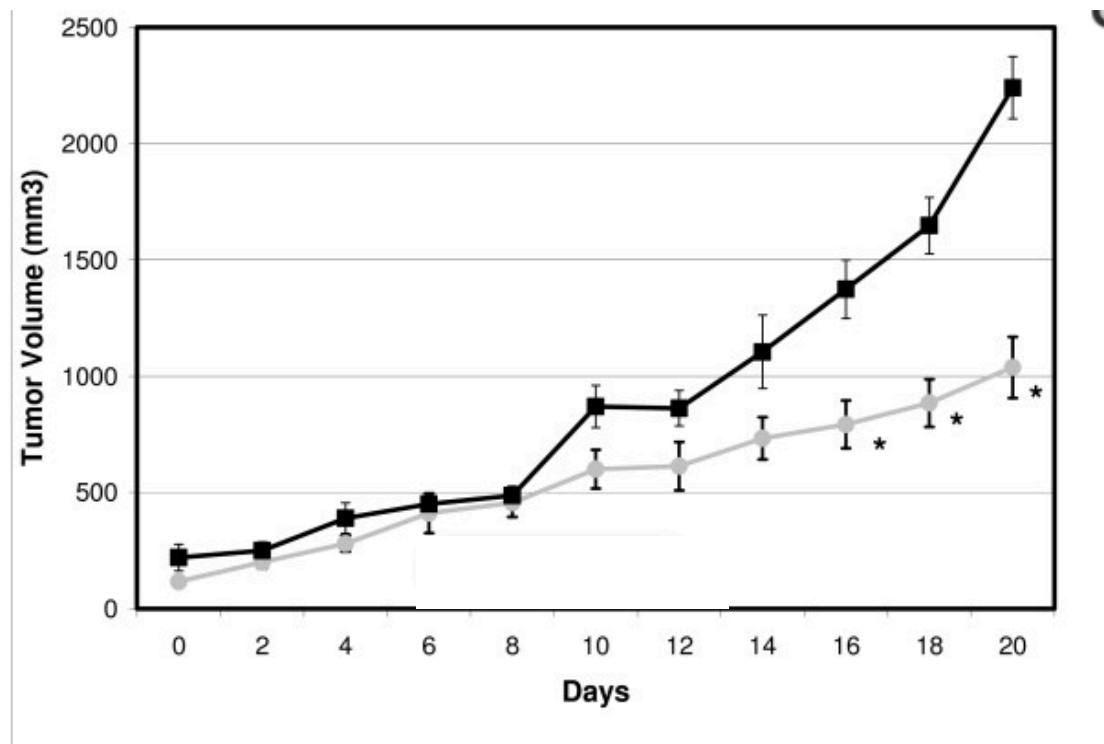

Figure 4: Black Line: Saline Control, Grey Line: 3mM zinc acetate. Figure showing a study carried out on mice for prostate cancer, where there is a reduction in tumour volume (mm³) over the 20-day period with zinc acetate, compared to the saline control (Gonzales et al, 2009).

Zinc in the extracellular environment of cells is highly important in the transformation of healthy to malignant cells. In normal cells it is broken down to Zn^{2+} molecules and it enters epithelial cells via ZIP transporters where it inhibits m-acotinase and prevents oxidation of citrate in the Krebs cycle. Secondly Zn^{2+} induces Bax to form pores in the outer mitochondrial membrane to allow cytochrome c to move through and interact with caspases to cause apoptosis. However in malignant cells, there is a reduced influx of Zn^{2+} and a lower intracellular zinc content and citrate is oxidized in the Krebs cycle. Without this Bax is unable to form pores to allow cytochrome c to enter, so the pro-apoptotic effect of zinc is abolished leading to energy efficient proliferating cells (Franz *et al,* 2013). This mechanism is shown in Figure 5. This gives a strong understanding of the importance of zinc and its pro-apoptotic effect in healthy cells.
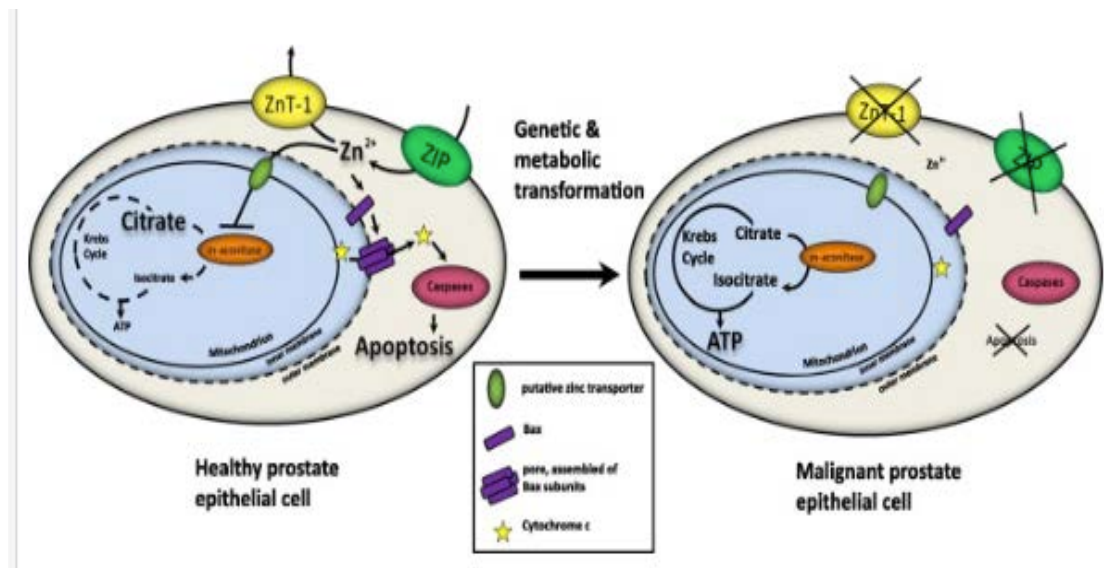

Figure 5: Zinc acetates role in the transformation from healthy cells to malignant cancer cells (Franz et al, 2013)

This mechanism highlights the importance of zinc in the extracellular environment. Zinc homeostasis is an important factor as zinc is essential for cell proliferation, differentiation and the regulation of mitosis and DNA synthesis (Bayersmann & Haase, 2001). Secondly, it is a structural component of many proteins including proteins involved in cellular signaling pathways and transcription factors, it is essential to maintain zinc levels correctly for the healthy function of a cell (Bayersmann & Haase, 2001).

Zinc is able to modulate cellular signal recognition and can stimulate/inhibit activity of transcription factors, which shows the importance of zinc in the healthy function of a cell (Bayersmann & Haase, 2001). The homeostatis of zinc and its ability to regulate cell function is potentially behind the mechanism of action behind zinc acetate as a potential cancer therapy agent. This is because, a disruption in the regulation of zinc may lead to the inability of the cell to function correctly, so increasing the uptake of zinc, may cause induced cell death.

1.4.3 Paracetemol

Paracetemol, also known as acetaminophen is one of the most popular and widely used drugs for treating pain and fever (Prescott *et al,* 2000). Paracetemol has been used clinically for over a century (Clissold *et al,* 1986) and has a clearly established role as an analgesic for mild or moderate pain (Prescott *et al,* 2000). Following administration it is absorbed from the gastrointestinal tract and distributes evenly throughout most tissues and fluid (Forrest *et al,* 1982). The action of paracetemol at a molecular level is not

clear (Graham & Scott, 2005) but is generally considered as a weak inhibitor of prostaglandin production leading to its analgesic properties (Clissold *et al,* 1986).

In therapeutic doses paracetemol is seen as a safe drug that produces fewer side effects than a drug such as aspirin (Clissold *et al,* 1986). However one of the main concerns associated with it is that in overdose it can cause severe hepatic necrosis (Forrest *et al,* 1982) or hepatotoxicity (Cllssold *et al,* 1986). This however only occurs when doses over 10g-15g are taken (Clissold *et al,* 1986).

In terms of cancer, paracetemol is commonly used to alleviate pain as it causes minimal added side effects (Stockler *et al,* 2004). However there is some concern with the carcinogenic potential of paracetemol due to it being the major metabolite of phencacetin, which has been identified as a human carcinogen by the International Agency for Research on Cancer and due to this was withdrawn from the market in many countries (Friis *et al,* 2002). Due to this link between malignant tumours, mainly of the urinary tract, and phenacetin, paracetemol studies have also focused on these cancers (Friis *et al,* 2002). Results for paracetemol in urinary tract cancer have been inconsistent (Friis *et al,* 2002). However any increase or decrease in cancer risk from the drug, due to its widespread use, would have huge public health implications and therefore is worth characterizing further (Friis *et al,* 2002).

Paracetemols use in cancer therapy is fairly uncharacterized and doesn't have a link into how the drug may possibly interplay in cancer therapeutics. This is a further reason to characterize and understand the mechanism, if any, that paracetemol has on cancer cells lines/primary cells.

1.5 Aims and Objectives

The aims of this project are 3 fold. Firstly I will screen zinc acetate, colchicine and paracetemol to determine if they have an anti-proliferative effect in head and neck cancer cell lines. Secondly I will explore and confirm their mechanisms of action using scratch wound assays and cell cycle analysis using flow cytometry. The results from these experiments should enable us to determine whether these drugs are useful as possible targets in repurposing programmes for head and neck cancer treatments.

2. Materials & Methods

2.1 Cell Lines

SCC040 (UPCI-SCC-040, DMSZ Serial #ACC660) oral squamous cell carcinoma cell lines were brought from the Leibniz institute DMSZ; a German Collection of Microorganisms and cell cultures. SCC040 is a HPV negative cell line established from an oral squamous cell carcinoma of a 50-year-old Caucasian male.

2.1.2 Maintenance of Cell Lines

Cell lines were maintained at 37°C with 5% CO_2 in Dulbecco's modified eagles medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma #F75524), 2mM L-glutamine (Invitrogen #25030-123) and 1% penincillin/streptomycin (Invitrogen #15070-063). Cell lines were incubated in a 75cm^3 flask. Cells were split 1:5 twice a week.

2.1.3 Preparation of Cell Lines

Media was removed from the flask and cells were washed with 5ml of Phosphate Buffer Saline (PBS). PBS was removed and 2ml of TrypLE™ express dissociation reagent (Life Technologies, Serial #12605-010) was added to the flask. Cells were incubated for 5-10minutes at 37°C and 5% CO_2. 4ml of DMEM was added to the flask and cells were then transferred into a 15ml sterile tube, they were then spun down in a Starstedt LC24 centrifuge (Serial #77759) for 4 minutes at 1100 RPM at room temperature to form a pellet. The media was removed and cell pellet was re-suspended in 10ml of DMEM for use.

2.2 Drugs

Colchicine (Serial #C258000), paracetemol (Serial #P0300000), zinc acetate (Serial #96459) and Staurosporine (Serial #S4400), were purchased from Sigma Aldrich. Cisplatin (Serial #1550) was purchased from Cambridge Bioscience.

The three target drugs, colchicine, paracetemol and zinc acetate were firstly made as a stock solution. Stock solutions of the drugs were made using water for Colchicine and Zinc Acetate, and DMSO for Paracetemol, Staurosporine and cisplatin. Colchicine stock solution was made at a concentration of 0.175mM, paracetemol was made at 1323mM and zinc acetate was made at 1600mM. This stock solution was then stored at -4°C and thawed for use. These stocks were 10000x the working concentration for assays. The final concentration of drugs used in each assay was 1x. For colchicine this was 0.0175µM, for paracetemol this was 132.3µM and for zinc acetate this was 160µM.

Staurosporine and cisplatin stocks were prepared using DMSO. Staurosporine stock concentration was 1600mM, and cisplatin was 10mM. To prepare stocks for each assay the stock solution concentrations were diluted using DMEM.

Cyclohexamide was brought from Sigma Aldrich (Serial #C4859). Cyclohexamide was ready diluted in DMSO at a stock concentration of 100mg/ml.

2.3 Proliferation Assays

Proliferation assays were carried out in 96 well plates. To count the cells, 10µl of the 10ml prepared suspension (See 2.1.3) were added 1:1 to 10µl of Trypan Blue stain (0.4%) (Life Technologies, Serial #T10282). 10µl of this was added to a Countess chamber slide (75 mm x 25 mm x 1.8 mm; depth: 100µm) (Life Technologies, Serial #C10228). Cells were counted using an Invitrogen Countess®; automated cell counter (Serial #C10227).

Cells were diluted to a concentration of 25,000 cells/mL and plated at 100µl per well to give a final concentration of 2500 cells per well. Cells were incubated for 24hours at 37°C with 5% CO_2.

Treatments were added at 2x concentration in 100µl media to give a final well volume of 200µl and a final concentration of 1x. Treatments were as follows; DMEM (Negative untreated control), DMSO (vehicle control for Paracetemol/Cisplatin), Water (Vehicle control for zinc acetate and colchicine), Zinc Acetate (160µM), Colchicine (0.0175µM), Paracetemol (132.3µM), Cisplatin (5µM), Cisplatin (10µM) and Cisplatin (15µM).

As a negative control, media only plates were prepared to mimic the cells plate for each time point. For this 100µl of media was treated with 100µl of each of the treatments. This value would be deducted from final fluorescence readings to eliminate the effects the drugs may have on auto fluorescence.

Separate plates were made for each time point (1x media plate and 1x cell plate) for each of the three time points (24hours, 48 hours, 72 hours).

To measure proliferation Alamar Blue cell viability reagent (Life Technologies, Serial #DAL1025) was added 1:10 (20µl per well) 4 hours before the time point (20hr for 24hr time point, 44hour for 48hour time point and 68hour for 72hour time point) and incubated with the reagent at 37°C with 5% CO_2.

Plates were de-lidded and read using a Wallac Victor[2] 1420 (Serial #1420-832) multi label counter plate reader for fluorescence at 550/590nm.

The assay was repeated three times, and within each assay cells were treated in triplicate. An average of the triplicate technical repeat readings and an average from the 'no cells' plate were taken. The average of the no cells plate was deducted from the cells plate for each treatment. These values were converted to percentages of the untreated control at the same point.

2.4 Migration Assay

From the 10ml suspension (prepared in 2.1.3), cells were counted and diluted to 100,000 cells per ml using DMEM. 2mL of cells were plated in a 12 well assay plate (200,000 cells per well). Cells were incubated for 24hours at 37°C and 5% CO_2 until a confluent monolayer was formed. Media was removed and 1ml of fresh DMEM was added to each well.

Treatments were added at 2x concentration in 1ml media to give a final well volume of 2ml and a final concentration of 1x. Treatments were as follows: DMEM (Negative untreated control), DMSO (vehicle control for Paracetemol/Cisplatin), Water (Vehicle control for zinc acetate and colchicine), Zinc Acetate (160µM), Colchicine (0.0175µM), Paracetemol (132.3µM), Cisplatin (15µM), Staurosporine (2µM), Staurosporine (4µM), Cyclohexamide (3µM) and Cyclohexamide (5µM).

After 24hours of drug treatment, a scratch wound was created using a pipette tip to scratch through the cell monolayer vertically. Cells were imaged at 0hour and 24hour using a Nikon Eclipse TS100 microscope at 10x magnification and a bright field light. Images were captured using Q-capture image software. 3 images for each treatment were taken.

The assay was repeated three times, and within each assay cells were imaged in triplicate. The area of the scratch wound was calculated using FIJI is just image J image processing package and an average of the triplicate images was calculated. This was compared to the area at 0hr to calculate the percentage closure from 0hours to 24hours in each treatment type. Readings were calculated as percentage closure compared to the untreated control.

2.5 Flow Cytometry; Cell cycle analysis

From the 10ml suspension (prepared in 2.1.3), cells were counted and diluted to 50,000 cells per ml using DMEM. 2ml of this was then plated into a 6 well

assay plate, and cells were incubated at 37°C and 5% CO_2 to settle and adhere for 24 hours.

Cells were then treated (3 wells per treatment) 1:1 in triplicate with 1ml of the following treatments: DMEM (Negative untreated control), DMSO (vehicle control for Paracetemol/Cisplatin), Water (Vehicle control for zinc acetate and colchicine), Zinc Acetate (160µM), Colchicine (0.0175µM), Paracetemol (132.3µM) and Cisplatin 15µM.

Cells were then incubated for 16hours or 48hours at 37°C and 5% CO_2.

Media from each triplicate well for each treatment was removed and stored in a 15ml falcon tube. Cells were then washed twice in 1ml of cold PBS to prevent cells clumping. PBS was removed and 500µl of TrypLE™ express dissociation reagent was added to the well and were incubated for 5-10minutes at 37°C and 5% CO_2. Triplicate wells for each treatment containing 500µl TrypLE™ were combined in this step and added to the previously removed media for each specific treatment.

Cells were then spun down in a Starstedt LC24 centrifuge (Serial #77759) for 4 minutes at 1100 RPM at room temperature to form a pellet. Media was removed from the falcon tube and the pellet was re-suspended in 500µl of cell cycle buffer (10µg/ml propidium iodide, 10^{-4} M sodium chloride and 1% triton x100 in distilled water). Samples were transferred into FACS tubes (BD Falcon™ 5ml Polystyrene Round-Bottom Tubes 12x75mm, Serial #352052)

and stored at 4°C and protected from the light. Samples were analyzed by flow cytometry using the Becton Dickinson FACS Calibur machine and Becton Dickinson Cell Quest software, after 1 hour.

Samples were vortexed before running. Samples were run for 20,000 events and resulting histogram plots were not gated. S phase was calculated using the following calculation:

S-phase = 100 – (G0/G1 x 2) + (G2/M x 2) + Sub-G1 + Doublets

The assay was repeated three times and an average of the percentage of cells in each cell cycle phase was taken.

2.6 Statistical Analysis of Results

Triplicate results for the Alamar Blue assay and Migration assay were analyzed for statistical significance using a multiple comparisons One-Way ANOVA and post-analyzed using Dunnett's multiple comparisons test, using the GraphPad Prism 6 software.

Flow Cytometry data was analyzed using a paired one-way ANOVA, using the GraphPad Prism 6 software.

3. Results

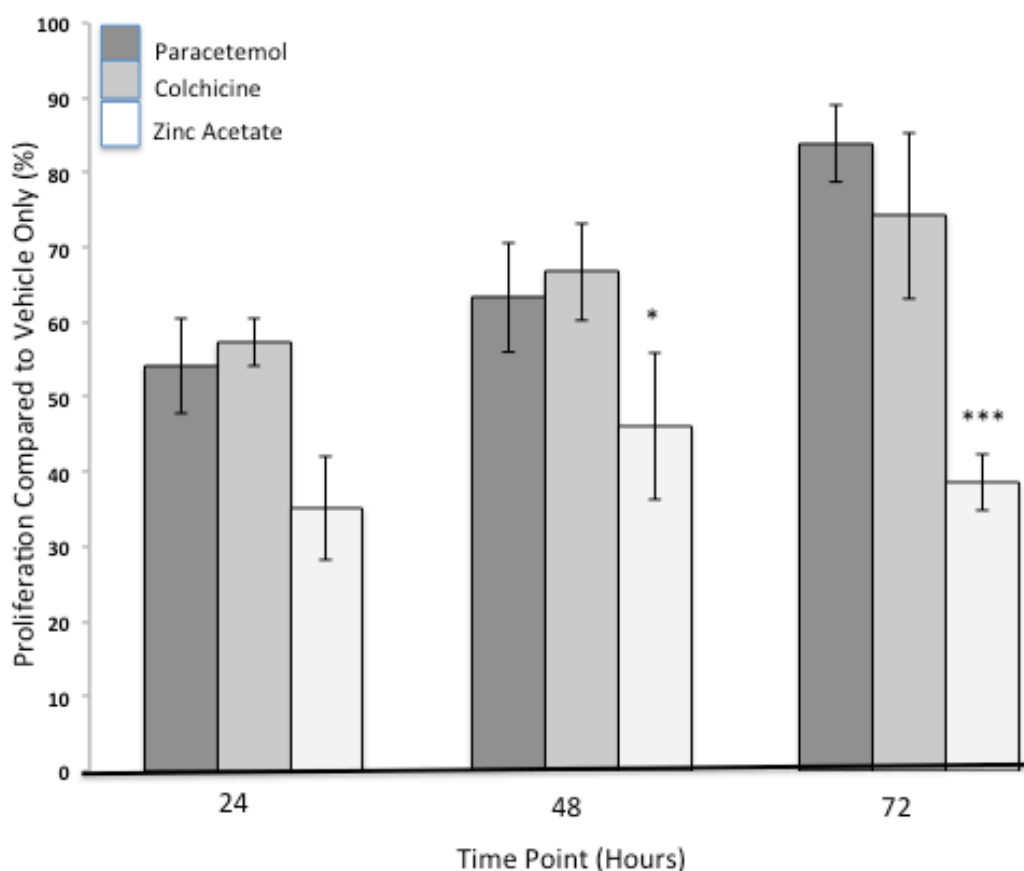## 3.1 SCC040 Alamar Blue Proliferation Assay

Figure 7: Percentage proliferation compared to the vehicle only control in SCC040 cell line, with standard error bars and statistical analysis using a one-way ANOVA. Drugs were statistically analyzed against their vehicle control (Water for colchicine and zinc acetate, DMSO for cisplatin and paracetemol). Results indicate a statistically significant anti-proliferative effect of zinc acetate at 48 hours ($p \leq 0.05^*$) and at 72 hours ($p \leq 0.001^{***}$) against the vehicle control (DMSO).

From *figure 7;* at 24 hours, from the statistical analysis, there is no significant difference between zinc acetate, colchicine, paracetemol and their vehicle controls (Zinc acetate and colchicine vehicle control is water, and paracetemol is DMSO). By 48 hours, paracetemol and colchicine both show no significant difference from their vehicle only control, DMSO and water, respectively. Zinc acetate however, shows a $34.11 \pm 8.98\%$ decrease in proliferation compared to water ($P \leq 0.05$). Finally at 72 hours Zinc acetate also shows a strong

statistical difference compared to its vehicle only control, with a 62.68 ±
3.75% decrease in proliferation compared to water (P≤0.001). This indicates
that from 48 hours to 72 hours the anti-proliferative effect of zinc acetate has
increased and is the only target drug to show a statistically significant
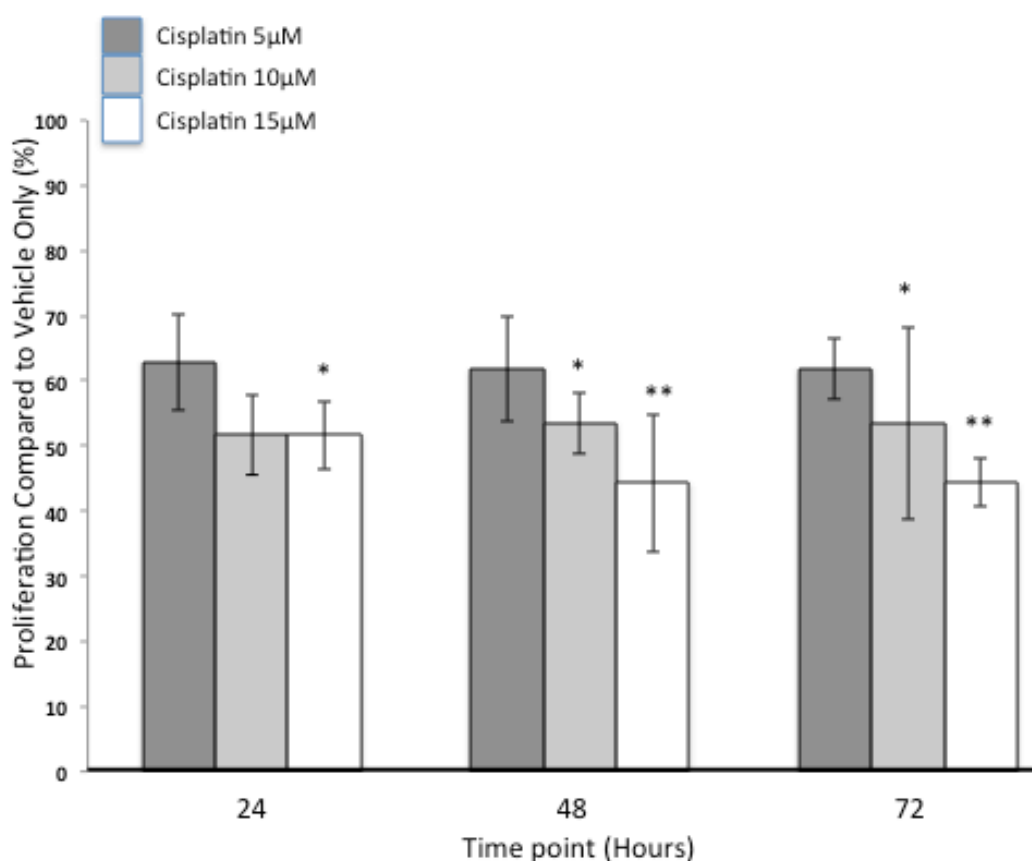prevention of proliferation, from the target panel.

Figure 8: Percentage proliferation of cisplatin treated cells, compared to
the vehicle only control in SCC040 cell line, with standard error bars and
statistical analysis using a one-way ANOVA. Results here the positive
controls for cisplatin 15µM at 24, 48 and 72 hours (p≤0.05*, p≤0.01** and
p≤0.01** respectively) and cisplatin 10µM at 72 hours (p≤0.05*) showed a
significant effect on proliferation against the DMSO vehicle control.

From *Figure 8,* at 24 hours, cisplatin 5µM and 10µM, both show no significant
difference from their vehicle control, DMSO. This shows that the DMSO is
acting on the cells to give their anti-proliferative effect, with the cisplatin
having no effect on this at this time point. However cisplatin 15µM, shows a

significant difference from its vehicle control ($39.10 \pm 5.34\%$ decrease in proliferation, P≤0.05). Indicating that cisplatin at this dose is having an anti-proliferative effect on the cells. At 48 hours, Cisplatin 5µM shows no significant difference compared to its vehicle control DMSO. However cisplatin 10µM and 15µM, both show significant differences in proliferation from DMSO (($40.65 \pm 4.68\%$ decrease, P≤0.05) and ($49.07 \pm 10.56\%$ decrease, P≤0.01) respectively). Finally at 72 hours, cisplatin 10µM and 15µM again showed a significant difference from their vehicle only control (($41.29 \pm 14.74\%$ fold decrease, P≤0.05) and ($60.47 \pm 3.69\%$ decrease P≤0.01) respectively).
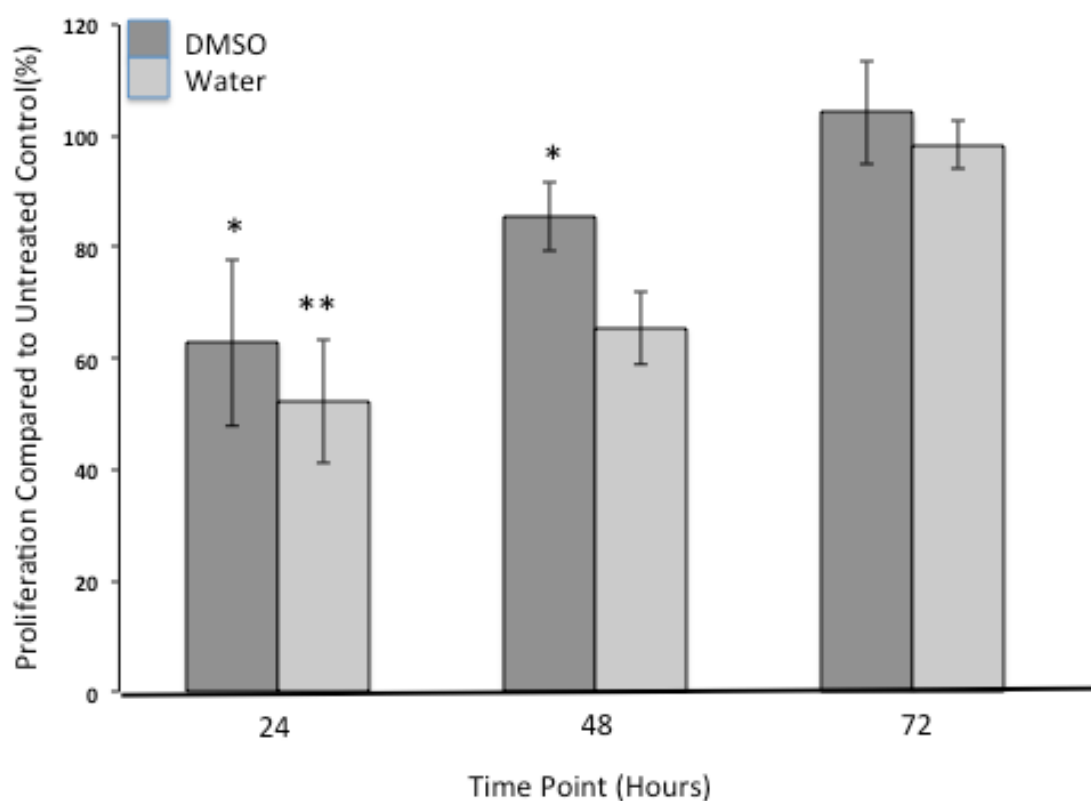

Figure 9: Percentage proliferation compared to the untreated control in the SCC040 cell line with standard error bars and statistical analysis using a one-way ANOVA. Results also show a significant effect of DMSO at 24 and 48 hours (p≤ 0.05*) and for water at 24 hours (p≤0.01**)

As seen in *figure 9* at the 24-hour time point, there is a significant difference between the untreated and DMSO controls (34.32 ± 15.01% decrease, P≤0.05), as well as the untreated and water controls (46.86 ± 11.12% decrease, P≤0.01). This indicates that at this time point, the vehicle controls were affecting cell proliferation. At 48 hours, there is no significant difference between the untreated control and the vehicle only controls. This shows that at this time point the DMSO and water are having no effect on the cells, as there is no difference compared to the untreated control. These results suggest that the cells at 24 hours are highly sensitive to any treatment as the cells are seeded at a low density and therefore are subject to being affected by any changes in their environment, such as the addition of low concentrations of DMSO.

3.2 SCC040 Migration Assay
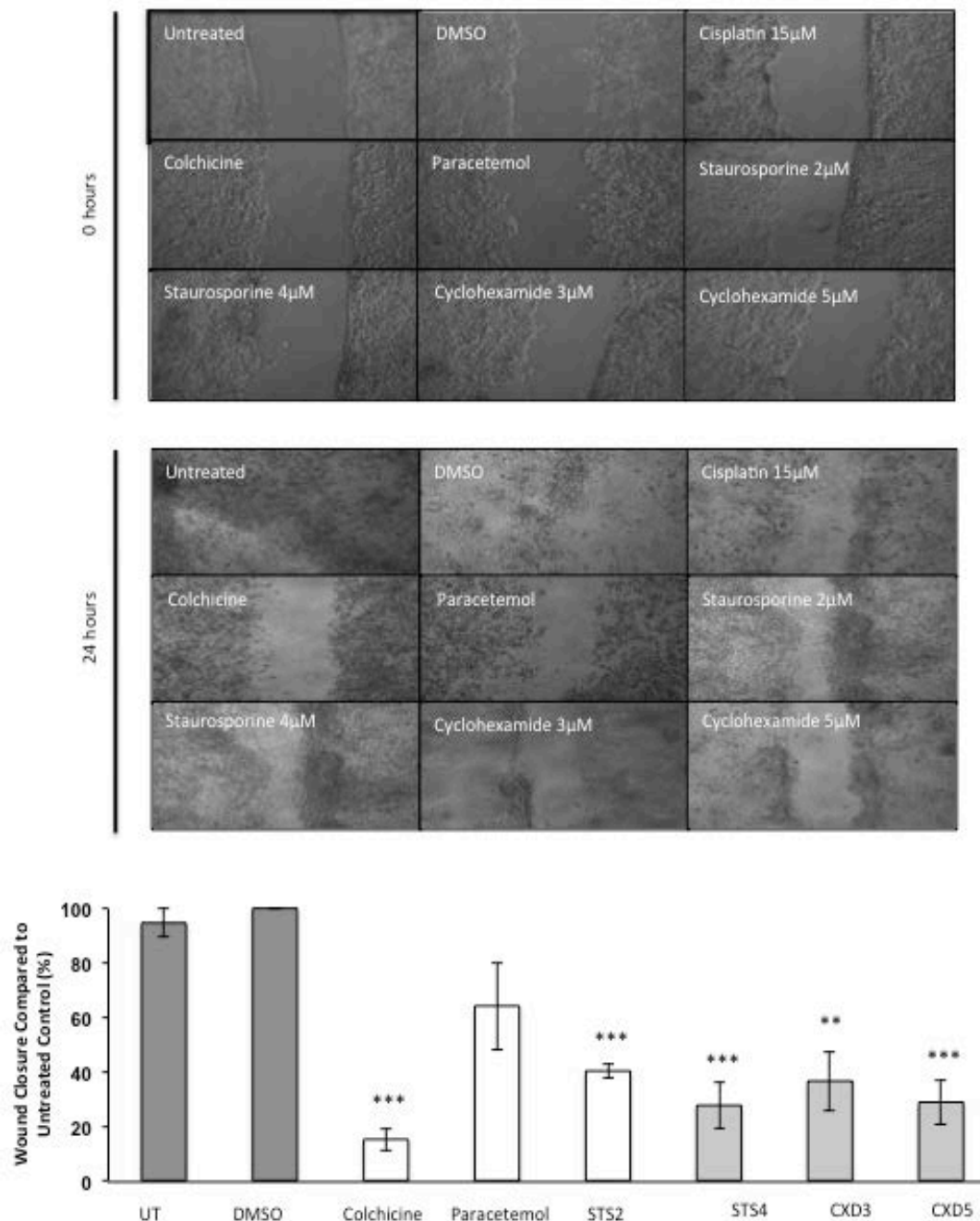
Figure 10: A. A representative panel of images for 0hr and 24hr time points in the SCC040 migration assay. B. Average wound closure at 24hours in SCC040 cell line, with standard error bars and one-way ANOVA statistical analysis compared to the untreated control. Results indicate a significant difference in wound closure between colchicine and UT(Untreated control). ($P \leq 0.001$). There is also a significant difference in wound closure between staurosporine 2μM /4μM (STS2/STS4) and UT ($P \leq 0.001$) and between cyclohexamide 3μM/5μM (CXD3/5) ($p \leq 0.01$ and $p \leq 0.001$ respectively) and UT.

From *Figure 10* it is possible to see that there is no significant difference between the two negative controls (DMEM and DMSO). Staurosporine and Cyclohexamide were also used as positive controls and show fairly similar

results in terms of percentage closure after 24 hours. Statistical analysis shows, Staurosporine 2µM and 4µM and Cyclohexamide 3µM and 5µM are all significantly different from the untreated control. Staurosporine 2µM shows a 54.42 ± 2.47% difference in wound closure (P≤0.001) and Staurosporine 4µM shows a 67.06 ± 8.4% difference in wound closure (P≤0.001) compared to the untreated control. Cyclohexamide 3µM shows a 58.11 ± 11.02% difference in wound closure (P≤0.01) and Cyclohexamide 5µM shows 65.87 ± 8.13% difference in wound closure (P≤0.001) compared to the untreated control. This shows the anti-migratory effect of these drugs on the SCC040 cell line and validates the assay as functioning correctly.

Paracetemol shows no significant difference in wound closure from the untreated and DMSO control, indicating that this drug has no effect on wound closure. Colchicine has a significant effect on wound closure compared to the untreated control at 24 hours (79.55 ± 4.02%, P≤0.001) This shows that colchicine inhibits the migration of SCC040 cells.

3.2.1 Migration Assay - Zinc Acetate Treatment
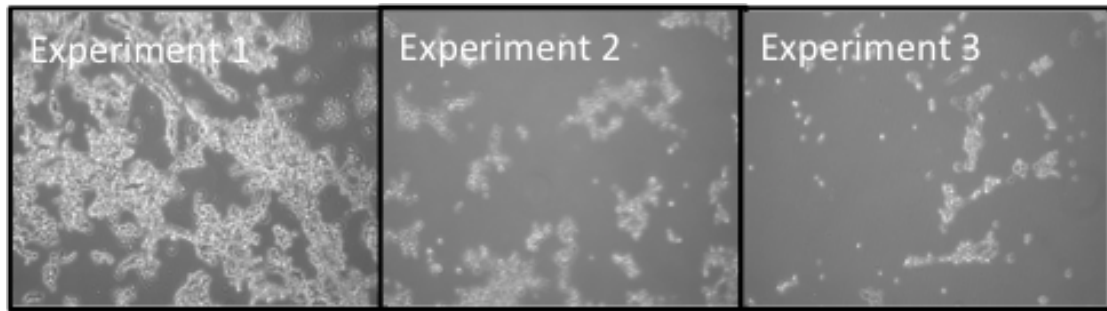
Figure 11: a panel of representative images from 3 migration scratch wound assays, with zinc acetate treatment on cells. From this panel, images show that the wound is no longer in tact and cells were no longer adherent, and were dead. This indicates zinc acetates action causes cell death by 24 hours.

Zinc acetate treatment was also carried out on SCC040 cells. These assays were performed in triplicate with triplicate images for each treatment in each experiment. The date for zinc acetate was not included because there was only one experiment where zinc acetate treated cells were able to be imaged in a wound shape, as in the other in the other experiments cells were dead as shown in *Figure 8.* This result therefore indicates that zinc acetate in most cases kills the cells by 24 hours, therefore its effect on migration cant be measured.

3.3 Cell Cycle Analysis Histograms via Flow Cytometry

3.3.1. SCC040 - 48hours Post Treatment Cell Cycle Analysis

A

B

Figure 12: A. Flow cytometry histograms profiles. B. Bar chart showing total events in each cell cycle phases. Results show that at 48hours, all treatments were dead. Untreated, DMSO and water all indicate a healthy cell cycle profiles.
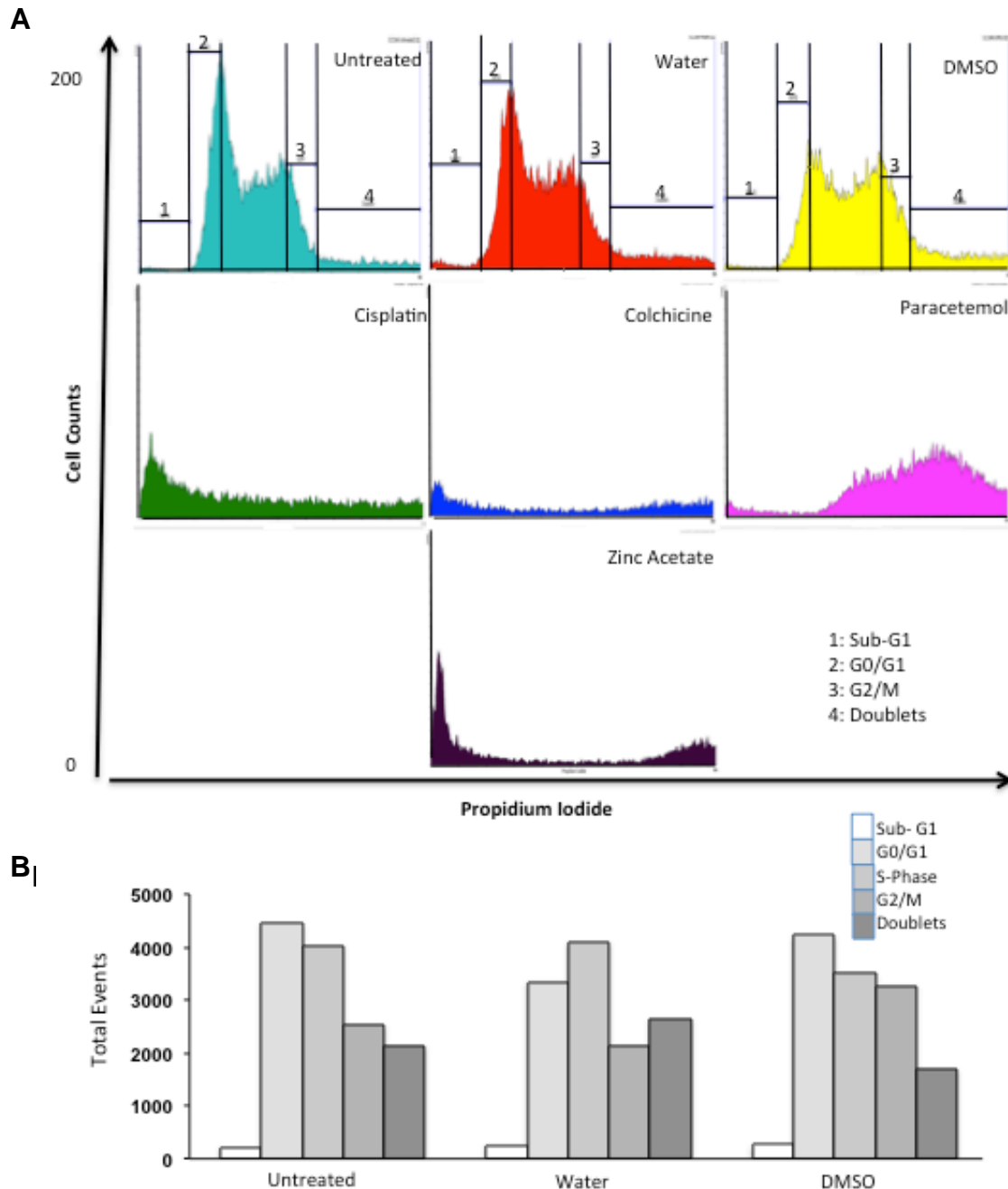
From *Figure 12* it is possible to see that in the treatments (colchicine, paracetemol, zinc acetate and cisplatin) that the cell cycle histogram plots are

indicating that the cells are dead by this time point. Untreated and the vehicle controls, DMSO and water, all show a healthy cell cycle by 48hours.

A ### 3.3.2 SCC040 – 16 hours post treatment cell cycle analysis
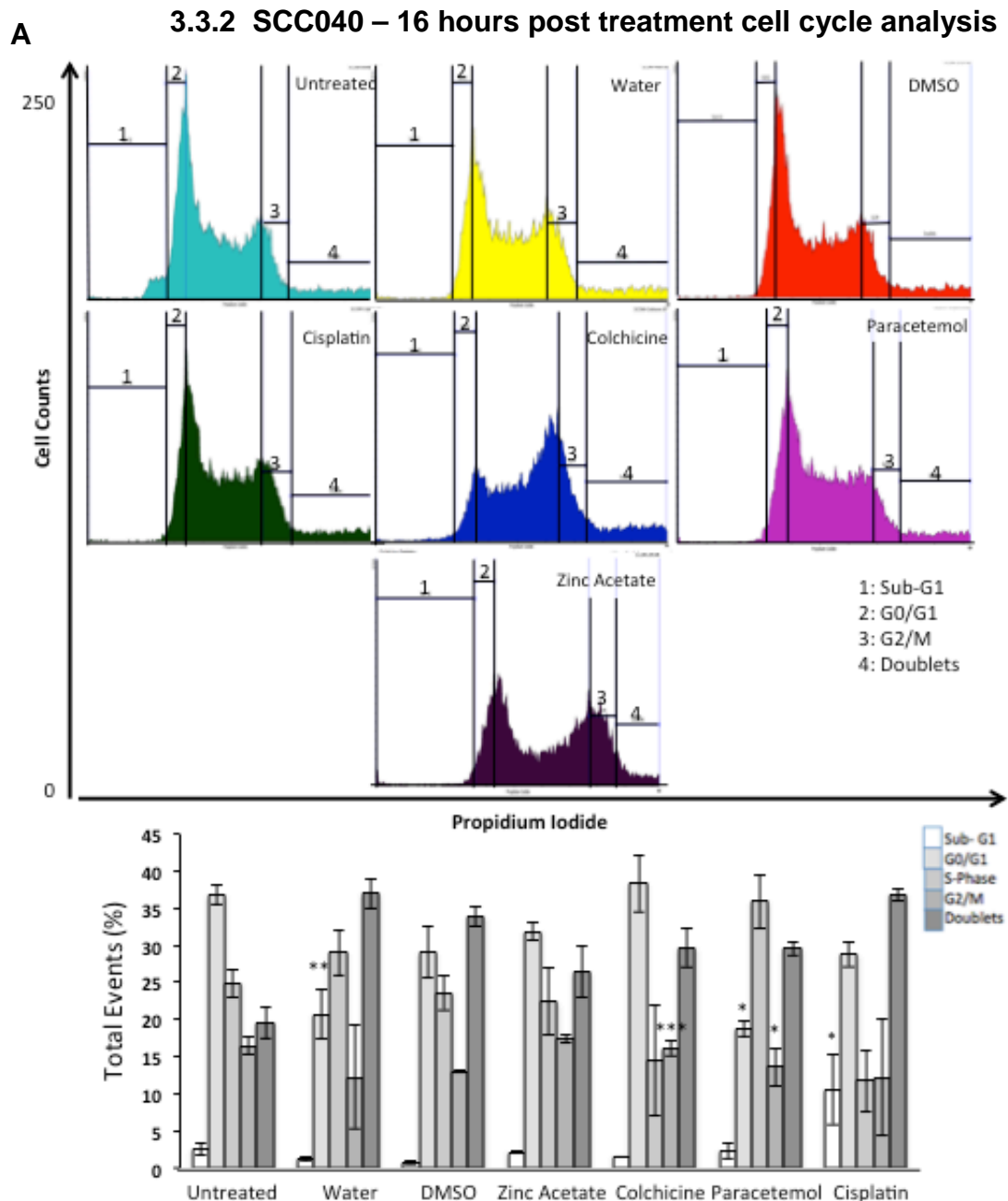
Figure 13: A. Flow cytometry histogram plots for each treatment, with gated markers for each cell cycle phase. B. Bar chart to show the total events (%) of cells in each phase with standard error bars and statistical analysis via a one way ANOVA. Results show there to be a significant difference between Water: G0/G1 phase and untreated G0/G1 (p≤0.01). There is also a significant difference between untreated G2/M and Colchicine G2/M (p≤0.001). In the paracetemol treatment, both G0/G1 and G2/M are signifincatly different from untreated G0/G1 and G2/M (p≤0.05). Lastly, in the cisplatin treatment the Sub-G1 phase is significantly different to the untreated sub-G1 phase (p≤0.05)

From *Figure 13* it is possible to see that there is a G2/M cell cycle block with the colchicine treatment at 16 hours ($p \leq 0.001$) compared to the untreated control Secondly paracetemol also shows 2 cell cycle shifts, at G0/G1 ($p \leq 0.05$) and G2/M ($P \leq 0.05$) compared to the untreated control. Cisplatin displays a statistically significant difference in the Sub-G1 phase compared to the untreated control ($p \leq 0.05$), indicating that there are a larger proportion of cells that are dead in the cisplatin treatment that in the untreated control. Lastly, water shows a significant difference in the G0/G1 phase of the cell cycle against the untreated control ($p \leq 0.01$). When compared to the untreated control, the other phases of the different treatments showed a non-significant difference in cell counts, which leads to the results indicating that there are only significant differences in some specific phases of the cell cycle.

4. Discussion

4.1 Anti-proliferative effects of Zinc Acetate

The results obtained for the Alamar blue proliferation assay bring forward a candidate with anti-proliferative effects in the SCC040 HNC cell line. Zinc has been previously characterized as an important factor in the regulation of cancer cell growth and proliferation by altering the extracellular environment (Lecane *et al,* 2005). For example, extracellular zinc when in abundance, both free or loosely bound can impact the cells metabolism, survival and growth due to various factors. These include, elevated levels of zinc inhibiting glycolysis, specifically via the glyceraldehyde phosphatedehydrogenase enzyme and also inhibition of the citric acid cycle via the a-ketoglutarate dehydrogenase complex, which all contribute to the healthy growth of cells (Lecane *et al,* 2005). This shows that, in the assay in this investigation, adding zinc acetate, may have increased the amount of zinc in the extracellular environments, which may inhibit these key pathways, that regulate cells normal growth and proliferation, leading to an anti-proliferative effect,

Zinc has been identified as a weak activator of extracellular signal regulated kinases (ERKs), which plays a role in the inhibition of cell growth, which occurs through the regulation of genes for proteins involved in the cell cycle such as $p21^{Cip/WAF1}$, however this mechanism is poorly defined (Park *et al,* 2002). From this the treatment with zinc used in this study may consequently lead to the activation or signal transduction of ERK pathways, which can in turn lead to an inhibited cell growth (Park *et al,* 2002). This supports the

findings in this study, where zinc may activate kinases to inhibit cell growth, as seen in the alamar blue assay.

In a previous study, when prostate carcinoma cells (LNCaP/PC-3 cell lines) were exposed to zinc, it resulted in necrosis of the cells, and the monolayer normally formed by the cells became detached and the viability of the cells was lost after 4-8 hours (Iguchi *et al,* 1998). This was previously seen in the migration assay when cells were treated with zinc acetate, showing that zinc acetate may have the same effect on various cell lines, due to its mechanism being linked with inhibition of key pathways that are needed for a healthy cells growth.

For many studies that have been carried out, they have looked at zinc alone, though zinc acetate is producing results similar to those found when only zinc was used. For example, when the effect of Zn^{2+} molecules was studied *in vitro* the proliferation of a mouse melanoma cell line was inhibited via the Zinc ions when used at 10-3M (Borovanksy *et al* 1985). This indicates that zinc may be broken down in the cell, to then bring forward its anti-proliferative effect.

In the study by Iguchi *et al* (1998) when prostate cancer cell lines were treated with zinc, necrosis of the cells was seen, which was determined by double staining with fluorescein-isothiocyanate-labeled annexin V and ethidium bromide. This may explain the results obtained in the Cell Cycle analysis for zinc acetate, as necrosis differs from apoptosis, and is characterized by no DNA fragmentation and the formation of apoptotic bodies, the methodology

used in this study would not be able to identify this possibility. Therefore to look at the necrotic effects of zinc acetate other methods of flow Cytometry based work such as Annexin V staining could be carried out.

4.2 Colchicine; an inhibitor of wound closure

From the results obtained in the scratch wound migration assay, it was possible to see that colchicine inhibited the migration of SCC040 HNC cells. In the alamar blue assay, colchicine had a non-significant effect on proliferation. This indicates that colchicine is has a more directed impact on the migration of cells than an anti-proliferative effect.

Colchicine is able to interact with tubulin and alter microtubule structure (Bhattacharyya *et al,* 2008). This is important because, microtubules are strongly implicated with the polarization of migrating cells, but specifically how they do this is unclear and their involvement remains to be controversial (Small *et al*, 2002). Past research has indicated a crucial role for microtubules in the maintenance and direction and migration of cells (Schliwa *et al,* 1999). Therefore altering the microtubule structure will have an effect on the cells ability to migrate.

In order to migrate cells require an interplay between microtubules and the actin cytoskeleton, which creates a polarization of the microtubules, which allows the microtubules growth at the leading edge of the cell, and shortening at the rear, for cell motility (Storer & Salmon, 1999). Cell movement itself is therefore regulated by polarization of the cell, the actin cytoskeleton and

adhesion complexes (Small & Kaverina, 2003). If this is primary interplay is inhibited, such as by colchicine, polarization of the cell cant take place, and in turn, motility is inhibited.

Colchicine is known to alter the structure of microtubules and therefore provides an understanding of its mechanism of action in the migration study carried out. In line with this in a study carried out by Ganguly *et al* (2013), on cells using a vinblastine, which also alters the function of microtubules, they found that there was little cell movement and cells were non directional. These studies suggest that suppressing microtubule function, destabilizes the leading edge of migrating cells and also the is interference with the tail retraction of the cell, and this destroys motility (Ganguly *et al,* 2013). In the same way as vinblastine, colchicine may therefore be altering the necessary microtubule network needed for migration as seen in this study's results.

Secondly, microtubule dynamics that are suppressed by drugs may affect locomotion as well as make the direction of movement more random. In a study by Ganguly *et al* (2012) under conditions where altered microtubule function was found cells along the edge of the wound in a scratch wound assay still elongated toward the wound, however the process was inhibited by their inability to retract the tail end, leading to a different directional movement, or no movement. This supports the results from the scratch wound assay carried out in this study, where the wound closure was inhibited, but this study also shows there is a possibility that colchicine alters directed movement and cells may have been migrating, but possibly away from the

wound. To evaluate this in this study, further research would need to be carried out using live cell imaging in order to study the movement and direction of the cells.

4.3 Colchicine cell cycle block

From the flow Cytometry data, it was possible to see that colchicine induced a potent G2/M block. Compounds that interfere with microtubules, such as colchicine, have already been implicated as chemotherapeutic agents from the treatment of cancer (Zhou & Ginannakakou, 2005). As seen in the results from this study, colchicine's interference with microtubule formation, results in spindle checkpoint arrest, leading to apoptotic cell death (Zhou & Ginannakakou, 2005). Microtubules are essential for mitosis, and colchicine is targets this in order to inhibit cell proliferation by blocking mitosis (Jordan & Wilson, 1998). However this result was not significantly seen in the proliferation assay from this study, though there is a decrease in cell proliferation seen in *Figure 7.*

In a supporting study by Blajeski *et al* (2002), when breast cancer cell lines were treated with 100nM colchicine, seven of the ten cell lines arrested in mitosis due to microtubule disruption and triggering of the mitotic checkpoint. This shows that in other cancers the same effect is being seen from colchicine treatment.

Colchicine interacts with tubulin and alters microtubule dynamics and this give a strong point of interest in HNC therapy, as although its use is limited due to

high toxicity in other therapies (Bhattacharyya *et al,* 2008), the results of this study give a possibility explore further, as its cell cycle blocking properties are now shown in HNC cell lines. This concept can therefore be taken forward and developed as a new treatment strategy that may be a more effective therapy option for HNC patients in the future (Zhou, & Ginannakakou, 2005).

4.4 Assay results combined findings

From the results gathered in this experiment, it was possible to see that zinc acetate was shown as an anti-proliferative drug and colchicine shown to hold anti-migratory properties. However in the proliferation assay, colchicine didn't show a statistically significant difference in proliferation, but in the cell cycle colchicine blocked the cell cycle in the G2/M phase at 16 hours and induced death in a large proportion of cells by 48 hours. However as seen in *Figure 10* there is a proportion of cells not seen on the left hand side of the histogram, therefore showing all cells are not dead and these cells have been analyzed in the proliferation assay and shown to proliferate over the 72 hours. In the same way, zinc acetate and paracetemol were mostly dead by 48 hours however a proportion of the cells in the histogram are in the living and therefore are able to proliferate as seen in the proliferation assay.

4.5 Further Research

The experiments conducted, looking into the anti-proliferative and anti migratory effects of the three target drugs, colchicine, zinc acetate and paracetemol could be expanded in further research. The results found regarding the anti-migratory effects of colchicine can be further developed

through determining the divide between the anti-proliferative effects of the drugs and anti-migratory effects. As the drugs are all shown to produce anti-proliferative effects in the HNSCC cell lines, a further expansion would be to use BrdU staining which is a marker for proliferating cells, that can identify cells proliferating from those that are migrating. Further to this analysis of invasion using Boyden Chamber invasion assays , which allow the movement of cells from one chamber into another can be used to determine is colchicine inhibits invasion secondary to migration.

Secondly the expansion of work in to the mechanism of action behind the targets can be taken further. Though the understanding of colchicine's mechanism of action has been partially determined, the use of flow cytometry using Annexin V and TUNEL could also be used to establish if the drugs are activating apoptosis. Alternatively the use of western blots could be carried out to determine if pro-apoptotic proteins are present such as caspases when treated with the target drugs. These further studies could then give a more all rounded understanding of the mechanisms that these drugs are producing an anti-proliferative effect via, as shown in the Alamar blue assay completed in this study.

Lastly the use of a control cell line, from another type of cancer, could be used in order to determine and confirm the specificity of these candidate drugs to head and neck cancer. Secondly additional cell lines could be used such as those that are HPV- and HPV+ or P53-/P53+ as these risk factors represent

separate HNC groups, so could be analyzed to see if results differ between groups or if response rates are better or worse.

5. Conclusion

In conclusion, from this study it has been possible to see that zinc acetate significantly inhibited proliferation of HNC SCC040 cells. Secondly through scratch wound healing assays, colchicine was identified as a potent inhibitors of migration. Cell cycle analysis using propidium iodide stain and flow cytometry indicated that colchicine blocked the cell cycle at G2/M phase which helped to further understand its mechanism of action in HNSCC cell lines. The results indicate that colchicine is a anti-migratory drug and G2/M block, whereas zinc acetate is an anti-proliferative drug. This has categorized these candidates into different sub sets subset of possible future therapy mechanisms. The research carried out in this study begins to explain the possibilities of the mechanisms of action behind the drug-repurposing candidates but has much room for expansion. However from this a basis of understanding of the mechanisms of action behind these candidates has been characterized. Further research can be carried out to investigate the mechanisms of action drugs have on other HNC cell lines or primary cells using other techniques such as BrdU staining or Annexin V staining and flow Cytometry. This would expand on this study and take a step forward in the drug-repurposing programme dedicated to these drugs, to understand their clinical relevance to a greater extent.

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