The effect of thiazinoquinones and poly (para-phenylene ethynylene) (PPE), poly (para-phenylene vinylene) (PPV) and poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers as anti-cancer or antimicrobial agents for drug repurposing.

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Declaration

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Abstract

The expensive nature of drug development and growth of antimicrobial resistance is resulting in the need for novel anti-cancer and antimicrobial treatments. While it is important to develop novel compounds, drug repurposing is also becoming an appealing option in finding new treatment. Two types of novel compounds, thiazinoquinones and poly (para-phenylene ethynylene) (PPE), poly (para-phenylene vinylene) (PPV) and poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers were evaluated as both anti-cancer drugs and antimicrobials. MTS proliferation assays and Annexin V/ propidium iodide assays were used to evaluate anti-cancer activity of both sets of the compounds. The antimicrobial activity of the PPE/PPV copolymers was evaluated by performing zones of inhibition, minimum inhibitory concentration and minimum bactericidal concentration assays. In addition, *Galleria mellonella* toxicity models were used to investigate the cytotoxicity of one of the PPE/PPV copolymers. Overall the thiazinoquinones did significantly reduce the survival rate of the cell population at the highest concentration in which, the sub category MC4 displayed more efficacy than the others. Thiazinoquinone MC4-176-1 decreased the surviving cell population to 0.2% at 100 μM. The MC4 sub category remained the most active in which MC4-176-2 reduced the viable population the most to 9.51%. In contrast to this, MC4-176-1 and MC8-28-1 had the highest early apoptotic population percentage. Overall the thiazinoquinones tested were prominently inducers of apoptosis in Jurkat cells. As the apoptotic pathways and the prevention of apoptosis is significant in the development of cancers, further investigation should be done into the mode of action of these compounds. The PPE/PPV copolymers were collectively ineffective at reducing the survival rate of the Jurkat cells below normal population levels so would not need to be investigated further however, they were found to be effective antimicrobials against a range of bacteria including *Escherichia coli,* Methicillin Resistant *Staphylococcus aureus* and *Enterococcus faecium*. The antimicrobial activity ranged in efficacy with each compound and bacteria; The zones of inhibition indicated a high efficacy of compound 003 and 033 against *L.monocytogenes* at 41.67mm and 51.67mm respectively. In addition compound 043 was overall a

more effective inhibitory and bactericidal antimicrobial against the range of bacteria in a planktonic state. Compound 043 was most effective at inhibiting the growth of *E. coli, MRSA* and *E. faecium* at concentrations of 19.5µg/ml, 83µg/ml and 78.1µg/ml respectively. The most bactericidal concentration of 043 was 39.1µg/ml against *E.coli*. Their lack of anti-cancer abilities suggests they may make a suitable topical antimicrobial as they do not cause cell damage and mode of action should be investigated. The galleria models indicated the compounds were not toxic at the highest level tested i.e. 50mg and should be taken through to use in a full infection model.

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Introduction

Drug repurposing

The research and development of drugs is a long, expensive and arduous task. The costs incurred for investigational compounds have been estimated to be around £172.7 million in a given developmental period of around 80.8 months (DiMasi et al., 2016). The repurposing of drugs that are currently in use are now being investigated to determine if they have other useful properties, for example the use of antimicrobials as remedies against cancer and *vice versa*. Repurposing drugs is particularly useful and its potential has been highlighted in the case of anticancer and antimicrobial treatments, due to the similarities in the disease mechanisms, for example progression of disease via increased cell proliferation and achievement of host wide metastasis (Benharroch and Osyntsov., 2012). Drug repurposing is viewed as a more efficient and cost-effective way to find new treatments (Soo et al., 2017). Significant examples of drug repurposing are Nelfinavir, an antiviral drug used in the treatment of human immunodeficiency virus (HIV), which has also demonstrated effectiveness in the treatment of Tuberous sclerosis complex (Dunlop et al., 2017). Another example is Mechlorethamine, a drug used in the treatment of many cancer types which has also been shown to have bacteriostatic and bactericidal activity against a number of bacterial species (Radojevic et al., 2012).

The hallmarks of cancer

Cancer is a disease arising from extensive changes to the genome and it is distinguished by mutations that produce oncogenes with a dominant gain of function and tumour suppressor genes with a recessive loss of function (Hanahan and Weinberg., 2000). The natural homeostatic ability of cells to proliferate or initiate apoptosis when necessary is essential for normal growth and development in the tissue but when this homeostasis is disrupted it leads to tumorigenesis (Hanahan and Weinberg., 2011). The first hallmarks of cancer were outlined by (Hanahan and Weinberg., 2000) and included sustained proliferative signalling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, resisting cell death and inducing

angiogenesis. Collectively most, if not all, of these attributes are manifestations present in cancer cells and are therefore an effective target for treatment (Hanahan and Weinberg., 2011).

Growth signalling and cancer

Sustaining proliferative signalling is a key factor for the development of cancer. A neoplasm occurs when cancer cells do not cease to proliferate which can be a direct result of the cell gaining independence from exogenously derived mitogenic growth signals (Engström et al., 2015). The growth signals are expressed mostly by growth factors that bind to receptors on the surface of the cell, typically containing intracellular tyrosine kinase domains. The receptors emit signals, which through intracellular pathways, control progression through the cell cycle (Balek et al., 2018). The intracellular communication in normal cells is not fully understood however, the signalling within cancer cells is better outlined (Dai et al., 2016). Cancer cells can acquire this independent capability in a number of ways; firstly they may produce growth factor ligands themselves resulting in autocrine proliferative stimulation. They may also indirectly self-stimulate by sending signals to surrounding normal cells which will then supply the growth factors (Feitelson et al., 2015). Alternatively, promoting cell proliferation can occur by increasing the amount of growth factor ligands on the cell surface, which results in the cell being hyper-responsive to normal levels of growth factor (Schneider and Wolf., 2009). Another way to promote proliferation is by stimulating secondary pathways within the cell to bypass the need for growth factor initially, for example a mutation effecting the B-Raf protein results in the stimulation of signalling between the Raf to mitogen activated protein (MAP) activated pathway (Duronio and Xiong., 2013). Negative feedback mechanisms are a homeostatic measure in which cells establish normal tissue growth. A disruption within these mechanisms can also result in the over proliferation of cells such as the disruption of the *Ras* genes which normally ensures proliferative action is intermittent (Fernandez-Medarde and Santos., 2011).

The ability to be independent of external growth factors is a key attribute for cancer cells, however they must also be able to bypass the action of tumour suppressor genes to successfully proliferate

(Kurayoshi et al., 2018). Tumour suppressor genes aid in the reduction of cell proliferation. Two common suppressor genes code for the RB (retinoblastoma- associated) and TP53 proteins which operate together to discern whether a cell should continue to proliferate, apoptose or activate senescence (Bykov et al., 2017). The RB protein is a key correspondent between the many signals involved in the cell cycle. RB1 protein prevents the cell cycle from continuing from G1 to S phase by regulating the function of cyclin and cyclin dependent kinase complexes (Huang et al., 2015). In cancer cells the inactivation of the RB protein enables the cell to continue proliferation without hindrance, especially in cases where external growth factors are involved as the RB protein typically transduces signals external to the cell. TP53 However receives intracellular signals to evaluate the cell status, if there are signs of irreparable damage or optimal conditions are not met then the cell cycle may be halted in G0 phase or apoptosis can be activated (Hainaut and Plymoth., 2013).

The immune system and cancer

Avoiding immune destruction, the innate immune system is equipped to eradicate the majority of small tumours that have developed (Finn., 2012). However, if a tumour is able to evade the immune response and given the opportunity to develop into a greater mass, it becomes undetectable by the immune system (Janssen et al., 2017). It has been noted that in patients that are immunocompromised there is a greater incidence of tumours as the innate immune system is impaired (Schulz., 2009). Each time a cell cycle is completed the telomeres at each end of the chromosome are slowly eroded away resulting in complete erosion and then senescence of the cells. In a tumour cell, an enzyme called telomerase is used which adds telomeres to the end of chromosomes resulting in continuous proliferation (Jafri et al., 2016).Tumour-promoting inflammation is an important factor which may occur with a microbial infection, autoimmune disease or in the presence of a toxin. This chronic inflammation can lead to the development of pro-tumour mutations and apoptosis resistance. The inflammatory mediator prostaglandin E2 is generated by COX2 which is over expressed in many cancer cells and therefore the tumour itself generates inflammation (Gao et al., 2014) . Inflammation at the tumour site is seen as both a

positive and negative incidence, the increase in inflammation increases the probability that the innate immune system will act on the cancer cells. However, leukocytes releasing growth factors and other indirect growth promoters at the same time may sustain the tumour (Grivennikov et al., 2010).

Metastasis

Activating invasion and metastasis is a significant factor of cancer related deaths (Hanahan and Weinberg., 2011). Metastasis can occur by several ways, there may be a breakdown of the adhesion between cells causing cells to break away from the tumour and the extracellular matrix may breakdown resulting in the invasion of the cancer cell into the surrounding tissue (Arvelo et al., 2016). These factors can cause the intravascation of the tumour cell into the circulatory system and from this there may be increased chemotaxis of the tumour cells by chemokines released from other sites such as the lungs or pancreas (Popper., 2016).

Tumour cells may induce angiogenesis using two cytokines which are vascular endothelial growth factor and basic fibroblast growth factor. The release of these cytokines results in the growth of new blood vessels at the site of the tumour leading to it eventually becoming vascularised which accelerates growth and metastasis (Ziyad and Iruela-Arispe., 2011). Genome instability and mutation is a key factor for the development of cancer. Genes such as p53 which intracellularly help to control cell proliferation can mutate and allow for the constant proliferation of cancer cells (Wang et al., 2015). The development of cancer may also be linked to genetic factors passed down in generations for example, BRCA1 and BRCA2 have been found to have a direct link to causing breast cancer (Adel Anis, 2015). These hallmarks are all potential targets for novel cancer treatments, each must be targeted differently depending on the cancer type (Hanahan and Weinberg., 2011).

Cancer cell death machinery – apoptosis and necrosis

With the development of new treatment, the mechanism of cell death is a significant factor to consider. The mechanism of cell death, for example apoptosis or necrosis, has an enormous effect on the efficacy and suitability of treatment in patients (Ricci., 2006). The most suitable mechanism is often debated as both has positive and negative arguments. Apoptosis or programmed cell death is

an essential part of a cells life cycle and has an important role in many vital processes (Pattani et al., 2015). This programmed cell death can be seen as a more suitable treatment as it a more controlled mechanism and is less likely to induce a negative reaction (Elmore., 2007). Apoptosis is activated by two main pathways, the extrinsic and the intrinsic pathways. The extrinsic pathway is mediated by death domain receptors that are a type of tumour necrosis factor (TNF) receptor such as TNFR1 and 2, FasL-Fas and TRAIL-DR4 or DR5. The extrinsic pathway is characterised by two models: the FasL/FasR and the TNF-alpha/TNFR1 models (Elmore., 2007). In these models, receptors cluster and then bind with the homologous trimeric ligand. After binding with the ligand, cytoplasmic adapter proteins are enlisted which display corresponding death domains that bind with the receptors. The binding of the Fas ligand to Fas receptor results in the binding of the adapter protein FADD and also the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP.FADD then associates with procaspase-8 via dimerization of the death effector domain. A death-inducing signalling complex (DISC) is then formed, resulting in the activation of procaspase-8 and the execution phase (Jin and El-Deiry, 2005).

The intrinsic pathway is initiated inside the mitochondria within the cell by positive and negative signals. When the cell is damaged by a positive stimulus such as radiation, exposure to toxins and hypoxia or by a negative stimulus such as lack of growth factors and cytokines it leads to apoptosis. Apoptosis is trigged intrinsically by the opening of the mitochondrial permeability transition (MPT) pore resulting in loss of mitochondrial membrane potential and release of two predominant groups of pro-apoptotic proteins (Elmore, 2007). The first group of proapoptotic proteins activated are cytochrome *c*, Smac/DIABLO, and HtrA2/Omi; The release of these activates the caspase-dependent mitochondrial pathway in which an apoptosome forms, Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9 (Jin and El-Deiry., 2005).

Alternatively necrosis as a mechanism of cell death is not a necessary part of the cell cycle and is accidental rather than programmed since necrosis is the breakdown of a cell that has been caused by an external factor and results in an inflammatory reaction (Vanlangenakker et al., 2008). In a

treatment perspective, the presence of an inflammatory reaction is the main concern of which mechanism is preferred. Inflammation at the site of a malignant tumour may be beneficial as it could cause more damage to the malignancy, however the inflammation can induce negative side effects in the patient which would make apoptosis the assumed more preferable mode of action (Fink and Cookson., 2005).

The serine/threonine kinase receptor-interacting protein 1 (RIP1) is an important factor in the activation of necrosis induced by ligand- receptor interactions (Galluzzi and Kroemer., 2008). In necrosis, the main effectors are calcium and reactive oxygen species (ROS). Necrosis is characterised by elevated cytosolic calcium levels which usually leads to extensive amounts of mitochondrial calcium, bioenergetic effects, and activation of proteases and phospholipases (Feoktistova and Leverkus., 2014). The presence of ROS results in damage to DNA and other proteins which then results in mitochondrial dysfunction, ion balance deregulation and loss of membrane integrity (Feoktistova and Leverkus., 2014). The loss of membrane integrity and its overall breakdown is also mediated by other proteins, such as acidsphingomyelinase (ASM), phospholipase A2 (PLA2) and calpains (Galluzzi and Kroemer., 2008). Lastly, necrosis leads to the release of factors that then result in action by phagocytes and a following immunological response (Festjens et al., 2006).

The cell cycle

In eukaryotic cells, cell division takes around 24 hours. The cell cycle is divided into two main stages, interphase and the mitotic phase. Interphase begins the cell cycle in G1 phase, During G1 phase the cell Is metabolically active, physically grows in size and copies its organelles. G1 is seen as the first gap phase, this follows into the S phase where the cell synthesises a full copy of the DNA in its nucleus while duplicating its centrosome which helps separate DNA during the M phase. G2 phase is the second gap phase, in this phase the cell continues to grow and generates more proteins and organelles whilst preparing for mitosis (Wenzel and singh., 2018). The M phase or mitotic phase only lasts around a hour but is the most significant phase of the cell cycle as it is when the cell divides its

copied DNA and cytoplasm to make two new cells. The M phase is usually followed by cytokinesis. (Wenzel and singh., 2018)

The cell cycle is regulated by extracellular signals such as growth factors and internal events during cell growth and DNA replication. A main point in the cell cycle where many cells may arrest their development is in late G1 phase, a phase also known as the restriction point. This restriction point is a key regulatory point at which many factors are assessed before the cell is allowed to continue into interphase. Throughout the cell cycle there are a number of checkpoints to ensure a cell will not divide in the presence of damaged or mutated genetic information. The checkpoints in the cell cycle consist of detection, sending a signal and an effector, and example of this is in G1 phase. In G1 phase DNA damage is self-signalling as it results in the activation of proteins by tumour suppressor genes such as p53, at this point the DNA may be repaired if the damage is minimal or apoptosis will be triggered.

Conventional cancer treatments

Historically, cancer treatments have targeted rapidly dividing cells via inhibition of DNA replication or cell division. Alkylating agents were some of the earliest groups of chemotherapeutics, with Cyclophosphamide still in use in many leukaemia treatment programs. However, not only do these agents effect normal cells, particularly rapidly dividing epithelial cells of the skin and digestive tract, but cells may also develop resistance to such agents via detoxification mechanisms and DNA repair mechanisms (Puyo et al., 2014).

There has been several treatments developed to target the individual hallmarks of cancer. Topoisomerase inhibitors were developed to target DNA replication therefore preventing cell division and causing cell death; Topoisomerase I inhibitors are mostly derived from camptothecin which is a plant extract (Pommier., 2006). This class of drug tends to prevent the re-ligation of single strand breaks in DNA during DNA synthesis. Examples of these are Irinotecan, which is used to treat colorectal and Topotecan is another compound used to treat cisplatin refractory ovarian carcinoma. (Lorusso et al., 2010)

Tyrosine kinase inhibitors are a targeted therapy against tyrosine kinase molecules which play an important role in regulating growth factor signalling and the signalling cascade (Arora, 2005). The inhibitors compete with the ATP binding site of oncogenic tyrosine kinases, the inhibitors are competitive and non-competitive depending on the type used. The PI 3-Kinase inhibitor wortmannin is a non-competitive inhibitor whereas LY294002 is a competitive inhibitor. DNA- dependent protein kinase inhibition results in abnormalities in DNA double- strand break repair (Arora, 2005). Imatinib is a tyrosine kinase inhibitor which was developed to treat chronic myeloid leukaemia (CML) (Smith., 2011) . Imatinib binds to BCR-ABL kinase domain, which is in an inactive conformation in a pocket reserved for the ATP binding site, thus preventing the transfer of a phosphate group to tyrosine on the protein substrate and the subsequent activation of phosphorylated protein (Sacha., 2013). As the result, the transmission of proliferative signals to the nucleus is blocked and leukemic cell apoptosis is induced (Iqbal and Iqbal., 2018).

Reactive oxygen species (ROS) is an important factor in many types of cancers, in which they can increase development and progression of a tumour. However, tumour cells also appear to need a specific balance of ROS as they release antioxidant proteins to maintain a lower level of ROS (Renschler., 2004). In addition, the location of the ROS generation and concentration is significant for the cellular functions of ROS in cancer. A treatment possibility is utilising this balance between ROS and antioxidants and increasing the level of ROS to damage the cell or also to increase antioxidants prior to maybe prevent the cancer growth (Liou and Storz., 2010).

Thiazinoquinones

With the development of organic polarography, organic compounds with redox abilities have been investigated. The organic compounds Thiazinoquinones, derived from a *Aplidium* sea squirt and found in New Zealand, have previously been evaluated and found to have the ability to form semiquinone radical species which can be reduced or oxidised in the presence of reactive oxygen species (ROS) and O_2 (Imperatore et al., 2017). This redox potential could suggest they may be useful as a cytotoxic agent. It has previously been seen that, although they are inducers of ROS, the scope

of their capabilities is dependent on their structure. For example it was seen regioisomers of the natural compounds had a higher inducing potential (Harper et al., 2015). Thiazinoquinones have previously been tested against Jurkat cells using a standard viability assay combining Annexin V and Propidium iodide stains. When tested it was found that they caused both apoptosis and necrosis, and the chemical property that defined the action was the placement of a geranyl side chain in ring-D of the molecules tested and the presence of the dioxothiazine ring determined the overall activity of the compounds (Harper et al., 2015). Novel isolates from other *Aplidium* species have also found to be active such as the Mediterranean ascidian *Aplidium* conicum of which the isolates Conicaquinones A and B were derived from. Conicaquinones A and B were found to be extensively cytotoxic to rat glioma cells (Aiello et al., 2003)

Antimicrobial treatments

Antimicrobial resistance (AMR) is a huge, global health concern. The overuse/misuse of antibiotics and antimicrobials has resulted in multidrug resistant strains of several pathogens. Therefore it has become necessary to look to other agents such as novel organic and inorganic compounds to find a new class of treatment. Research into finding novel treatments for antimicrobial resistant bacteria is now a paramount concern of the World Health Organisation of whom recently released a list of their most concerning bacteria such as *Escherichia coli* and *Staphylococcus aureus* (World Health Organization., 2017). There is evidence to suggest antimicrobial resistance is a result of several factors such as the over prescription of antibiotics and less regulated dispensing of medications in other countries such as China (Li., 2014). Antimicrobial resistance occurs as a result of a bacterium, virus and other microorganisms surviving in the presence of a treatment which then allows for the continuation of a more resistant population. This development of resistance is enabled through the presence of a low concentration of treatment such as when a patient does not finish a course of treatment leaving some of the pathogenic population behind. This developed tolerance may be passed on through the same species or different species of microorganism via transfer of plasmids through conjugation (Bennett., 2009). The overuse of antibiotics in the food industry is another

contributing factor to the development of antimicrobial resistance, antibiotics are used as growth promoters in meat production by adding them to the food of animals to prevent loss of livestock and (Rinsky et al., 2013). This presence of antibiotics in the diet of animals has been suggested to result in a low concentration of the antibiotic being present in food and the environment such as plants and water. This increase in frequency of low concentration antibiotics in many organic systems has resulted in more resistance (Hamscher and Bachour, 2018).

Novel synthetic compounds are being developed around the globe deriving from many different organic and inorganic sources (Balakrishna et al., 2017). These compounds can be of an active nature such as with a cationic charge which allows them to be used for many different purposes (Pappas et al., 2016).

Poly (para-phenylene ethynylene) (PPE) and poly (para-phenylene vinylene) (PPV) compounds

Cationic compounds are already used and have been effective for over half a century without a development in antimicrobial resistance where they have been utilised in hand washes and other topical antimicrobials. A recent example of such is Poly (para-phenylene ethynylene) (PPE)- and poly (para-phenylene vinylene) (PPV)-poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers which exhibited significant activity especially against *Enterococcus faecium*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* and A*cinetobacter baumannii* (Damavandi et al., 2018).

Aim

To determine the effect of thiazinoquinones and poly (para-phenylene ethynylene) (PPE), poly (para-phenylene vinylene) (PPV) and poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers as anti-cancer or antimicrobial agents for potential use in drug repurposing.

Objectives

- Determine the antimicrobial activity of PPE and PPV cationic compounds
- Determine the anticancer activity of PPE and PPV cationic compounds

- Determine the anticancer activity of thiazinoquinones compounds

  Use a basic *Galleria monella* biotoxicity model to determine the toxicity of the compounds

Methods

Chemicals

The PPE/PPV copolymer compounds were a kind gift from Dr David Barker at the University of Auckland New Zealand. These were prepared in his laboratory according to (Damavandi et al., 2018). The cationics had the following structures (Figure 1).

The Thiazinoquinones were also a kind gift from Prof Brent Copp at the University of Auckland New Zealand. These were prepared in his laboratory according to Harper et al., (2015).

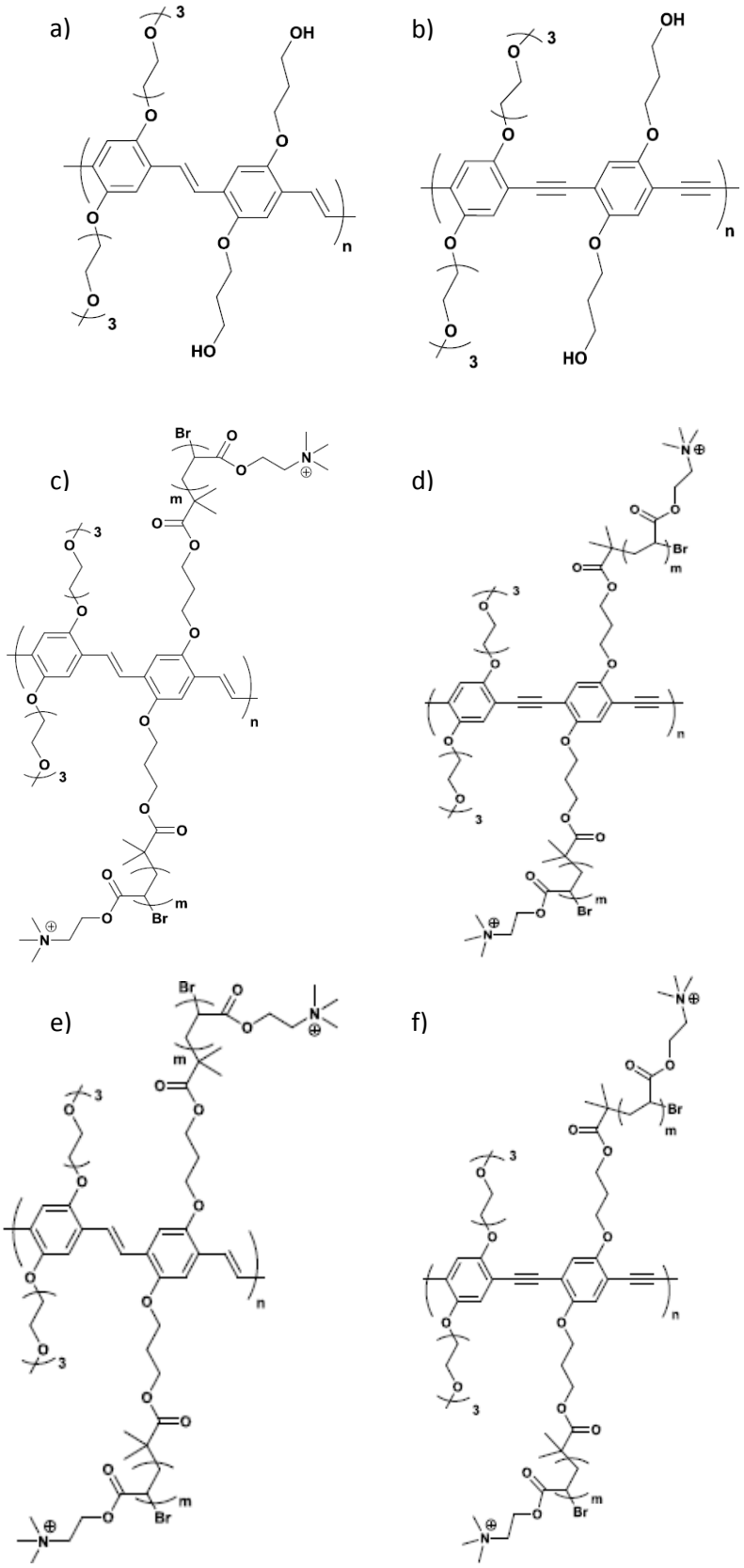

Figure 1. Structure of the cationic compounds a) 011, b) 021, c) 013, d) 023, e) 033 and f) 043.

The Thiazinoquinone compounds structure was not provided as the patent application is in progress however the molecular weight was provided.

Table 1: Thiazinoquinones and their molecular weight

Compound:	Molecular weight:
MC4-176-1	455.5234
MC4-176-2	455.5234
MC4-180-1	455.5234
MC4-180-2	455.5234
MC8-28-1	727.9915
MC8-28-3	727.9915

The thiazinoquinones that were tested were prepared in sterile conditions by adding the necessary amount of DMSO (Sigma, UK) to make up a concentration of 1M whereas, the PPE and PPV compounds were weighed out at 1mg/mL and suspended in distilled water in sterile conditions. An exception to this was the backbone PPE/PPV compounds 011 and 021 which required 10% dimethyl sulfoxide (DMSO) (Fisher, UK) to dissolve the sample.

Microbiology

Bacterial species

The bacteria used were as stated below:

-*Enterococcus faecium* (NCTC 7171) (on Columbia blood agar with horse blood)

-*Escherichia coli* (NCTC 10418) (on Tryptone soya agar)

-*Acinetobacter baumannii* (NCTC 12156) (on Tryptone soya agar)

-*Staphylococcus epidermidis* (NCTC 11047) (on Tryptone soya agar)

-*Multi-drug resistant Staphylococcus aureus* (NCTC 13552) (on Tryptone soya agar)

-*Klebsiella pneumoniae* (NCTC 9633) (on Tryptone soya agar)

-*Pseudomonas aeruginosa* (NCTC 10332) (on Tryptone soya agar)

-*Listeria monocytogenes* (Scott A) (on Tryptone soya agar)

All microbiological agars and broths were supplied by Oxoid, (UK) and were prepared according to the manufacturer's instructions.

Bacterial inoculation preparation

Under aseptic conditions, 10mL of a suitable broth was transferred to a 25mL universal. A single, pure colony of bacteria was transferred to the broth then incubated for 24 hours in a 37°C 5% CO_2 incubator. After the 24 hours of incubation, the samples were placed in the centrifuge for 10 minutes at 3000rpm and the supernatant was removed leaving the pellet within the universal. The bacterial pellet was re-suspended in 10mL of distilled water and mixed in a vortex mixer, the process was then repeated once to wash down the cells. When the cell washing process was completed, 1mL of the suspension was transferred to a 1.5mL semi-micro cuvette and read in a spectrophotometer (Jenway, UK) at 540nm and was diluted if necessary by adding water until reaching an optical density of 1.0 which equated to 5.0 x 10^8 CFU/mL.

Zones of inhibition

The zones of inhibition were investigated by pipetting 100µL of the prepared bacteria at 5.0 x 10^8 CFU/mL. One hundred microliters was pipetted onto the surface of the agar plates and spreading with a sterile spreader. The sample was left to dry on the surface of the plate for 5 minutes then uniform wells were taken from the agar using a size 5 cork borer providing a well with a 9mm diameter. One hundred microliters of the appropriate compound were added to the wells. The agar plates were then kept in a CO_2 incubator for 24 hours. When removed from the incubator, the diameter of the zones around each well was measured twice and an average was taken.

Minimum inhibitory concentration (MIC)

To perform a minimum inhibitory concentration assay, firstly a bacterial suspension was prepared at the correct optical density which was and was then diluted at a ratio of 1:2 with double strength brain heart infusion broth or tryptone soya broth. A redox indicator was added to provide a visible colour change indicating cellular respiration; 0.015 w/v of sterilized triphenyl tetrazolium chloride

(TTC) (Sigma, UK) was added per 10mL of the bacterial mixture. A flat bottom 96 well micro-titre plate (ThermoScientific, UK) was used; one hundred microliters of the compounds that were tested were pipetted into the first column and pure double strength broth of the same volume was pipetted into the 12th column as a control. Using a multichannel pipette, 100µL of the bacterial mix with TTC was transferred to columns 11 through to 1; when pipetting into the first column with the compounds, the pipette was pumped to mix effectively and from there 100µL was then transferred consecutively from the previous well (1 to 10), at 10 the 100µL was removed to keep column 11 as a control. The plate was kept in a 5% CO_2 incubator for 24 hours. When the plate was removed from the incubator the MIC value for each compound was identified as the last well before turning blue, which indicated bacterial growth.

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was investigated by first preparing the suitable agar plates, diving them into the appropriate amount of sections with a black marker underneath and labelling them by concentration and compound. The only wells investigated were those up to and including the first negative result in the minimum inhibitory concentration assays i.e. the first well that turned blue. Ten microliters of the individual wells were transferred to the plate's individual sections; the plates were then incubated overnight in a 5% CO_2 incubator. When removed from the incubator the section that demonstrated the lowest number with no colonies present represented the MBC value.

Galleria mellonella Biotoxicity model

The *galleria mellonella* larvae were used to provide a basic infection model. The galleria were weighed and an average was taken providing a weight of 0.250mg of which was used to account for when calculating the correct injecting concentration. The first set of galleria were counted and divided into groups according to how they were to be treated: untreated control, water and DMSO control, 10mg and 50mg then each group was placed into a petri dish with filter paper. To clean the needle in between groups, 70% ethanol and distilled water was dispensed into petri dishes. A pipette tip was secured to the lab work bench to provide a surface of which to hold the Galleria

safely and securely, using safety gloves the galleria were placed and held on their back and the needle was injected into their second back right pro-leg. They were then incubated at 37˚C for 24, 48 and 72 hours.

As the highest concentration used did not kill the galleria, this was then used in a infection model. The bacteria used was *Enterococcus faecium* at an optical density of 0.1 at 600nm in sterile PBS, providing a cell count of $1x10^8$ per ml so when 10µl of bacterial suspension is injected into the galleria the bacterial concentration is at $1x10^6$. The second set of galleria samples included a PBS control, *E. faecium* control, 50mg compound control, *E .faecium* and 50mg compound combined and water and DMSO control. When the galleria was inoculated with the bacteria they were incubated for 30 minutes and then injected in the second back right leg with the compound and incubated for 24, 48 and 72 hours and monitored.

Cellular Techniques

Media preparation

To prepare the appropriate media for the Jurkat cells, BioWhittaker Roswell park memorial institute media (RPMI) (Lonza,UK) was warmed to 37˚C in a water bath. Fifteen millilitres of Fetal Bovine Serum and 5mL of BioWhittaker Pen-Step (5000 U Penicillin/mL:5000 U Streptomycin/mL) (Lonza,UK) was defrosted and added to the RPMI in sterile conditions. The combined media was kept in fridge at 4°c to preserve until use.

Cell culture

In a laminar flow, under sterile conditions, Jurkat cells were cultured by adding 2mL of the cell sample to 8mL of the combined media (RPMI with FBS and PenStrep), this ratio was used when less cell population was required. When preparing to complete an assay, a ratio of 3mL of the cell sample to 7mL of the combined media was used to increase cell yield. These ratios are appropriate for this cell line as there is still sufficient cytokines and cell interaction present at the lower concentration of cell sample but not too high a concentration of which could result in cell death from a lack of

available nutrients. When the cells had been prepared in the appropriate ratio, the cells were kept in an incubator at 37°C with 5% CO^2

Cellular assays

Two types of cellular assays were performed, MTS assays and Annexin V/Propidium Iodide assays, and were both prepared initially in the same way; To determine the viable number of cells an MTS proliferation assay was used and to quantify and characterize cell death an Annexin V/ Propidium Iodide assay was used. To perform these assays a cell count of $1x10^5$ was used per well. To attain this two x 100µL samples of Jurkat cells were counted using a haemocytometer (Hawksley, UK under a white light microscope (Leica) with trypan blue (Corning) and an average was taken. The cell count was used to calculate the volume of cell sample needed to provide $1x10^5$ cells per well, when this volume was calculated the sample was then spun in a centrifuge at 500G for 5 minutes to pellet the cells. The pelleted cells were re-suspended in the required amount of fresh RPMI media with FBS and PenStrep and 100µL was added to the essential number of wells in a flat bottom 96 well plate (ThermoScientific). A serial dilution was performed where the first concentration was made up of 20µL of the drug stock and 480µL of RPMI. From the first concentration. Fifty microliters was taken and added to 450µL of RPMI and this method was continued until 6 dilutions had been obtained. One hundred microliters of each of the concentrations were added in triplicate to the corresponding wells.

MTS assay

Eight live cell control wells were prepared which contained 100µL of the cell sample with an additional 100µL of RPMI only (positive control). Another four wells were also prepared with 100µL of the cell sample and 100µL of ethanol to provide a dead cell (negative) control. The negative control wells were isolated by filling the surrounding wells with RPMI to prevent any evaporating ethanol causing false positive results. When all of the wells were prepared the plate was transferred to a 37°C incubator with 5% CO_2 for 24 hours. After the incubation period, the plate was removed and the samples were pipetted into 1.5mL Eppendorf tubes (StarLab, UK) and centrifuged at 500G

for 5 minutes to obtain the cell pellet. The supernatant was removed and the cells were resuspended in 100µL of fresh RPMI and 20µL of MTS.The samples were transferred back to the 69 well plate and incubated for 2 hours at 37°C with 5% CO_2. After the plate was incubated, it was placed in a SynergyHT microplate reader (Biotek, UK) at 490nm and the results were analyzed.

Annexin V/PI assay

In The Annexvin V/ PI assays, the mechanism of cell death is identified by staining. Annexin 5 FITC binds to phosphatidylserine which is expressed on the cell surface when a cell is undergoing apoptosis. Propidium iodide will stain the nucleus and other DNA containing organelles but cannot cross the membrane of viable cells so can therefore be used to detect if a cell is viable or necrotic. The combination of both stains also can evaluate the stage the cell is at during apoptosis e.g early or late as late apoptosis would yield a positive result from both stains as both phosphatidylserine is expressed and the membrane will be broken down allowing the propidium iodide to stain the organelles.

For the control wells, there were 12 live cell controls with 100µL of the cell sample and an additional 100µL of RPMI only. When all of the wells were correctly prepared the plate was transferred to a 37°C incubator with 5% CO_2 for 24 hours. After the incubation period the plate was removed and the samples were transferred to 1.5mL Eppendorf tubes and centrifuged at 500G for 5 minutes. The supernatant was removed and re-suspended in 300µL of BioWhittaker Dulbecco's Phosphate Buffered Saline – 0,0095M (PO4) Without Ca and Mg (DPBS) (Lonza, UK) then was centrifuged again at 500G for 5 minutes. After the last centrifugation, the supernatant was removed and the sample was resuspended in 400µL of 1X Binding Buffer and transferred to 5mL round bottom tubes (Falcon). The 5µL of FITC Annexin V and 10µL of Propidium Iodide was added and the samples were analysed on a FACS BD Verse flow cytometer which detects the number of cell populations corresponding to each stain.

The annexin V/ PI assay was also repeated with DMSO controls to ensure the thiazinoquinones were causing the change in cell death mechanisms and differentiation and not the DMSO that was used to dissolve the compounds.

Results

Statistical tests used throughout the cellular analysis was dunnett's tests and one- way anovas.

Thiazinoquinones

To evaluate if and what type of cell death the thiazinoquinones were causing, it was necessary to first investigate the effect of DMSO on cell death. The annexin V/ PI analysis indicated and average Viable population of 81.2% ± 2.2, Early Apoptotic: 6.1% ± 1.4, Late Apoptotic: 9.4% ± 2.2 and Necrotic: 3.3% ± 1.8.

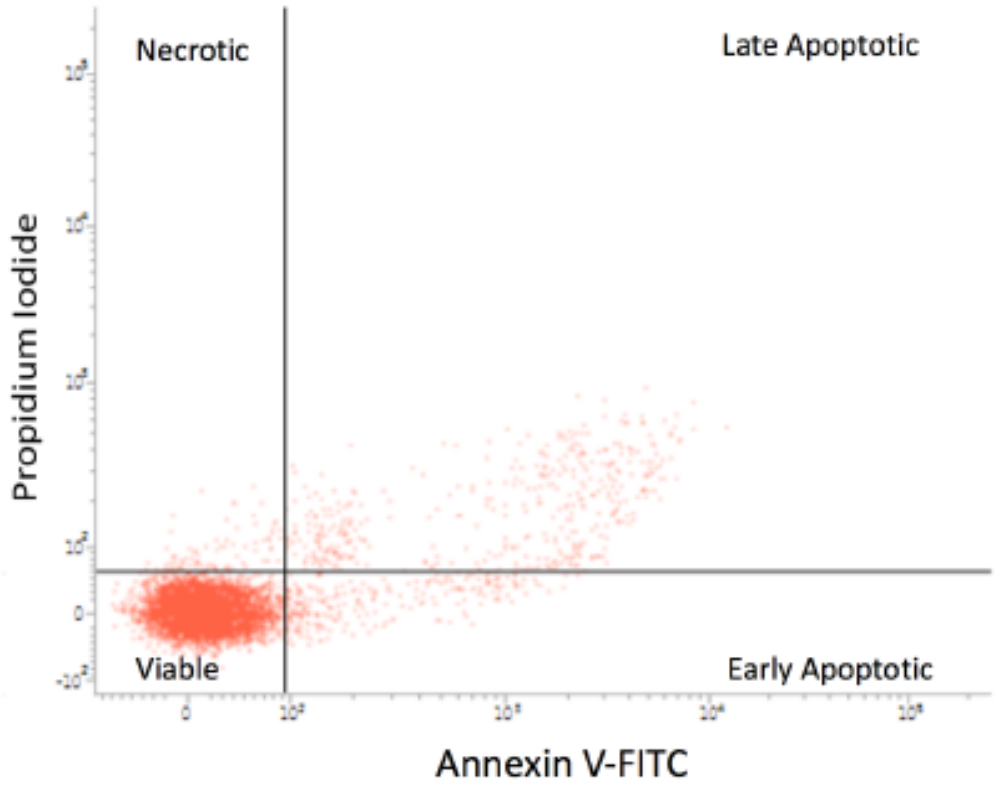

Figure 2: A representative plot of the three DMSO control samples tested.

At 100µM the compound MC4-176-1 was effective at decreasing the survival of the Jurkat cells. The efficacy decreased from 0.001µM (80.1% survival) to 1µM (117.7% survival) (Figure 1). However, at 10µM (77.3% survival) the efficacy began to increase and was significantly effective at 100µM (0.2%

survival).The error bars indicated that the compound had little effect at lower concentrations. At 100µM the compound MC4-176-2 was effective at decreasing cell survival but the efficacy fluctuated between 0.001µM at 77.6%, 0.01µM at 120%, 0.1µM at 88.1% and 1µM at 141%. The error bars indicated that the lower concentrations had a similar effect and the increase in activity around 1µM could be due to cell stress. At 10µM cell survival decreased to 38% and at 100µM the result was found to be significant at 2.1% survival. Compound MC4-180-1 at 0.001µM displayed a survival rate of 128.5%, 0.01µM at 126.8%, 0.1 µM at 156.6%, 1µM at 142.3% and 10µM at 138.2%. At 100µM the compound significantly displayed a 19.55% cell survival rate. Compound MC4-180-2 at 100µM had a survival rate of 30.21%. At lower concentrations the efficacy reduced resulting in 84% (0.001µM), 92.8% (0.01µM), 108% (0.1µM), 118.5% (1µM), 120% (10µM) cell survival. At 1µM the survival rate appeared to increase however this again may be due to cell stress. Compound MC4-180-2 at 100µM had a survival rate of 30.21%. At lower concentrations the efficacy reduced resulting in 84% (0.001µM), 92.8% (0.01µM), 108% (0.1µM), 118.5% (1µM), 120% (10µM) cell survival. At 1µM the survival rate appeared to increase however this may be due to cell stress. Thiazinoquinone MC8-28-1 at 100µM had a cell survival rate of 40.71%. The lowest concentration 0.001µM had a survival rate of 122%, while the results indicate a slight increase in efficacy at 0.01µM (103.6%), the following concentrations of 0.1µM and 1µM yielded a cell survival of 126.9% and 126.2% respectively indicating that there was little change between the lowest concentrations. At 10µM the efficacy at reducing cell survival still remained low with a cell survival of 103.6 %. At 100µM there was a significant survival rate of 45.32%, at lower concentrations the efficacy decreased. At the lowest concentration of 0.001µM cell survival was at 102.6% then increased to 113.3%, 118.6%, 116.1% and 124.4% (0.01µM, 0.1µM, 1µM and 10µM respectively). Overall the results demonstrated that the highest concentration of 100µM was the most effective at decreasing the survival rate of the jurkat cells. The MC4 sub-category of the thiazinoquinones had the most efficacy at 100µM decreasing the survival rate of the cells; MC4-176-1 and MC4-176-2 had a survival rate of 0.2% and 2.1% respectively.

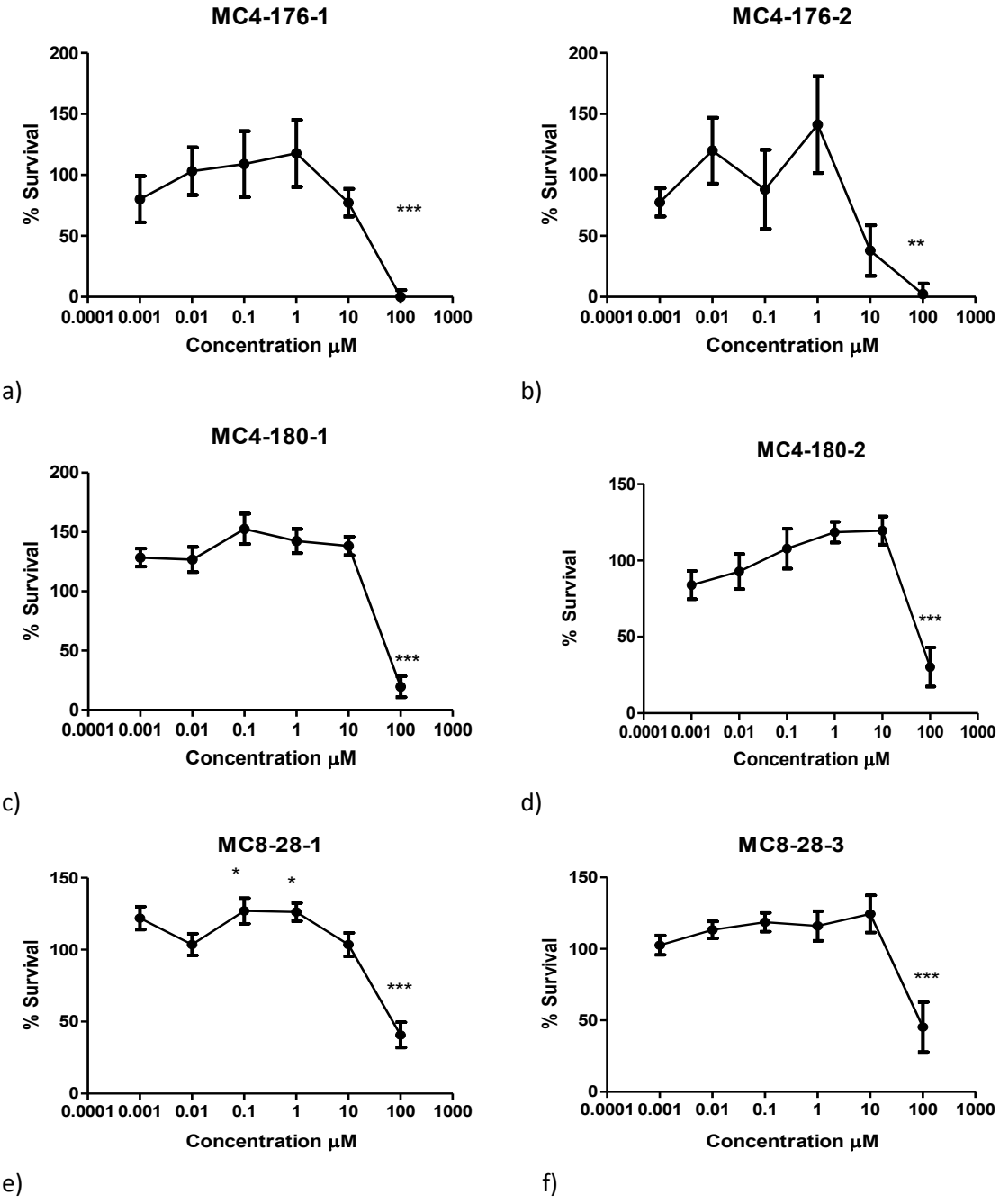

Figure 3. MTS proliferation analysis for thiazinoquinones a) MC4-176-1 b) MC4-176-2 c) MC4-180-1 d) MC4-180-2 e) MC8-28-1 and f) MC8-28-2. At 100μM cell survivability decreased and had little effect at the lower concentrations. N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

Annexin V/Propidium Iodide assays

The viable population decreased with each compound at 100µM (Figure 2). MC4-176-2 and MC4-180-1 were the most effective at reducing the viable population to 9.51% and 15% respectively. However, MC8-28-3 and MC4-180-2 were least effective and had the highest viable population with 100µM of the compound at 68.4% and 56.3% respectively.

The cell population undergoing early apoptosis increased with each compound at 100µM (Figure 3). Between the concentrations of 0.001µM, 0.01µM, 0.1µM, 1µM and 10µM the percentage of the cell population undergoing early apoptosis fluctuated between the 9.95% and 29.4% showing no significant increase. Compounds MC4-176-1 and MC8-28-3 had the least early apoptotic population of the thiazinoquinones at the highest concentration whereas MC4-180-1 and MC8-28-1 had the highest early apoptotic population at 82.4% and 75.2% respectively.

The thiazinoquinones MC4-176-1 and MC4-176-2 were most significant at increasing late apoptosis to 31.2% and 27.53% at 100µM (Figure 4). The compounds MC8-28-1, MC8-28-3, MC4-180-2 and MC4-180-1 had very little to no effect on the late apoptotic population only increasing it to < 1.32%.

The necrotic cell population was low with all of the thiazinoquinones (Figure 5); MC4-176-1 and MC4-176-2 at 100µM had the highest percentage of necrosis at 11.4% and 5.7%. The other compounds had little effect on the necrotic population only reaching to < 0.81%.

Overall, the thiazinoquinones had a greater effect on the cell population undergoing early apoptosis and the viable population. The viable population decreased with each compound at 100µM where MC4-176-2 and MC4-180-1 were most effective at reducing the viable population to 9.51% and 15% respectively. MC4-180-1 and MC8-28-1 had the highest early apoptotic population at 82.4% and 75.2% respectively.

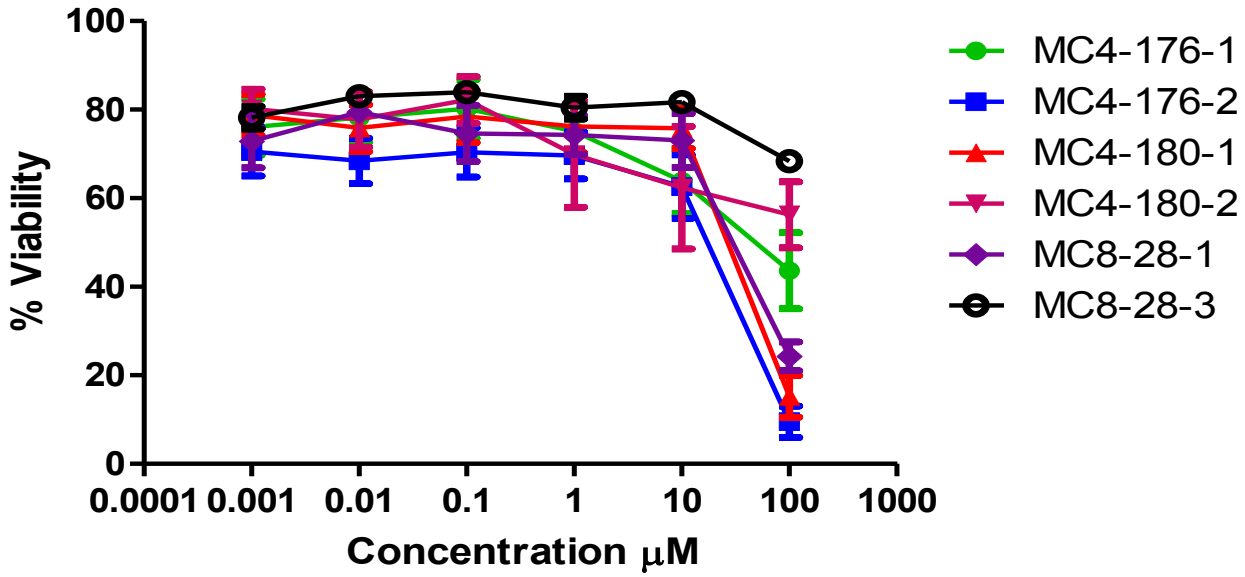

Figure 4: Annexin V and Propidium iodide assays demonstrating the viable population in the presence of thiazinoquinone compounds.

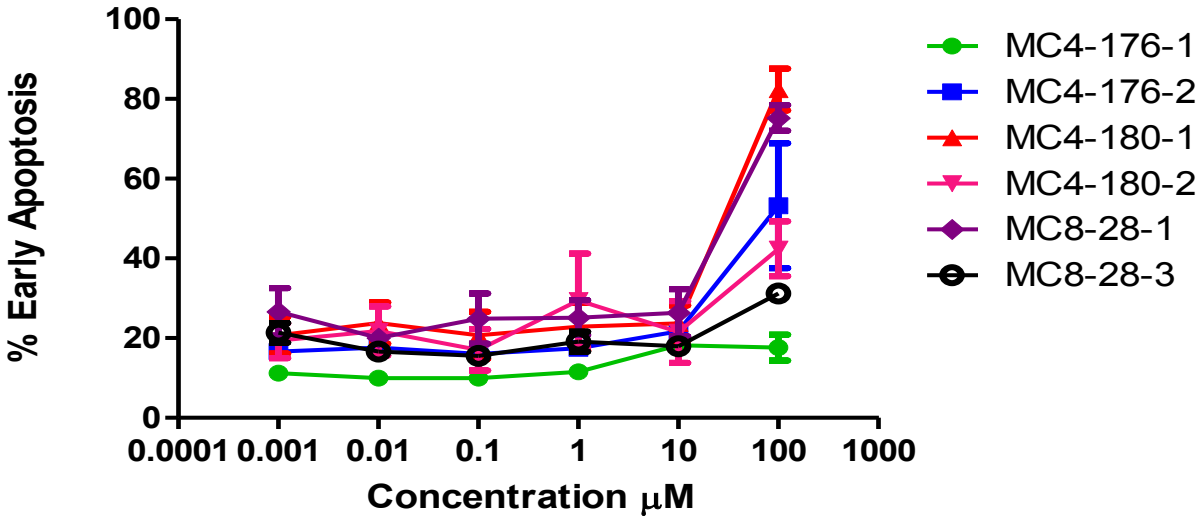

Figure 5: Annexin V and Propidium iodide assays indicating the cell population undergoing early apoptosis with each compound.

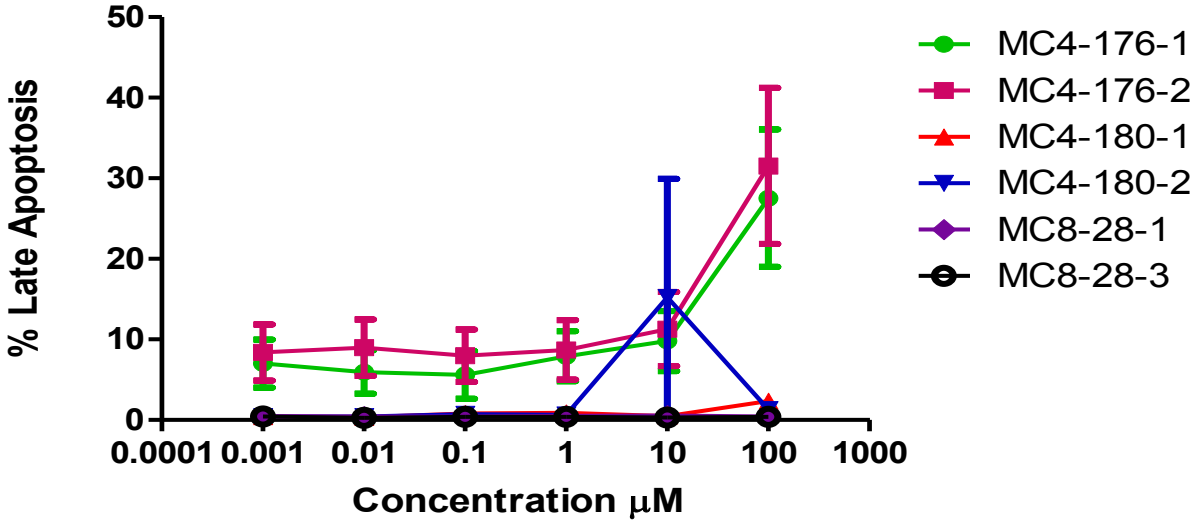

Figure 6: Annexin V and Propidium iodide assays indicating the cell population undergoing late apoptosis at 100μM with thiazinoquinones.

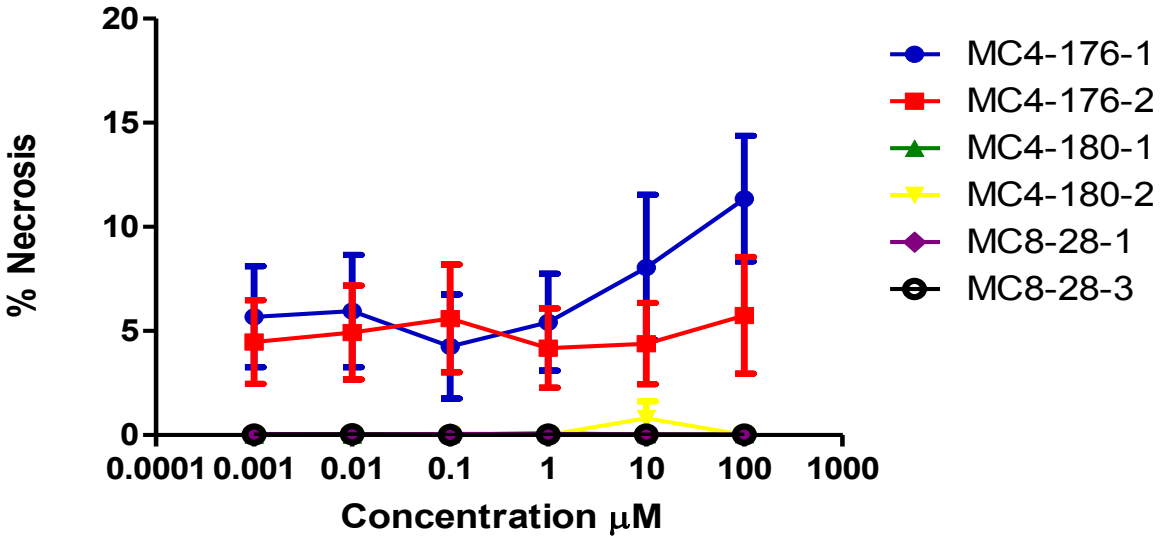

Figure 7: Annexin V and Propidium iodide assays of the cell population undergoing necrosis with thiazinoquinones.

PPV/PPE Compounds for use in cancer treatments

In the presence of compound 011, the cell population survival rate fluctuated between the concentration following no direct trend (Figure 11). There was an increase in cell survival rate between the concentrations 0.001µM, 0.01µM and 0.1µM from 73.1%, 89.9% and 109.8% respectively. From 0.1µM the survival rate dropped at 1µM (97.1%) and 10µM (89.7%) to then increase again slightly at 100µM to 92.6%. With compound 021, the cell population had a partially significant reduction at 0.001µM to 61.1%. Between 0.01µM - 100µM, the cell survival fluctuated. At 0.01% the cell survival rate was at 103.7% which decreased to 86.3% at 0.1µM but then began to increase again from 1µM to 10µM with 92.6% and 111.6% respectively. The cell population decreased again at 100µM to 85.3%. The cationic compound 013 did not significantly reduce the survival rate of the cell population. The survival rate of the population fluctuated with each concentration. At 0.001µM the survival rate was at 102.2% but when the concentration was increased to 0.01µM, 0.1µM, 1µM, 10µM and 100µM the survival rate was increased to 128.7%, 114.2%, 109.7%, 132.2% and 124.7% respectively. The survival rate of the Jurkat cells in the presence of compound 023 decreased from 0.001µM (105.3%) to 0.01µM (69.1%) and then at 0.1µM the survival rate increased to 84.1% of which then remained unchanged with an increase in concentration until 100µM which had a survival rate of 97.7%. Compound 033 reduced cell survival at 100µM significantly to 57.4%. However the lower concentrations did not reduce the cell survival below 100%. The survival rate fluctuated between 0.001µM (119.7%) - 10µM (115.9%). In the presence of compound 043, the cell population decreased from 0.001µM to 0.01µM; 119.7% to 82.1% respectively. At concentration 0.1µM the cell survival rate increased to 97.9% but then decreased again with the most effective being 1µM(63%) and 10µM (59.2%). The highest concentration of 100µM saw a slight increase from 10µM to 71.3%.

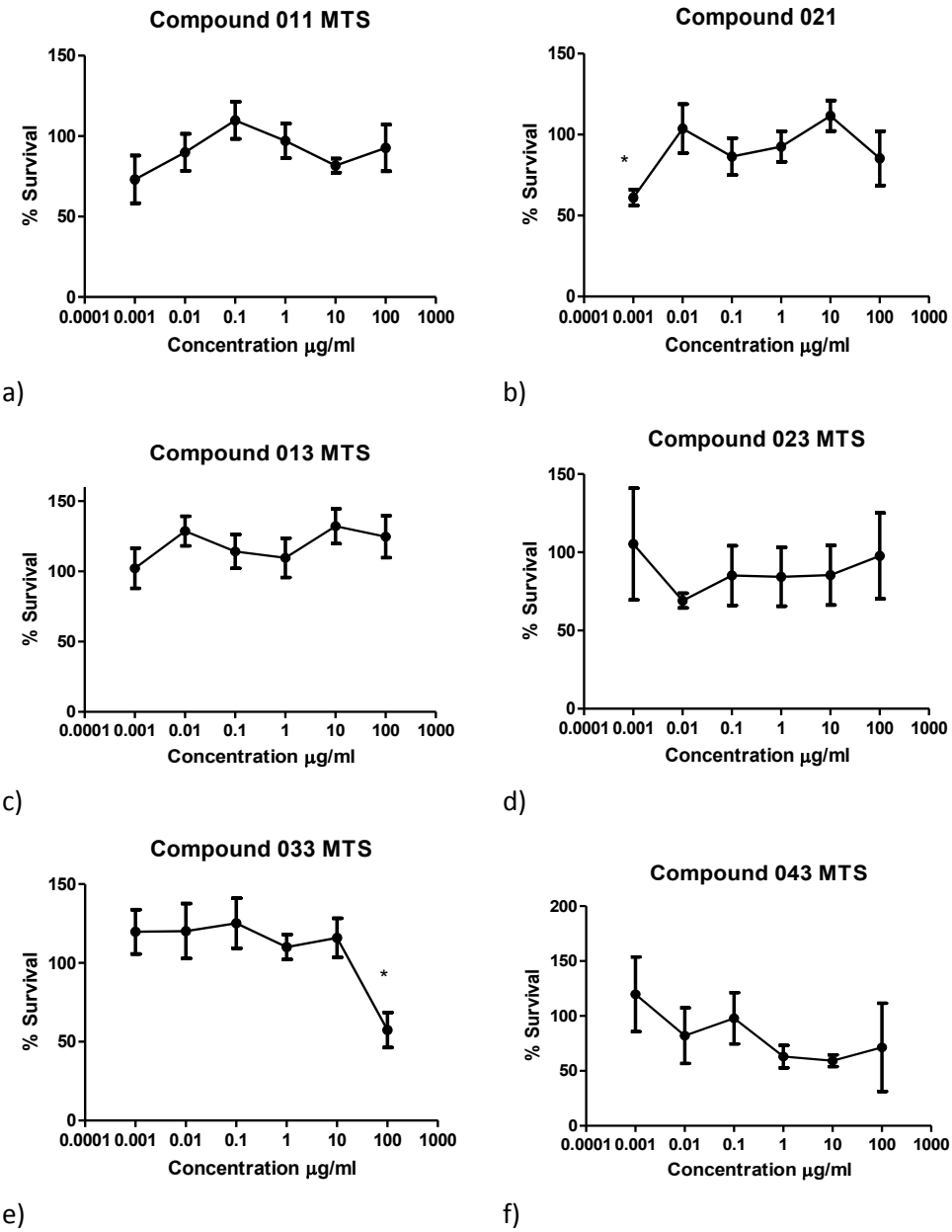

Figure 8: MTS analysis of the compounds a) 011, b) 021, c) 013, d) 023, e) 033 and f) 043 against Jurkat cells. N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

Zones of inhibition

To begin evaluating the antimicrobial efficacy of the PPE/PPV compounds, a standard zone of inhibition test was performed with 3 of the compounds. The results indicated that compounds 003 and 033 had the highest efficacy of the 3 compounds. Compound 033 had the best activity against the range of bacteria tested, the highest inhibitory effect being against *L. monocytogenes* at 51.67mm and the lowest being 26.30mm against *K. pneumoniae.* Compound 023 had no activity against the range of bacteria.

Table 2: Zones of inhibition of bacteria against PPE/PPV compounds in mm

Bacteria	003	023	033
L. monocytogenes	41.67	0	51.67
E. coli	23.67	0	30.67
P. aeruginosa	0	0	0
S. epidermidis	29	0	30.33
MRSA	27	0	35.33
A. baumanni	0	0	0
K. pneumoniae	0	0	26.30
E. faecium	29	0	30.33

Minimum inhibitory concentration assays (MIC) and minimum bactericidal concentration assays (MBC) to determine antibacterial activity of cationic compounds

Following the minimum inhibitory concentration assays, compound 043 was most effective at inhibiting the growth of the range of bacteria tested in comparison to the other compounds (Table 2). The bacteria *E.coli, MRSA* and *E. faecium* was inhibited most by compound 043 at concentrations of 19.5µg/ml, 83µg/ml and 78.1µg/ml respectively. Compounds 005, 004 and 024 were the least effective at inhibiting the range of bacterial growth.

Table 3. Minimum Inhibitory Concentration of bacteria with PPE/PPV compounds in µg/ml.

Compound	*E. coli*	MRSA	*P. aeruginosa*	*A. baumanni*	*K. pneumoniae*	*E. faecium*
003	1250	2812.5	No inhibition	5000	5000	3750
013	5000	1562.5	5000	5000	No inhibition	78.1
023	312.5	781.3	5000	1875	5000	117.2
033	1250	781.3	No inhibition	937.5	5000	78.1
043	19.5	83	1250	234.4	2500	78.1
011	1250	625	937.5	312.5	1250	1250
021	1250	625	937.5	468.8	1250	1250
005	No inhibition	5000	No inhibition	No inhibition	No inhibition	No inhibition
004	2500	No inhibition	No inhibition	2500	No inhibition	No inhibition
024	5000	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition

Following the minimum bactericidal concentration assays (Table 2), compound 043 was the most bactericidal of the range of compounds against *E.coli, MRSA* and *A. baumanni* at concentrations of 39.1µg/ml, 166µg/ml and 625µg/ml respectively. Compound 005,004 and 024 was the least effective against the range of bacteria and of these only 005 was bactericidal at 2500µg/ml against MRSA.

Table 4. Minimum Bactericidal Concentration of bacteria with PPE/PPV compounds in µg/ml.

Compound	*E.coli*	*MRSA*	*P. aeruginosa*	*A. baumanni*	*K. pneumoniae*	*E. faecium*
003	5000	5000	No inhibition	5000	No inhibition	No inhibition
013	5000	5000	No inhibition	5000	No inhibition	625
023	2500	2500	No inhibition	2500	5000	1250
033	2500	2500	No inhibition	2500	5000	3125
043	39.1	166	2500	625	3750	937.5
011	5000	3125	3750	1875	3750	5000
021	2500	1562.5	2500	1875	2500	5000
005	No inhibition	2500	No inhibition	No inhibition	No inhibition	No inhibition
004	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
024	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition

Galleria biotoxicity model

Galleria mellonella larvae were used as an *in vitro* model for treating *E. faecium* infections with compound 043. The first test group indicated the Galleria larvae could withstand the highest concentrations tested as all larvae survived and therefore would make a good infection model. The second group of larvae survived less than the first group, with the infection model group dying within 24 hours and after 48 hours the infection model group had also died.

Table 5: The status of the Galleria test groups after 24 hours.

Test Group	Alive	Dead
Control (Not injected)	10	0
Water and DMSO control	10	0
10mg	10	0
50mg	10	0

Table 6: The status of the Galleria test groups after 48 hours.

Test Group	Alive	Dead
Control (Not injected)	10	0
Water and DMSO control	10	0
10mg	10	0
50mg	10	0

Table 7: The status of the Galleria test groups after 72 hours.

Test Group	Alive	Dead
Control (Not injected)	10	0
Water and DMSO control	10	0
10mg	10	0
50mg	10	0

Table 8: The status of the infection model galleria test groups after 24 hours

Test Group	Alive	Dead
Control	9	1
Water and DMSO control	9	1
E. faecium control	8	2
50mg control	2	3
E. faecium and 50mg	0	10
PBS control	9	1

Table 9: The status of the infection model galleria test groups after 48 hours

Test Group	Alive	Dead
Control	9	1
Water and DMSO control	7	3
E. faecium control	3	7
50mg control	1	4
E. faecium and 50mg	0	10
PBS control	8	2

Table 10: The status of the infection model galleria test groups after 72 hours

Test Group	Alive	Dead
Control	9	1
Water and DMSO control	5	5
E. faecium control	0	10
50mg control	0	5
E. faecium and 50mg	0	10

Discussion

With the challenges presented with the prevalence of antimicrobial resistance and the difficulties in navigating new treatments to tackle each hallmark of cancer, it is necessary to investigate novel compounds and repurpose current treatments. In an effort to investigate both new compounds and drug repurposing, two sets of compounds were evaluated: Thiazinoquinones and Poly (para-phenylene ethynylene) (PPE)- and poly (para-phenylene vinylene) (PPV)-poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers.

Efficacy of thiazinoquinones as anti-cancer agents.

The thiazinoquinones investigated were MC4-176-1, MC4-176-2, MC4-180-1, MC4-180-2, MC8-28-1 and MC8-28-3. Overall, the standard viability test results demonstrated that the highest concentration of 100 µM was the most effective at decreasing the survival rate of the Jurkat cells for all thiazinoquinones. Of these, the MC4 sub-category of the thiazinoquinones had the highest efficacy at decreasing the survival rate of the cells when tested at 100µM, with MC4-176-1 and MC4-176-2 demonstrating survival rates of 0.2% and 2.1% respectively. The least effective compound appeared to be MC8-28-2, at 100µM the cell survival was only reduced to 45.32%. The change in efficacy also correlates to the change in molecular weight of the compound, As the molecular weight increases the efficacy decreases. This decrease in efficacy with increase in size suggests the smaller thiazinoquinones may be more effective as they are more effective at crossing the cell membrane (Yang and Hinner., 2014). Some of the tested compounds did show some efficacy at lower concentrations, including MC4-176-1 which had a 77.3% cell survival rate at 10µM and MC4-176-2 which had a 38% cell survival rate at 10µM. However, the error bars demonstrated that most of the fluctuations observed at concentrations lower than 100 µM were not significant. The larger error bars at the lower concentrations may be caused by compound variables such as light activation increasing compound efficacy by increasing possibly increasing semiquinone radicals and reactive oxygen species (Imperatore et al., 2017). Other reasons for the larger error bars may be variable

procedure conditions such as temperature increase or decrease could result in cell damage and cell stress prior to testing resulting in different cell survival rates as they may have already mounted a protective response to toxicity or activated death signalling pathways. In the presence of a toxic agent, cells mount a protective response depending on the stress causing agent such as temperature (Fulda et al., 2010).The changes observed at the lower concentrations may also be due to these cell stress responses, at higher concentrations these changes would not be observed as they would have undergone cell death much faster (Seelige et al., 2017).

As previously referred to, the study into the relationship between the structure of these compounds and their efficacy indicated there was a significant reliance on the structural isomerism of the compound. In the previously tested compounds it was found that the geranyl side chain position influenced the mechanism of cell death it induced. In addition work by others found that such compounds were also found to be a moderate inducer of ROS but this particular feature did not correlate with the structure (Harper et al., 2015). This suggests that there is another structural factor that determines the efficacy independent of the geranyl side chain, further study would be done into this to determine and possibly improve efficacy.

The viable population decreased with each compound at 100µM; MC4-176-2 and MC4-180-1 were most effective at reducing the viable population to 9.51% and 15% respectively. However, MC8-28-3 and MC4-180-2 were least effective and had the highest viable population with 100µM of the compound at 68.4% and 56.3% respectively. The viable population decrease may be due to natural cell death as there was no significance at the lower concentrations even with the most effective thiazinoquinones however, there was significance ($p < 0.001$) at 100 µM with MC4-176-2, MC-4-180-1 and MC8-28-1 indicating the compounds were effective.

The cell population undergoing early apoptosis increased with each compound at 100µM, between the concentrations of 0.001µM, 0.01µM, 0.1µM, 1µM and 10µM the percentage of the cell population undergoing early apoptosis fluctuated between the lowest 9.95% and the highest 29.4% showing no significant increase. Compounds MC4-176-1 and MC8-28-3 had the least early apoptotic

population of the thiazinoquinones at the highest concentration whereas MC4-180-1 and MC8-28-1 had the highest early apoptotic population at 82.4% and 75.2% respectively. The thiazinoquinones were most successful at decreasing population viability and increasing early apoptosis.

The thiazinoquinones MC4-176-1 and MC4-176-2 were most significant at increasing late apoptosis to 31.2% and 27.53% at 100µM. The compounds MC8-28-1, MC8-28-3, MC4-180-2 and MC4-180-1 had very little to no effect on the late apoptotic population only increasing it to <1.32%.

The necrotic cell population was low with all of the thiazinoquinones, MC4-176-1 and MC4-176-2 at 100µM had the highest percentage of necrosis at 11.4% and 5.7%. The other compounds had little effect on the necrotic population only reaching to <0.81%. Overall, the thiazinoquinones had a greater effect on the cell population undergoing early apoptosis and the viable population. The viable population decreased with each compound at 100µM where MC4-176-2 and MC4-180-1 were most effective at reducing the viable population to 9.51% and 15% respectively. MC4-180-1 and MC8-28-1 had the highest early apoptotic population at 82.4% and 75.2% respectively. The group of thiazinoquinones tested were predominantly more apoptosis inducing. In previous studies this group of compounds has been found to induce apoptosis, however with the changes in structure and molecular weight it is evident their mode of action changes (Carbone et al., 2012). This set of thiazinoquinones had little to no necrotic activity. The percentage of early apoptotic cell population may be partially due to natural cell death as there was no significance ($P > 0.05$) at the lower concentrations even with the most effective thiazinoquinones.

Efficacy of Poly (para-phenylene ethynylene) (PPE)- and poly (para-phenylene vinylene) (PPV)-poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers as anti-cancer agents:

In the presence of compound 011, the cell population survival rate fluctuated between the concentrations following no direct trend. There was an increase in survival rate between the concentrations 0.001µM, 0.01µM and 0.1µM from 73.1%, 89.9% and 109.8% respectively. From

0.1µM the survival rate dropped at 1µM (97.1%) and 10µM (89.7%) to then increase again slightly at 100µM to 92.6%

With compound 021, the cell population had a partially significant reduction at 0.001µM to 61.1%. Between 0.01µM - 100µM, the cell survival fluctuated. At 0.01% the cell survival rate is at 103.7% which decreases to 86.3% at 0.1µM but then begins to increase again from 1µM to 10µM with 92.6% and 111.6% respectively. The cell population decreases again at 100µM to 85.3%.

The cationic compound 013 did not significantly reduce the survival rate of the cell population. The survival rate of the population fluctuated with each concentration; At 0.001µM the survival rate was at 102.2% but when the concentration was increased to 0.01µM, 0.1µM, 1µM, 10µM and 100µM the survival rate was increased to 128.7%, 114.2%, 109.7%, 132.2% and 124.7% respectively.

The survival rate of the jurkat cells in the presence of compound 023 decreased from 0.001µM (105.3%) to 0.01µM (69.1%) and then at 0.1µM the survival rate increased to 84.1% of which then remained unchanged with an increase in concentration until 100µM which had a survival rate of 97.7%. Compound 033 reduced cell survival at 100µM significantly to 57.4%. However the lower concentrations did not reduce the cell survival below 100%; The survival rate fluctuated between 0.001µM(119.7%) - 10µM (115.9%). In the presence of compound 043, the cell population decreases from 0.001µM to 0.01µM; 119.7% to 82.1% respectively. At concentration 0.1µM the cell survival rate increased to 97.9% but then decreased again with the most effective being 1µM(63%) and 10µM (59.2%). The highest concentration of 100µM saw a slight increase from 10µM to 71.3%.

The PPE/PPV copolymers had little to no effect on reducing the survival rate of the jurkat cells. When evaluated previously a correlation between the compounds charge indicated that the cationic compounds were active against bacteria species in contrast to the anionic versions. The mode of action may be reliant on this charge and it may not have an effect on the jurkat cells (Damavandi et al., 2018). The delivery of a compound to a jurkat cell is effected by the hydrophobicity of the cell; hydrophobicity effects intracellular delivery which may be why this set of compounds collectively

had little to no effect on reducing the survival rate (Posey et al., 2016). These compounds could be further investigated for this use for example if the compounds were irradiated with UV wavelength before to charge its reactivity with the cell membrane(Ji et al., 2011).

Efficacy of Poly (para-phenylene ethynylene) (PPE)- and poly (para-phenylene vinylene) (PPV)-poly[(2-(methacryloyloxy)ethyl) trimethylammonium chloride] (PMETAC) graft copolymers as a antimicrobial agent:

Compound 043 was most effective at inhibiting the growth of the range of bacteria tested in comparison to the other compounds. The bacteria *E.coli, MRSA* and *E. faecium* was inhibited most by compound 043 at concentrations of 19.5µg/ml, 83µg/ml and 78.1µg/ml respectively. Compounds 005, 004 and 024 were the least effective at inhibiting the range of bacterial growth.

Compound 043 was the most bactericidal of the range of compounds against *E.coli, MRSA* and *A.baumanni* at concentrations of 39.1µg/ml, 166µg/ml and 625µg/ml respectively. Compound 005,004 and 024 was the least effective against the range of bacteria and of these only 005 was bactericidal at 2500µg/ml against MRSA. In previous investigations it has been noted that there is a correlation between the charge of the compounds and the efficacy, the cationic compounds 003,013,023,033 and 043 showed higher efficacy than the anionic 004 and 024 (Damavandi et al., 2018). The efficacy may also be related to the hydrophobicity/hydrophilicity of the compounds, the structural differences of each compound would result in different physicochemistry and therefore would result in different interactions with the different physicochemistry of each bacterial species (Lee et al., 2018).

The first set of galleria models indicated that with the addition of the compound the larvae was able to survive with a concentration of 50mg. The larvae survived past 72 with all of the intial controls therefore indicated a infection model may be successful, however the second set of results showed otherwise and the inoculated larvae with the compound died before 24 hours. This initally suggested an antagonistic reaction within the galleria itself however, upon further examination of the larvae it

may have been that the needle had damaged the larvae too much on inoculation as the area surrounding the puncture was black (Kwadha et al., 2017). The second set of results should be repeated, and more compounds should be tested.

The mode of action of both compounds should be taken to further studies to evaluate their possible use in future treatments.

Conclusion

The thiazinoquinone sub category MC4 was more effective than the other thiazinoquinones. MC4-176-1 decreased the surviving cell population to 0.2% at 100 μM. Overall the thiazinoquinones did reduce the survival rate of the cell population but only significantly at the highest concentration. When using the stains annexin V and propidium iodide, similar results were also found. The MC4 sub category remained the most active in which MC4-176-2 reduced the viable population the most to 9.51%. In contrast to this, MC4-176-1 and MC8-28-1 had the highest early apoptotic populations therefore the efficacy may not be effected by the size of the molecule but should be taken further and cell cycle analysis should be performed. Overall the thiazinoquinones tested were prominently inducers of apoptosis in Jurkat cells. The PPE/PPV copolymers were collectively ineffective at reducing the survival rate of the Jurkat cells below normal population levels so would not need to be investigated further however, they were found to be effective antimicrobials against a range of bacteria including *E.coli,* MRSA and *E. faecium*. Their lack of anti-cancer abilities suggests they may make a suitable topical antimicrobial as they do not cause cell damage and should be investigated further, the *Galleria* infection model should be repeated and mode of action should be further investigated.

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Appendix

Table 11: The significance of each compound, at different concentrations, at reducing the viable population (Figure 3). N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

	Drug Concentration					
	0.001µM	0.01µM	0.1µM	1µM	10µM	100µM
MC4-176-1	N.S	N.S	N.S	N.S	N.S	***
MC4-176-2	N.S	N.S	N.S	N.S	N.S	***
MC4-180-1	N.S	N.S	N.S	N.S	N.S	***
MC4-180-2	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-1	N.S	N.S	N.S	N.S	N.S	***
MC8-28-2	N.S	N.S	N.S	N.S	N.S	N.S

Table 12: The significance of each compound, at different concentrations, at increasing the late apoptotic population (Figure 5). N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

	Drug Concentration					
	0.001µM	0.01µM	0.1µM	1µM	10µM	100µM
MC4-176-1	N.S	N.S	N.S	N.S	**	*
MC4-176-2	N.S	N.S	N.S	N.S	N.S	***
MC4-180-1	N.S	N.S	N.S	N.S	N.S	***
MC4-180-2	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-1	N.S	N.S	N.S	N.S	N.S	***
MC8-28-2	N.S	N.S	N.S	N.S	N.S	N.S

Table 13: The significance of each compound, at different concentrations, at increasing the early apoptotic population (Figure 4). N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

	Drug Concentration					
	0.001µM	0.01µM	0.1µM	1µM	10µM	100µM
MC4-176-1	N.S	N.S	N.S	N.S	N.S	***

MC4-176-2	N.S	N.S	N.S	N.S	N.S	***
MC4-180-1	N.S	N.S	N.S	N.S	N.S	***
MC4-180-2	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-1	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-2	N.S	N.S	N.S	N.S	N.S	N.S

Table 14: The significance of each compound, at different concentrations, at increasing the necrotic population (Figure 6). N= 6 per concentration, per compound. Significant differences between cell survival with compound and a normal cell sample are indicated by ($P \leq 0.05$ = * , $P \leq 0.01$ = ** and $P \leq 0.001$ = ***)

	Drug Concentration					
	0.001µM	0.01µM	0.1µM	1µM	10µM	100µM
MC4-176-1	N.S	N.S	N.S	N.S	N.S	N.S
MC4-176-2	N.S	N.S	N.S	N.S	N.S	N.S
MC4-180-1	N.S	N.S	N.S	*	N.S	N.S
MC4-180-2	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-1	N.S	N.S	N.S	N.S	N.S	N.S
MC8-28-2	N.S	N.S	N.S	N.S	N.S	N.S