Genotoxic effects of NSAIDs and hydrocortisone in bulk and nano forms in lymphocytes from patients with haematological cancers.

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work

Abstract

Chronic inflammation is intimately linked with cancer development and progression and therefore reducing or eliminating inflammation represents a logical treatment and prevention strategy. Studies have shown that anti-inflammatory agents have anti-tumour effects in cancers, with reduced metastases and mortality. Current use of anti-inflammatory agents in the treatment and prevention of cancer is limited by their toxicity and side effects. The emerging field of nanotechnology allows the fundamental properties of a drug to be altered, creating a product with improved reactivity and bioavailability, leading to more targeted treatments and reduced dosage. In the present study, the genotoxic effects of three commonly used anti-inflammatory drugs; aspirin, ibuprofen and hydrocortisone, in their bulk and nano forms were evaluated on peripheral blood lymphocytes of healthy donors using the comet assay and the micronucleus assay. In order to determine any anti-cancer effects, these agents were also tested in peripheral blood lymphocytes in patients with haematological cancers. The glucocorticoid hydrocortisone was also evaluated for anti-oxidant capacity. Our results demonstrate that the nano versions of each drug produced a different response than the bulk counterpart, indicating that a reduction in particle size had an impact on the reactivity of the drug. Our results also indicate that the nano versions of each drug were less genotoxic than the bulk formulation, further emphasising the potential of nanoparticles as an improvement to current treatment options. We also found an anti-oxidant effect with hydrocortisone, with a more profound effect seen with the nano formulation.

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Firstly I must thank Professor Diana Anderson for the opportunity she has given me. It has been a great honour to have worked alongside such a distinguished and inspirational individual. Her dedication to science has motivated me throughout my studies. She is a fantastic scientist and wonderful supervisor. Whenever I had a problem, her door was always open. Without her, none of this would be possible

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And finally, my wonderful hubby. You have been my rock. You always believed in me and sacrificed so much. This is for you darling.

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Chapter 1

General Introduction

1 Introduction

Cancer is one of the largest public health concerns with more than 331 000 people diagnosed in the UK in 2011 and an estimated 1 in 3 people in the UK will develop cancer during their lifetime (Cancer Research UK, 2014). Studies have revealed that the health and economic burden of cancer in the European Union to be €126 billion per year (Luengo-Fernandez *et al.*, 2013). With a worldwide estimate of 8.2 million cancer deaths in 2012, the need to find cost-effective preventative and treatment measures is vital. The emerging field of nanotechnology has created a new dynamic in medicine and pharmaceutical production with many intrinsic properties of substances being amplified at the nanoscale. This widens the scope for existing pharmaceutical agents currently being used in cancer treatment as their effects may be enhanced with a reduction in particle size. Promising results have already been obtained using non-steroidal anti-inflammatory drug (NSAID) nano particles (NPs) when compared to the bulk substance for the treatment and prevention of cancer (Najafzadeh et al., 2016). In this study, the comet and micronucleus assays were used to determine the genotoxic effect of aspirin nano-suspensions (Asp N) and ibuprofen nano-suspensions (Ibu N) when compared to bulk suspensions in peripheral blood lymphocytes of healthy individuals and patients with respiratory diseases. Results showed a decrease in DNA damage, of both healthy individuals and those with disease states, when treated with Asp N and Ibu N when compared to the bulk suspensions. A decrease in DNA damage of lymphocytes treated with Asp N when compared to untreated lymphocytes from healthy individuals was also noted. Although these results are promising for NP treatment, other studies suggest an increase in toxicity with a decrease in

particle size. Osman *et al*. (2010) assessed the toxicity of zinc oxide and titanium dioxide NPs revealing an increase in DNA damage with increasing NP concentrations. The enhanced ability of NPs to cross lipid membranes could result in a shift in their toxicity, and therefore it is necessary to evaluate the effect of NPs on target cells. Previous work in our laboratory has examined at NP formulations in peripheral blood lymphocytes (PBL) from patients with various types of cancer including lung, prostate and breast cancer. This methodology however has not been applied to cancerous tissue samples and therefore we used the blood from patients with haematological cancers as a cancer tissue model for genetic toxicity.

1.1 Haematological Cancers

Cancer is characterised aberrant cell growth, facilitating the potential to invade surrounding tissue or metastasise to other parts of the body. It is a multifactorial process, resulting from the accumulation of mutations leading to the dysfunction of normal cellular function. Haematological malignancies are a diverse group of cancers originating in the bone marrow or lymphatic system. As the circulatory and immune systems are closely linked, the effects of the disease are likely to be reflected in other systems. The main categories include leukaemia, lymphoma and myeloma, however many sub-groups exist. The greatest incidence in haematological cancers are seen in the elderly, however, the exact aetiology is unknown. There is a number of risk factors that can increase the likelihood of the development of a haematological malignancy including exposure to ionising radiation, chemicals and dusts, smoking, viral infection, genetic predisposition and Downs syndrome. Haematological malignancies accounted for 8.4% of all malignant disease diagnoses in England during 2001-

2010 with the incidence of Hodgkin lymphoma, non-Hodgkin lymphoma and myeloma increasing over that period, with no haematological cancers experiencing a decline in incidence (PHE, 2014).

1.2 Hydrocortisone and cancer therapy

Hydrocortisone (HC) is an agent belonging to the steroid hormone family, namely glucocorticoids (GCs). It is commonly used in the treatment of lymphomas, leukaemia's, myelomas and for relief of certain symptoms arising from other cancer. HC mediates its action either directly or indirectly through the glucocorticoid receptor (GR) which, upon activation, is able to alter signalling pathways within the cell and translocates to the nucleus where it acts as a transcription factor influencing processes such as proliferation, differentiation and apoptosis (Schlossmacher *et al*., 2011). The anti-inflammatory and immunosuppressive properties of GCs have also been crucial to cancer treatment. The link between cancer and inflammation has been extensively reviewed (Rakoff-Nahoum, 2006) in which they conclude that chronic inflammation can predispose an individual to cancer through exposure to inflammatory mediators that leads to increased cell proliferation, mutagenesis and can influence tumour progression and metastasis. GCs are able to both inhibit initial events in the inflammatory response as well as promote resolution of inflammation. These are as a result of the transcriptional effects of GR agonism which results in the activation and repression of a number of different genes in leukocytes. GR mediated transrepression (protein-protein binding of GR with target) of pro-inflammatory transcription factors, nuclear factor kappaB (NF-kB) and activating protein 1 (AP1) results in the down regulation of inflammatory mediators (Clark, 2007). Transcriptional activation of a number of

anti-inflammatory mediators, including dual specificity phosphatase 1 (DUSP1), by GR has also been implicated in the anti-inflammatory effect of GCs (Coutinho and Chapman, 2011). The above effects have resulted in HC being incorporated into chemotherapeutic regimes; however, prolonged use has resulted in the development of resistance to the drug. The mechanism of this resistance is a result of genetic alterations in the GC gene resulting in aberrant GR being expressed that is unable to bind GCs and therefore their therapeutic potential is reduced or lost (Moalli and Rosen, 1994). This emphasises the need to modify current pharmaceuticals to prolong their use in cases of resistance and therefore nanotechnology may provide the answer to overcome the resistance and tolerance to HC and in doing so may enhance its intrinsic properties.

1.2.1 Hydrocortisone nano suspensions in current use

HC is widely used as a treatment for various conditions including eye and ear inflammation, allergic reactions and inflammatory skin disorders. The introduction of nanotechnology has provided an opportunity to enhance existing treatments. Reis *et al.* (2013) and Katas *et al.* (2012) demonstrated that using biodegradable polymeric NPs and chitosan NPs to deliver HC respectively, greatly improved the treatment of atopic dermatitis, a chronic inflammatory skin disorder. They found that hydrocortisone-loaded NPs were more stable against degradation and therefore lead to prolonged release of the drug with a more targeted approach increasing specificity and bioavailability. The uniform particle size exhibited led to an increase in skin penetration and a reduced toxicological profile. Both studies illustrate the potential of these NPs as delivery systems for anti-inflammatory drugs that could improve efficacy and reduce adverse effects.

Similar results were reported by *Ali et al*. (2011) in the use of HC nanosuspensions for ophthalmic delivery. In current topical treatments, poor solubility displayed by HC resulted in low corneal permeability and bioavailability. During this study, HC nanosuspensions were developed using microfluidic nanoprecipitation and wet milling procedures. When tested in rabbits, the nanosuspensions demonstrated enhanced bioavailability and sustained action when compared to the HC solution. This highlights the potential to decrease the frequency of administration and therefore improve patient compliance of ocular treatments. Albumin NPs coated with HC were also shown to increase the drug concentration in the pre-corneal target area whilst enabling the targeting of HC away from the inner compartments of the eye where intraocular pressure resulted in adverse side effects in the inflamed eye (Zimmer *et al.*, 1994).

1.3 NSAIDs

In 400 B.C the Greek physician Hippocrates prescribed the extract of willow bark for the treatment of fever and inflammation. It was later found that the active ingredient responsible for the therapeutic effects was salicin, which led to the production of salicylic acid and its derivatives, known as NSAIDs (Rao and Knaus, 2008). NSAIDs are now some of the most commonly used drugs worldwide with analgesic, antipyretic and anti-inflammatory applications. More recently it has been shown that NSAIDs are potential therapeutic agents in the treatment and prevention of cancer and certain neurological disorders (Bacchi et al., 2012). The use of NSAIDs is not without adverse effects such as gastrointestinal (GI) complications, cardiovascular events and renal toxicity,

which severely hinders their potential. The two NSAIDs used in this study include acetylsalicylic acid (aspirin) and the aryl-propionic acid, ibuprofen.

1.3.1 Mechanism of NSAID action

The mechanism of NSAID action was initially described by Vane (1971) as an inhibitor of prostanoids through inhibition of the cyclo-oxygenase (COX) enzyme and this was then further expanded to include the presence of COX-2 by Simmons et al. (2004). Prostaglandins (PGs) are produced via the COX pathway (fig. 1-1) through the metabolism of fatty acids and are implicated as mediators during inflammation, pain, fever and the development of cancer and certain neurological disorders (Bacchi et al., 2012). COX has two isoforms produced by different genes that are similar in structure however have different intracellular locations and substrate/inhibitor selectivity. COX-1 is constitutively expressed and functions as a housekeeping enzyme, responsible for the regulation of a number of cell functions, including preserving the integrity of the gastric mucosa and maintaining platelet and kidney function. The COX-2 enzyme is highly inducible by pro-inflammatory cytokines and growth factors such as interferon γ, tumour necrosis factor α and interleukin 1, revealing a role for COX-2 in inflammation and the control of cellular growth (Vane et al., 1998). Arachidonic acid, a fatty acid embedded in cell membranes, is the precursor to PG and thromboxane synthesis. Through the inhibition of these COX enzymes, a reduction is PG synthesis is observed, subsequently reducing in pain and inflammation through the decrease in vasodilating PGs (PGE_2 and PGI_2) (Simmons et al., 2004).

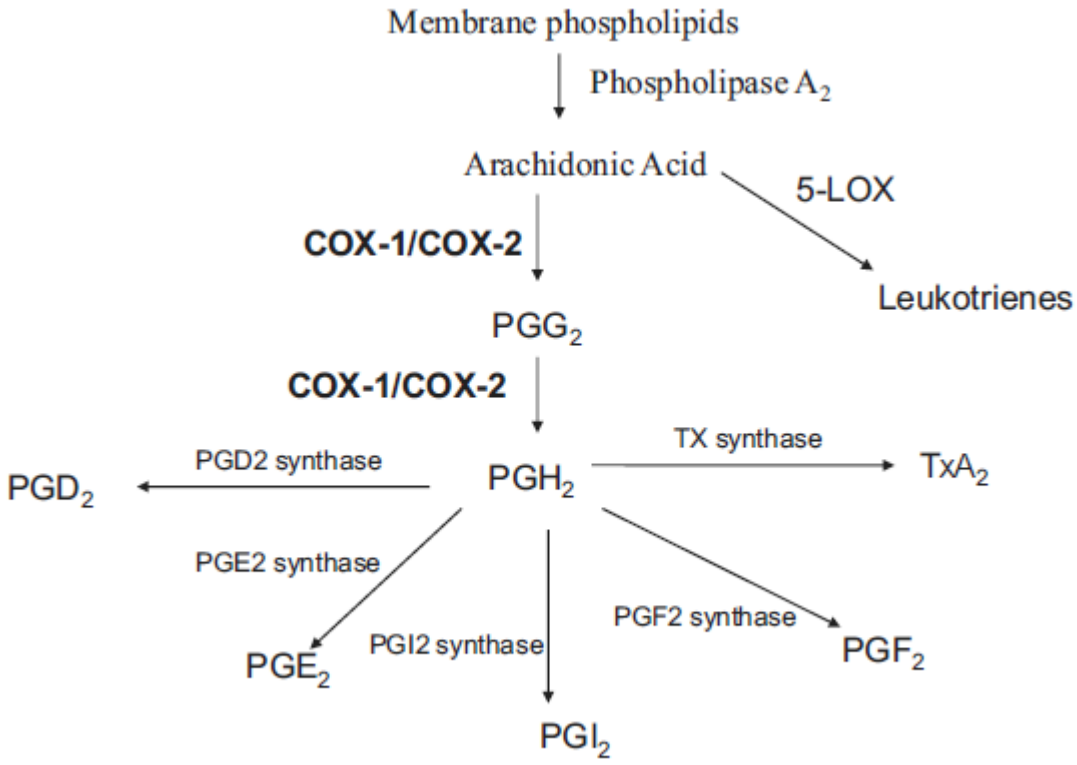

Figure 1-1 Prostaglandin synthesis

Prostaglandin synthesis showing the COX pathway. Figure from Bacchi et al. (2012)

1.3.2 NSAIDs and Cancer

The association of aspirin with anti-metastatic effects was initially demonstrated when the relationship between platelet levels and metastasis was investigated. It was found that reduced platelet levels inhibited the spread of a number of different experimental malignancies and therefore a direct correlation between the ability of a tumour to promote platelet aggregation and its capacity for metastasis was revealed (Gasic et al., 1968). Gay and Felding-Habermann (2011) demonstrated that platelets were able to guard circulating tumour cells from the immune system and promote their arrest at the endothelium enabling the establishment of secondary tumours. Due to the association of

thrombocytopenia with reduced metastasis, aspirin and other drugs that inhibit platelet aggregation were found to significantly reduce the ability of a tumour to metastasise (Gasic et al., 1973), illustrating the potential of these drugs to limit the progression of cancer. Bennett et al. (1977) established that prostaglandins (PGs) were important for the growth of tumours and that abnormal PG synthesis is characteristic of malignant cells and therefore the benefit seen with aspirin treatment was mediated through the inhibition of the COX enzyme which are responsible for prostaglandin synthesis. Randomised trials for the prevention of cardiovascular disease entailed a daily aspirin dose (≥75mg daily) versus control which enabled the effect of daily aspirin on the risk of cancer metastasis to be determined. Results from these trials showed that daily aspirin reduced the risk of distant metastasis by 30%-40%, with a 70% reduced metastasis risk and an overall reduction in deaths in patients with adenocarcinoma that did not have metastasis at diagnosis and remained on treatment for the duration of the trial (Rothwell et al., 2012). This suggests that the effect of aspirin on invasive cancer and cancer death occurs relatively early in carcinogenesis and that aspirin may also have an effect on the growth, spread and initiation of tumours (Chan and Cook, 2012).

Through meta-analysis of randomised controlled trials, Flossmann and Rothwell (2007) were able to demonstrate that the allocation to ≥300mg of aspirin or related NSAIDs daily for 5 years or more reduced the incidence of colorectal cancer after a latency of 10 years. Long—term follow up of individual patient data indicated that daily aspirin reduced the risk of death due to colorectal cancer by 20% and this was maintained at a 20-year follow up (Rothwell et al., 2010b). Further studies indicated that the benefit from aspirin treatment

increased with the duration of treatment and also extended to other common cancers including oesophageal, brain and lung cancer, with a latent period of 5 years (Rothwell et al., 2011). Recently, Streicher et al. (2014) reported the relationship between aspirin use and decreased pancreatic cancer incidence and mortality which is significant in light of the 5 year survival rate of less than 5% for pancreatic cancer. Analysis of observational studies on aspirin and the risk of cancer by Bosetti et al. (2012) confirmed the protective effect for colorectal cancer and other cancers of the digestive tract with a modest risk reduction for breast and prostate cancer. Data on lung cancer was inconclusive however and they failed to find a protective association with pancreatic, endometrial, ovarian, bladder and kidney cancer. Conflicting results were seen from Sturmer et al. (1998) and Cook et al. (2005) who failed to find an association between aspirin use and the risk of cancer, however low aspirin dose with short treatment time and alternative day use respectively could possibly account for these findings.

The discovery that expression of COX-2 was upregulated in cancerous tissue and associated with enhanced invasiveness, increased mutagenesis and proliferation mediated by PGE_2 and the production of reactive oxygen species (ROS) lead to interest in NSAIDs as a means of treating and preventing different types of cancer (Kanaoka et al., 2007, Sobolewski et al., 2010). The use of selective COX-2 inhibitors was of great interest, however the association of the anti-cancer effects of platelet deactivation through COX-1 inhibition further added to the effectiveness of NSAIDs as a cancer preventative (Nash et al., 2002). Aspirin is currently the only drug to permanently inhibit COX-1 and COX-2 activity. Other anti-cancer effects consist of both COX-dependent

(inhibition of sphingosine-1-phosphate (S1-P) production and activation of the NSAID-induced gene (NAG-1)) and independent mechanisms (regulate the activation of nuclear factor kappa B (NF-κB)) and are extensively reviewed in (Stolfi et al., 2013, Ruegg et al., 2003).

1.4 Nanotechnology

Nanotechnology is the study, understanding and manipulation of matter at dimensions between 1 and 1000 nanometers. At this nanoscale, matter exhibits properties distinct from the bulk material including biological, physical and chemical characteristics which provides a platform where all these disciplines converge. It is only recently that scientists have gained the ability to visualise these particles and in doing so acquired the means to take advantage of their unique properties that occur at this scale. Nanoparticles (NPs) have a greater surface area to volume ratio than larger scale materials which allows for more contact between the nanoparticle and the surrounding material resulting in an increased surface energy and improved reactivity (Chan, 2006). These quantum effects seen as a result of particle size affect properties such as electrical and heat conductivity, chemical reactivity and dissolution (Chan, 2006). The endless possibilities generated by nanotechnology have caused much interest in medicine, electronics, engineering and power generation resulting in a rapidly developing and innovative field. Working at the nanoscale provides the opportunity to modify fundamental properties of the material including delivery, immunogenicity and diffusivity. This manipulation at the nanoscale provides the opportunity to produce medicine with greater dissolution with alternative routes of administration and more targeted delivery systems which can reduce therapeutic toxicity and extend the drugs circulatory half-life leading to reduced

health-care costs (Zhang *et al.*, 2007). The implications of nanomedicine are vast with the potential to improve diagnosis, imaging and treatment of disease however the safety of these particles has not been extensively studied with concerns raised over toxicity and environmental impact. Nanoparticles can be inhaled, ingested, injected and absorbed through the skin and the increased use of nanoparticles in manufacturing and industry leads to an increase in exposure rate therefore the adverse effects of nanoparticles to living cells requires further attention and investigations (Oberdörster *et al*., 2005). An understanding of the genotoxic effects of specific nanoparticles on human cells and the mechanisms of this toxicity is vital when considering reducing a bulk compound to the nanoscale.

1.4.1 Applications of nanoparticles in medicine

By reducing the size of compounds, therapeutic properties not exhibited by the bulk compound may be present at the nanoscale despite having the same chemical composition and formula. This broadens the application potential of current agents and therefore much research is focused understanding the behaviour of material at this scale. Nanotechnology has greatly contributed to the development of drugs in cancer therapy and neurological disorders due to the ability of the particles to cross normally impermeable barriers such as the blood-brain barrier and tumour pores. The increased surface area of the particle enhances the solubility and rate of dissolution resulting in increased bioavailability and rapid onset of therapeutic action (Chan, 2006). The increased reactivity of drugs allows for reduced concentrations to be used thereby reducing the potential adverse reactions to potent drugs. Damm *et al.* (2008) demonstrated that silver nanoparticles had a much higher antimicrobial efficacy

than silver micro particles. The rate of silver ion release in the nanoparticles was deemed to be an order of magnitude higher than the micro particle due to the larger surface area of the nanoparticle and as such silver nanoparticles are used in infection prevention and wound healing.

Significant applications of nanoparticles lie in biochemical imaging, drug delivery, gene therapy and tissue engineering (Salata, 2004). The modification potential of the particles allow them to act as a platform for the assembly of multifunctional structures that can be used in drug targeting, sensing and imaging. The size and shape of the nanoparticles can be altered according to the desired drug payload and can also influence the cellular uptake and retention of drugs. Nanoparticle drug-delivery systems can convey drugs more efficiently and conveniently than current methods and in doing so increase patient compliance and drug shelf life with increased drug pharmacokinetics. Semiconductor and metallic nanostructures provide a mechanism in which optical properties can be altered by a change in size resulting in a change in emission wavelengths which facilitates biomedical imaging (Doane and Burda, 2012, Parveen et al., 2012). The properties of NP's as a result of their specific surface area result in an enhanced ability of adsorption, concentration and protection of DNA and RNA making them ideal gene delivery vectors (Sun *et al*., 2014) with modified gold nanometre gene vectors being extensively studied in relation to tumour treatment due to their transfection efficiency.

1.4.2 Hydrocortisone nano suspensions in current use

HC is widely used as a treatment for various conditions including eye and ear inflammation, allergic reactions and inflammatory skin disorders. The introduction of nanotechnology has provided an opportunity to enhance existing

treatments. Reis *et al.* (2013) and Katas *et al.* (2012) demonstrated that using biodegradable polymeric NPs and chitosan NPs to deliver HC respectively, greatly improved the treatment of atopic dermatitis, a chronic inflammatory skin disorder. They found that hydrocortisone-loaded NPs were more stable against degradation and therefore lead to prolonged release of the drug with a more targeted approach increasing specificity and bioavailability. The uniform particle size exhibited led to an increase in skin penetration and a reduced toxicological profile. Both studies illustrate the potential of these NPs as delivery systems for anti-inflammatory drugs that could improve efficacy and reduce adverse effects. Similar results were reported by *Ali et al*. (2011) in the use of HC nanosuspensions for ophthalmic delivery. In current topical treatments, poor solubility displayed by HC resulted in low corneal permeability and bioavailability. During this study, HC nanosuspensions were developed using microfluidic nanoprecipitation and wet milling procedures. When tested in rabbits, the nanosuspensions demonstrated enhanced bioavailability and sustained action when compared to the HC solution. This highlights the potential to decrease the frequency of administration and therefore improve patient compliance of ocular treatments. Albumin NPs coated with HC were also shown to increase the drug concentration in the pre-corneal target area whilst enabling the targeting of HC away from the inner compartments of the eye where intraocular pressure resulted in adverse side effects in the inflamed eye (Zimmer *et al.*, 1994).

1.4.3 Size dependent toxicity

Alterations are seen in the physical and chemical properties of particles, as their size is deceased from the bulk material to the nanoscale with materials

becoming more reactive with an enhancement of their intrinsic properties causing changes in their biological effects. Engineered gold nanoparticles are associated with amplified electrical, chemical, mechanical, thermal and optical properties which differ drastically from the bio-inert bulk counterpart (Yah, 2013), however, this increased reactivity is accompanied by an increase in toxicity. This phenomenon was demonstrated when non-toxic gold particles were reduced to their nanoform resulting in increased toxic effects in living cells (Chen *et al*., 2009). Treatment of BALB/C mice with gold nanoparticles (AuNPs) from 8-37nm resulted in fatigue, alterations in the colour of their fur, loss of appetite and weight loss which was found to be caused by damage to the liver, lungs and spleen. The majority of the mice treated died within 21 days. Treatment of mice with AuNPs of 3-5nm and 50-100nm proved to be non-toxic due to the initiation of a sufficient antibody response and diffusion-restricted effects respectively. Modifications to the surface of AuNPs (8-37nm) with immunogenic peptides resulted in reduced toxicity suggesting that the toxicity exhibited by AuNPs was due to their inability to elicit an immunological response and ability of the nanoparticles to diffuse freely into the cells. Gerber *et al*. (2013) and Yah (2013) extensively reviewed the toxicity associated with AuNPs that includes implicating AuNPs as developmental hazards to mammals through embryo lethality and morphological effects in zebra fish and as a genetic mutagen in the germ line of Drosophila that may be inherited by progeny. Gerber *et al*. (2013) also described increased toxicity associated with a stressed liver environment by accelerating stress-induced apoptosis and stimulating the inflammatory response further highlighting the importance of investigating the particle size, surface chemistry and charge that are crucial to

the development of toxicity. Further evidence of the difference in toxicity displayed by nanoparticles and their bulk counterparts was seen in copper oxide (CuO) nanoparticles, which are increasingly being used in biocides. During acute and chronic toxicity testing in *Daphnia magna*, ten times higher toxicity was seen in CuO NPs than CuO micro particles with a similar affect seen in *Vibrio fischeri* (Rossetto *et al*., 2014). Mice treated with carbon nanotubes exhibited dose-dependent epithelioid granulomas, interstitial inflammation, peribronchial inflammation and necrosis whereas treatment with carbon black induced no toxic effects. The effect exhibited by the carbon nanotubes was comparable to effects seen with treatment with the positive control quartz, which is considered an occupational health hazard in chronic inhalation exposures (Lam *et al.*, 2004). This highlights the importance of classifying materials based on their bulk and nano characteristics separately.

1.4.4 Nano particles and Cell interactions

Large-scale dissipative particle dynamics simulations revealed three main pathways in which NPs enter cells namely spontaneous penetration for very small NPs (diameter <4.5nm), endocytosis and semi-endocytosis for larger NPs (diameter >4.5nm) (Chen et al., 2013). The spontaneous penetration pathway is comprised of three methods of NP internalisation including direct penetration, inverted micelle-like penetration and cooperative chain-like penetration. The adsorption and internalisation of NP were also found to affect the integrity of vesicles as clustering of NP on the vesicle surface lead to various morphological changes and even vesicle rupture. Other adverse effects of NP internalisation indicated that the hydrophilic pore produced during direct penetration may allow leakage of intracellular water. Shang *et al.* (2014)

described the interaction of NP with biomolecules present in bodily fluids such as proteins, sugars and lipids. These biomolecules can coat the surface of the NP and form a protein corona which is able to adapt to varying conditions and concentrations of biomolecules and therefore their composition is not static which establishes the identity for the NP. This is influenced by various properties of the NP including surface chemistry, size, shape and charge. The protein corona facilitates varying interactions with cells and their receptors which could result in the NP being internalised through pinocytosis when a strong interaction is made with surface receptors. Once internalised, NPs may be able to reach the nucleus through diffusion across the nuclear membrane or be actively transported through a nuclear pore allowing direct interaction with DNA (Magdolenova *et al*., 2013). This demonstrates that the size and surface properties of NPs have a strong influence on the method of internalisation, uptake efficiency and cytotoxicity when interacting with living cells.

1.4.5 Nanoparticle toxicity

The mechanisms of NP toxicity are extensively reviewed by Magdolenova *et al.* (2013) and Manke *et al*. (2013) and can be described as either direct/indirect primary or secondary genotoxicty. This includes generation of reactive oxygen species (ROS), oxidative stress, inflammation, genetic damage and inhibition of the cell cycle and apoptosis. Conflicting results have been published detailing NP genotoxicity therefore for the mechanisms of toxicity to be interpreted accurately, the physico-chemical properties of NPs must be established including particle shape, size, composition, crystalline structure, solubility, surface area and properties as well as the agglomeration potential (Magdolenova *et al.* 2013).

1.4.6 Primary genotoxicity

Direct primary genotoxicity involves the direct interaction of NPs with DNA or chromosomes. NPs that are able to enter the nucleus (fig 1-2) may interfere with DNA replication, transcription and repair through either mechanical disruption or chemically binding to the DNA molecules causing structural damage and DNA instability. During mitosis these NPs could introduce breakage to chromosomes and interfere with the progression of mitosis. Li et al. (2013) were able to determine that NPs with a high binding affinity for DNA produced a strong inhibitory effect on replication. They were able to demonstrate that quantum dots and hematite NPs were able to bind to DNA and alter its conformation and that silver and ZnO NPs in particular formed a very compact DNA conformation. This could result in nuclear proteins that are required for transcription, translation or repair not being able to access the relevant DNA binding sites due to NPs occupying the sites directly or through conformational changes of the DNA restricting access or altering spatial relationships. The genotoxic effects that arise from NPs do not always require the NP to be in direct contact with the DNA and therefore can induce toxicity indirectly. Indirect primary genotoxicity may arise through a number of different mechanisms including interaction with nuclear proteins, interaction with the mitotic machinery, disruption of the cell cycle, ROS generation, transition metal formation and inhibition of the antioxidant defence (Magdolenova et al., 2013). Silica NPs were shown to form aberrant inclusions in the nucleus containing various proteins including topoisomerase 1, CBP and polyQ. The result of these protein aggregates was inhibition of replication, transcription and cell proliferation (Chen and von Mikecz, 2005).

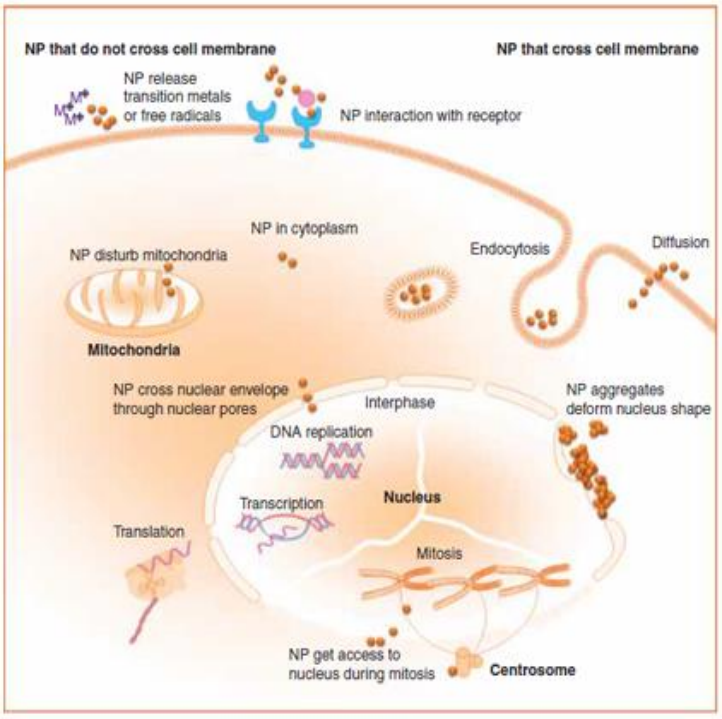

Figure 1-2 Internalisation of NP and access to the nucleus

Diagram illustrating the different routes that NPs become internalised into the nucleus of cells (Magdolenova *et al.* 2013).

1.4.6.1 Nanoparticle mediated oxidative stress

Studies have shown a direct correlation between ROS levels and consequent oxidative stress in NP toxicity (fig 1-3) (Manke *et al*. 2013). Oxidative stress can result from either excessive ROS generation or depletion of cellular antioxidant capacity. NP mediated ROS generation is said to be linked to the surface properties and size of the NP. Dissolution of the NP and the subsequent release of metal ions and the presence of transition metal on the surface are able to catalyse ROS generation (Li *et al.*, 2008). ROS generation, whether endogenous or exogenous, result in the production of free radicals including the superoxide anion and hydroxyl radical that are capable of interacting with and causing damage to DNA and other cellular compartments (Akhtar et al., 2012, Fu et al., 2014). The interaction of ROS with DNA may result in oxidised base lesions and strand breaks leading to the introduction of mutation or cell death

(Magdolenova *et al.* 2013). Toxic levels of these free radicals cause membrane damage and electron chain dysfunction leading to the mitochondrial apoptotic pathway. NPs can contribute to this ROS generation by blocking the electron transport chain of the mitochondria or catalysing the transfer of electrons to molecular oxygen (Manke *et al*. 2013). Ahmad *et al*. (2012) described the relationship between ROS generation and cellular death following the treatment of HepG2 cells with silica NPs. Protein and mRNA levels of apoptotic genes p53, bax and caspase-3 were elevated after exposure to silica NP and a reduction in expression of antioxidant GSH and the anti-apoptotic gene bcl-2 was seen leading to apoptosis. The alterations induced by the silica NPs were attenuated by treatment with the ROS scavenger vitamin C leading to an increase in cell viability and demonstrating that ROS play a vital role in NP medicated cytotoxicity. There is also a clear association between NP medicated ROS generation and the disruption of cellular calcium homeostasis. Calcium plays an important role in cellular metabolism, signal transduction and gene expression and therefore intracellular calcium levels are tightly regulated with an increase in intracellular calcium resulting in cellular dysfunction, metabolic imbalance and cell death. Huang *et al*. (2010) demonstrated a concentration-dependent increase in intracellular calcium in cells treated with ZnO NPs that resulted in cell death. This affect was partially attenuated with the treatment of the antioxidant N-acetylcysteine (NAC) and cytotoxicity completely abolished illustrating the effect of oxidative stress on calcium homeostasis.

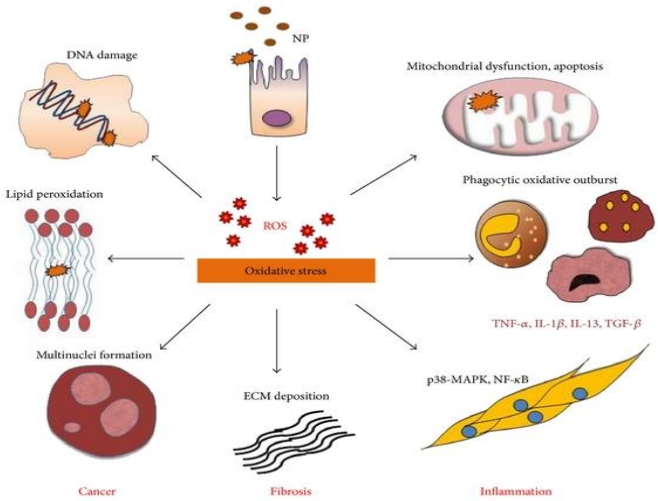

Figure 1-3 Pro-oxidant pathway

Pro-oxidant pathway for NP-induced toxicity: NP exhibit oxidative stress dependent toxicity (Manke et al. 2013).

1.4.7 Secondary genotoxicity

NPs have also been shown to generate ROS and RNS (reactive nitrogen species) in phagocytes through the initiation of inflammation that is able to overwhelm antioxidant and DNA repair mechanisms (Schins, 2013) and therefore suggests that secondary genotoxicity contains a threshold that must be overcome with a significant dose and duration of exposure (Schins, 2002). Trouiller *et al*. (2009) found that changes in cytokine expression in peripheral blood of mice were a result of an inflammatory reaction caused by TiO_2 NPs. This pro-inflammatory activity leads to the activation of phagocytes resulting in oxidative bursts to eliminate NPs, leading to genotoxicity. Pro-inflammatory gene transcription factors (MAP kinases, NF-$_K$B and AP-1) have also been activated in response to NP treatment (Donaldson *et al*., 2012). Park and Park (2009) reported activation of peritoneal macrophages after treatment of mice and the RAW264.7 cell line with silica NPs leading to the release of increased

levels of IL-1β, TNF-α and nitric oxide. This correlated with an increased mRNA expression of inflammatory genes from with the macrophages including IL-6, TNF-α, nitric oxide and COX-2. Treatment of the RAW264.7 cell line revealed ROS generation with a decreased expression of antioxidant glutathione (GSH) further indicating that NPs generate ROS which in turn trigger the expression of pro-inflammatory mediators leading to the activation of immune cells and the resulting ROS generation contributes significantly to genotoxicity.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Ethical approval

This study received approval from Leeds East Research Ethics Committee (REC number: 12/YH/0464), the University of Bradford Research Ethics Sub-Committee on Research in Human Subjects (Ref: 0405/8) and the Research Support and Governance office, Bradford Teaching Hospitals, NHS Foundation (Ref: RE DA 1202).

2.2 Whole-blood collection and processing

Whole blood was collected by venepuncture into lithium heparin Vacuette containers from healthy individuals and patients with haematological cancers. Healthy control individuals were recruited from the University of Bradford. Cancer patients were recruited from the Haematology clinic of Dr Lisa Newton, Consultant Haematologist from Bradford Teaching Hospitals NHS Foundation Trust. Characteristics of patients and healthy individuals are shown in appendix 2. Blood samples were collected after obtaining informed consent (Appendix 3) from the patients and volunteers; a questionnaire (Appendix 4) was also completed prior to sample collection and an information sheet provided (Appendix 5). After collection, blood samples were diluted with RPMI-1640 media (1:1 dilution) and supplemented with dimethyl sulfoxide (DMSO, 10% of the final volume). The sample was then separated into 1 ml aliquots and frozen at -80 °C until required.

2.3 Chemicals

All chemicals used in this study including source and CAS number are summarised in table 2-1. The drugs aspirin, ibuprofen and hydrocortisone were

tested at two particle sizes each, in their bulk form and its nano-sized counterpart to evaluate the effect of particle size on toxicity.

Table 2-1 Chemicals and sources

Chemical	Supplier	CAS Number
Aspirin	Sigma-Aldrich, UK	50-78-2
Cytochalastin-B	Sigma-Aldrich, UK	14930-96-2
Dimethyl sulfoxide	Sigma-Aldrich, UK	67-68-5
DPX Mountant	Sigma-Aldrich, UK	-
Ethanol	Sigma-Aldrich, UK	64-17-5
Ethidium bromide	Sigma-Aldrich, UK	1239-45-8
Formaldehyde	Sigma-Aldrich, UK	50-00-0
Foetal Bovine Serum	Gibco, Thermo Fisher Scientific	N/A
Giemsa stain	VWR	51811-82-6
Glacial Acetic Acid	Sigma-Aldrich, UK	64-19-7
Hydrocortisone	Alfa Aesar	50-23-7
Hydrogen peroxide	Sigma-Aldrich, UK	7722-84-1
Ibuprofen	Albermarle sprt	15687-27-1
Low melting point agarose	Invitrogen, UK	39346-81-1
Methanol	Fisher Scientific, UK	67-56-1
Mitomycin C	Sigma-Aldrich, UK	50-07-7
$Na_2EDTA \cdot 2H_20$	Fisher Scientific, UK	6381-92-6
NaCl	Sigma-Aldrich, UK	7647-14-5
NaOH	Fisher Scientific, UK	1310-73-2
Normal melting point agarose	Invitrogen, UK	9012-36-6
Penicillin-Streptomycin	Invitrogen, UK	-
Phosphate Buffered Saline	Sigma-Aldrich, UK	N/A
Phytohemagglutinin, M form	Gibco, Thermo Fisher Scientific	9008-97-3
Potassium chloride	VWR	7447-40-7
RPMI-1640 Medium	Sigma-Aldrich, UK	N/A
RPMI Medium 1640 + GlutaMAX-I	Gibco, Thermo Fisher Scientific	N/A
Sodium phosphate dibasic	Sigma-Aldrich, UK	7558-80-7
Sodium phonsphate monobasic	Acros Organics	7558-80-7
Tris Base	Fisher Scientific, UK	77-86-1
Triton X-100	Fisher Scientific, UK	9002-93-1

2.4 Preparation of nano-material

Suspensions of aspirin, ibuprofen and hydrocortisone were prepared with solid loads of 5%, 4% and 3% (W/W) respectively. The suspending medium consisted of hydroxypropyl methylcellulose (0.5% w/w), sodium lauryl sulphate (0.1% W/W) and polyvinylpyrrolidone K-30 (0.5%, w/w) in deionised water (Plakkot et al., 2011). Milling of the suspensions was carried out on a Lena Nanoceutics technology DM-100 machine (Sulaiman, 2007). Each suspension (250 ml each) was milled for 60 minutes using 150 ml of 0.2 mm yttrium stabilised zirconium beads (Glen Mills, USA). Solutions were stored in opaque glass bottles at 4 °C for the duration of the experiment and sonicated for 15 minutes prior to use.

2.5 Zeta potential

Zeta potential is one of the fundamental parameters known to affect the stability of a suspension. It is a measure of the magnitude of the electrostatic potential between particles. The zeta potential was measured using a Zetasizer Nano ZS (Malvern Instruments, UK) after the suspensions were diluted 1:100 using deionised water at 25 °C. Clear disposable zeta cells were used with automatic measurement duration between 10 and 100 runs with all measurements made in triplicate.

2.6 Particle size

The Dynamic Light Scattering technique of the Zetasizer Nano ZS (Malvern Instruments, UK) was used to determine the particle size of aspirin, ibuprofen and hydrocortisone nano-suspensions. All measurements were carried out in triplicate using disposable sizing cuvettes at room temperature. The particle size of the suspensions were measured immediately after milling and then

rechecked at the end of experiments to ensure the particle size has not significantly changed during the course of the experiment. The particle size of the bulk powder was measured using laser diffraction (Sympatec Helos, UK). Approximately 20mg of each drug was transferred to the sample vial. Whilst the feeder was running at 40 mm/s, the primary pressure was adjusted to 4 bars with triplicate measurements taken using an R_2 lens (0.25/0.45, 87.5 µm)

2.7 The alkaline comet assay

The alkaline (pH >13) version of the comet assay (SCGE) was used to determine the extent of DNA damage and was performed in accordance with the International Workgroup on Genotoxicity Testing guidelines (Tice et al., 2000) and as described by Singh et al. (1988). Stored whole blood from 12 healthy volunteers and 19 patients with haematological cancers were used in this assay. Frozen samples were allowed to thaw at room temperature prior to use.

2.7.1 Cell Treatment

90 µl of whole blood was incubated for 30 minutes at 37°C with various test agents, made up to a final volume of 1ml with RPMI 1640 media. The test agents included a positive control of 50 µM hydrogen peroxide, aspirin at 500 µg/ml, ibuprofen at 500 µg/ml and hydrocortisone at 50 µg/ml. Optimal doses from each drug were determined from preliminary studies in our laboratory (unpublished data). Aspirin, ibuprofen and hydrocortisone were tested in their bulk and nano forms at corresponding doses. To test the effect of hydrocortisone in a highly oxidising environment, 50 µg/ml of hydrocortisone (both bulk and nano) were co-treated with 50µM hydrogen peroxide. An untreated sample was used as a negative control. Immediately after incubation,

samples were centrifuged at 3000 rpm for 5 minutes and 900 µl of the supernatant discarded. The remaining pellet was then re-suspended in 0.5% low melting point agarose (40°C in PBS) and 100 µl of the suspension was added to coded slides pre-coated with agarose (1% normal melting point agarose). Coverslips were placed on the slides and gels allowed to set for 5 minutes on ice. Coverslips were removed and slides incubated overnight in freshly prepared lysis solution (2.5 M NaCl, 100mM EDTA, 10 mM Trizma base, 10% DMSO and 1% Triton X-100, pH 10) at 4°C in order to disrupt proteins, RNA and membranes. After lysis, the slides were transferred to a horizontal electrophoresis tank and incubated in freshly prepared alkaline electrophoresis buffer (10M NaOH and 200mM EDTA, pH >13) for 30 minutes at 4°C to facilitate the relaxation of supercoiled DNA, after which electrophoresis was performed at 25V, 300mAmps for 30 minutes. Slides were flooded with neutralisation buffer (0.4 M Tris-HCl, pH 7.5) 3 times for 5 minutes.

2.7.2 Slide Staining and Scoring

DNA was stained with the addition of 60µl ethidium bromide (20µg/ml) to each slide and a coverslip applied. Slides were stored in a sealed humidified box at 4°C until scored. Slides were then coded to ensure blind scoring and one hundred nuclei per slide were scored using a fluorescent microscope at 200 X magnification, equipped with a CCD camera using Komet 6 software, Kinetic Imaging (Andor Technology Ltd, Belfast). Olive Tail Moment (OTM) and % Tail DNA were both used to reduce variability in results.

2.8 The Cytokinesis Block Micronucleus (CBMN) Assay

The cytokinesis-block micronucleus assay was used to measure DNA damage and cytostasis induced by the bulk and nano forms of aspirin, ibuprofen and

hydrocortisone. It was also used to compare the inherent DNA damage present in samples from cancer patients when compared to healthy individuals. Whole blood samples were collected from 5 healthy individuals and 5 haematological cancer patients. Cell culture was performed on fresh blood under sterile conditions.

2.8.1 Cell Treatment

Under sterile conditions, 4.5 ml of basic culture media (RPMI-1640 containing L-Glutamine and 25 mM HEPES, 15% fetal bovine serum and 1% penicillin-streptomycin solution) was added to T25 cm^3 vented Corning culture flasks and frozen at -20 °C until required. The flasks were equilibrated at 37 °C (5% CO_2) for 30 minutes prior to use. At the start of cell culture, 400 µl of fresh whole blood was added to each flask, together with 130 µl phytohaemagglutinin (PHA). Flasks were gently mixed and incubated for 24 hours at 37 °C (5% CO_2). Chemical treatments were added at the following concentrations: Asp B and Asp N at 250 µg/ml, Ibu B and Ibu N at 125 µg/ml, HCB and HNC at 50 µg/ml and mitomycin C at 0.4 µM was used as a positive control. A negative control was also included, treated with RPMI-1640. The flasks were incubated for 20 hours at 37 °C (5% CO_2). In order to block cytokinesis, 30 µl of a 1mg/ml solution of cytochalasin-B was added to each flask and further incubated for 28 hours at 37 °C (5% CO_2). Contents of the flasks were transferred to 15 ml Falcon tubes.

2.8.2 Cellular Fixation and Slide Preparation

The tubes were centrifuged for 8 minutes at 800 rpm. Using a vacuum pump, the supernatant was removed and discarded, leaving 500 µl in the tube. 5 ml of a hypotonic solution (90 mM KCl at 4 °C) was slowly added to each flask whilst

gently mixing on a vortex mixer and incubated for 15 minutes at 4°C. The tubes were then centrifuged again and the supernatant discarded, leaving 500 µl. At this point the cells were ready for fixation. Fixation begins with the addition of 5 ml freshly prepared Carnoy's solution (1 part glacial acetic acid to 3 parts methanol), drop by drop whilst mixing gently on a vortex mixer followed by the addition of 3 drops of 38% formaldehyde to each tube. The tubes were centrifuged again as above and fixation repeated a further two times without the addition of formaldehyde. The supernatant was discarded, leaving 100 µl in the tube. Between 200-600 µl of fresh Carnoy's solution was added to each tube, depending on the cell density and pellet size, checked using a phase contrast microscope. Four slides were prepared per treatment group with two 20 µl drops of cell suspension placed equal distances from the edges on each slide and left to air-dry overnight. Slides were stained with Giemsa in Sorenson buffer (5% Giemsa, phosphate buffer) for 20 minutes before being rinsed for approximately two minutes and left to air-dry overnight. Coverslips were mounted on to the stained slides using two drops of DPX Mountant on a heating block at 40 °C and left to set overnight.

2.8.3 Cell Scoring

A total of 1000 cells were scored per treatment group using bright-field microscopy at 400x magnification according to the criteria recommended by (Fenech, 2007, Fenech et al., 2003). The nuclear division index (NDI) is a measure of the proliferative status of the viable cells scored which provides an indication of the cytostatic effects and mitogenic response induced by the different treatments used. The NDI was calculated using the following calculation NDI = M1 + 2(M2) + 3(M3) / N where M1 = mononucleated cells, M2

= binucleated cells, M3 = multinucleated cells and N = total number of viable cells scored. Micronuclei (MN) were scored from binucleated (BiNC) and mononucleated cells (MonoNC). Nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were only scored for BiNC.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad InStat 3.10 and GraphPad Prism software. Data were analysed by Kruskall Wallis one-way analysis of variance, followed by Dunnett's multiple comparisons test (SCGE and CBMN assay) and the Mann-Whitney test, followed by the Wilcoxon matched pairs signed ranks test (anti-oxidant comet assay). *P* values of < 0.05 were considered statistically significant.

Chapter 3

General Results

3 General results

3.1 Particle size and stability

The particle sizes of aspirin, ibuprofen and hydrocortisone nano-suspensions were determined using Dynamic Light Scattering (DLS). This is a well-established method for measuring the size distribution of submicron particles in solution. Following illumination of a sample with a laser, fluctuations of the scattered light are analysed and the size of the particles determined. This was performed prior to cell treatment and at monthly intervals thereafter for nano formulations to ensure particles remained in solution without aggregation. The mean particle size of the bulk powders are listed in Table 3-2 and were obtained from the powders as received from the supplier. The mean particle size before and after treatment of nano suspensions was; aspirin: 289nm and 299nm; ibuprofen: 323nm and 340nm; and hydrocortisone: 248nm and 253nm, respectively (Table 3-1). The zeta potential was also measured to ensure the stability of the suspensions. The zeta potential is a measure of the magnitude of the electrostatic potential between particles with a higher zeta potential indicating a more stable solution able to resist aggregation whilst a low zeta potential demonstrates the tendency of a suspension to flocculate. The zeta potential of the aspirin nano-solution was - 6.1mV (Table 3-1) indicating relative instability and the potential to aggregate therefore fresh suspensions were prepared monthly. Ibuprofen had a higher zeta potential (-2.1, table 3-1) indicating that it was more stable and although checked monthly, did not require fresh monthly preparations. The zeta potential data was not available for the hydrocortisone nano preparation however the particle size was checked monthly and appeared as stable as ibuprofen. Laser Diffraction was used to

determine the mean particle size of the bulk compounds. Aspirin had a mean particle size of 78.30μm, ibuprofen 52.80μm and hydrocortisone 5.98μm. The following data have been generated by Dr M. Isreb, School of Pharmacy, The University of Bradford.

Table 3-1 Average particle size (x90) and the volume mean diameter of the bulk powder (as received) of aspirin, ibuprofen (n=3) and hydrocortisone.

BulkPreparation	Average particle size (μm)	Volume Mean Diameter(μm)
Aspirin	78.30 ± 0.23	44.57
Ibuprofen	52.80 ± 4.37	20.50
Hydrocortisone	5.98	

Table 3-2 Mean particle size, polydispersity index and zeta potential of nano-suspensions taken before and after cell treatment

Suspension	Time of measurement	Mean particle size (nm)	Polydispersity index (PDI)	Zeta Potential (mV)
Aspirin nano-suspension 5%	Before cell treatment	289 ± 3	0.3 ± 0.03	-6.1
	After cell treatment	299 ± 6.3	0.3 ± 0.05	
Ibuprofen nano-suspension 4%	Before cell treatment	323 ± 6.4	0.2 ± 0.01	-2.1
	After cell treatment	340 ± 1.2	0.3 ± 0.001	
Hydrocortisone nano-suspension 3%	Before cell treatment	248	0.2	
	After cell treatment	253	0.3	

Chapter 4

Genotoxicity of aspirin, ibuprofen and hydrocortisone bulk and nano particles on peripheral blood lymphocytes in the comet assay

4 Genotoxicity of aspirin, ibuprofen and hydrocortisone bulk and nano particles on peripheral blood lymphocytes in the comet assay

4.1 Introduction

4.1.1 The comet assay

The comet assay (single-cell gel electrophoresis) is one of the most commonly used assays for measuring genotoxicity and DNA repair. DNA damage and subsequent migration during electrophoresis exhibit the appearance of a comet with fragments of DNA migrating at a different speed to the nucleus. The higher molecular weight DNA in the nucleus forms the head of the comet with leading fragments of damaged DNA migrating at a faster rate forming the appearance of a comet tail. The tail consists of relaxed DNA loops with the tail intensity indicating the extent of DNA breaks and tail length indicating the length of the DNA loops (Collins *et al*., 1997). The assay was originally developed by Ostling and Johanson (1984), however, the neutral conditions described limited the scope of the assay and therefore Singh *et al*. (1988) introduced a modification to the technique that involved electrophoresis under alkaline conditions (pH>13). The alkaline comet assay resulted in increased DNA migration and therefore provided increased sensitivity for identifying genotoxic compounds. This increased migration is associated with increased levels of single strand beaks (SSB), incomplete excision repair sites; double strand breaks (DSB) and the conversion of alkaline labile sites (ALS) to SSB. Collins (2004) noted that SSB are rapidly repaired and not regarded as a significantly mutagenic lesion and therefore high levels of breaks in the comet assay could indicate either high levels of damage or efficient repair. The specific repair mechanisms can be evaluated by treating the cells with lesion-specific repair endonucleases. The

comet assay provided clear advantages in genotoxicity studies when compared to the unscheduled DNA synthesis (UDS) assay and the alkaline elution assay. These included sensitivity for detecting low levels of DNA, small sample requirement, ease of use, cost and time effective, and flexibility in sample type (Tice *et al*., 2000). The comet assay is highly sensitive in detecting SSB, DSB and ALS; however, it is unable to readily detect DNA crosslinks. DNA crosslink are unique in their ability to stabilize DNA and inhibit its migration (Brendler-Schwaab *et al.*, 2005) and this can normally be identified by the degree of DNA migration being less than that for the negative control. These DNA crosslinks are highly relevant in mutagenesis and therefore on these occasions will require further work in the form of extending the electrophoresis time or treating a control and treated sample with an additional genotoxic agent and the migration in the presence and absence of this agent compared (Tice *et al*. 2000). Merk and Speit (1999) however revealed that this modified comet protocol while able to detect DNA-protein crosslinks; it was not well suited for the evaluation of DNA-DNA crosslinks and therefore leads to an underestimation of genotoxicity. The comet assay has also been employed in molecular epidemiology as a potential indicator of oxidative stress and carcinogenesis in human subjects. However, many lifestyle effects need to be considered when evaluating data as physical exercise and ageing have been found to contribute to increased DNA migration. Results are reported as olive tail moment (OTM) which represents the amount and distribution of DNA in the tail, together with the percentage of total DNA in the tail.

4.2 Experimental Aim

In this experiment, we treated cryopreserved lymphocytes from healthy donors and patients with haematological cancers with aspirin, ibuprofen and hydrocortisone in the bulk and nanotised state. With the potential of nano-medicine to enhance the efficacy of drugs, we sought to evaluate whether this increase in activity could lead to an increase in genetic insult. Previous studies from our laboratory using the comet assay indicate that the lymphocytes of patients diagnosed with cancer have increased sensitivity to genetic insult when exposed to ultra violet light (UVA) (Anderson et al., 2014). Further work revealed that this sensitivity extended to insult by DNA damaging agents (unpublished work) however the effect of these agents has not been assessed directly on cancerous cells. Performing he comet assay on lymphocytes from patients with haematological cancers enabled us to assess the direct result of the interaction of our target formulations with cancerous tissue.

4.3 Materials and Methods

All chemicals used in the comet assay are listed in Table 2.1. Methods are as described in section 2.3 and 2.7. Aspirin and ibuprofen (bulk and nano forms) were tested at 500 µg/ml each and hydrocortisone at 50 µg/ml. Bulk powders were freshly prepared before each experiment and solubilised in a suspending medium consisting of hydroxypropyl methylcellulose (0.5% w/w), sodium lauryl sulphate (0.1% W/W) and polyvinylpyrrolidone K-30 (0.5%, w/w) in deionised water. Nano particle solutions were more stable than the bulk solutions and therefore could be refrigerated at 4°C and sonicated for 15 minutes prior to use. The particle size was checked monthly to ensure particles did not agglutinate.

4.4 Results

4.4.1 Genotoxicity of aspirin, ibuprofen and hydrocortisone (bulk and nano particles) on peripheral blood lymphocytes from healthy donors.

The alkaline comet assay was used to determine the genotoxic potential of aspirin, ibuprofen and hydrocortisone on peripheral blood lymphocytes from healthy donors. Bulk and nano-formulations were investigated to determine if a reduction in particle size was accompanied by an increase in genetic toxicity. Results are described as OTM and percentage of tail DNA.

The results from the OTM (figure 4-1), indicated an overall increase in genetic toxicity in the treated samples when compared to the negative control however, only the Asp B and Ibu B were significant (p = 0.0428 and 0.0178, respectively) (table 4-1). In each drug tested, the genotoxicity of the nano formulation was less than that of the bulk compound. The greatest reduction in genotoxicity from the bulk to nano form was seen in aspirin with an OTM of 1.53 seen in the bulk experiment and 1.09 in the nano form, which is only marginally higher than that of the negative control (1.00). Although not significant, the reduction of DNA damage seen in hydrocortisone from the bulk compound to the nano form was less than that of aspirin and ibuprofen, indicating that the reduction in particle size for hydrocortisone did not have as much effect as the other two drugs. Ibuprofen was the most genotoxic agent, with both the bulk and nano forms OTM exceeding that of the other compounds. A similar trend was seen in the percentage of DNA in the tail (figure 4-2) with a significant increase seen in AspB ($p < 0.05$), IbuB ($p < 0.05$) however, a significant increase in tail DNA was also seen in HC B ($p < 0.05$) (table 4-1) which was not seen in the OTM.

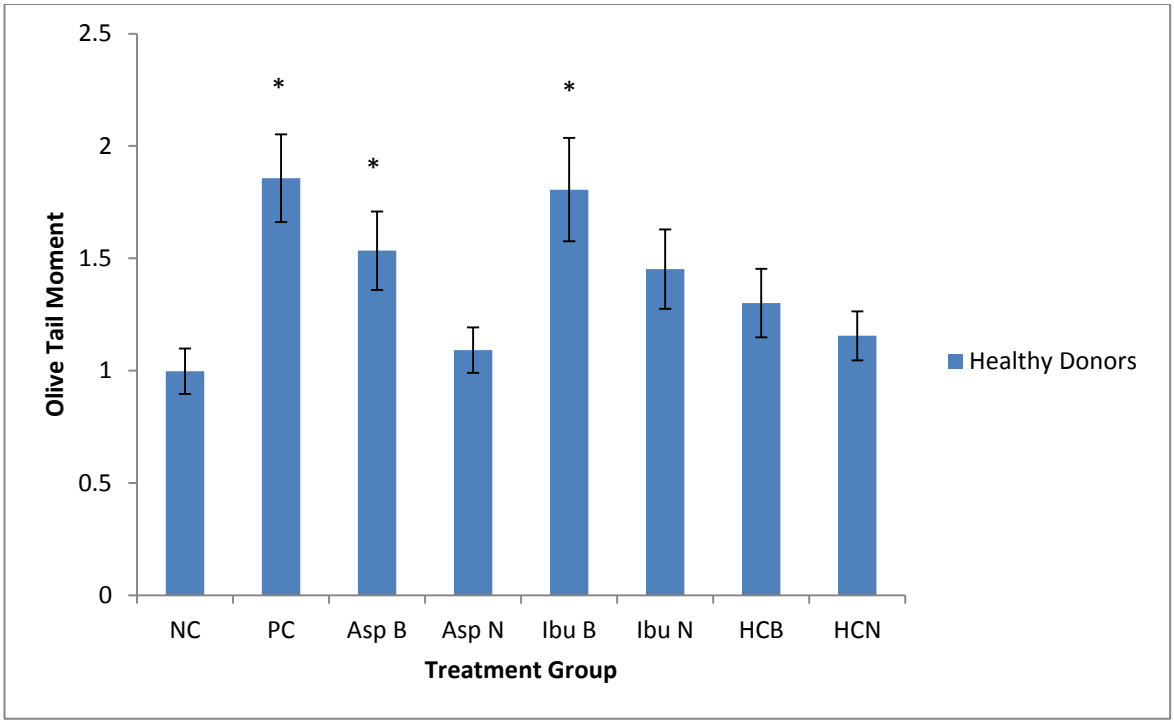

Figure 4-1 Effect of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on lymphocytes from healthy donors using Olive Tail Moment in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

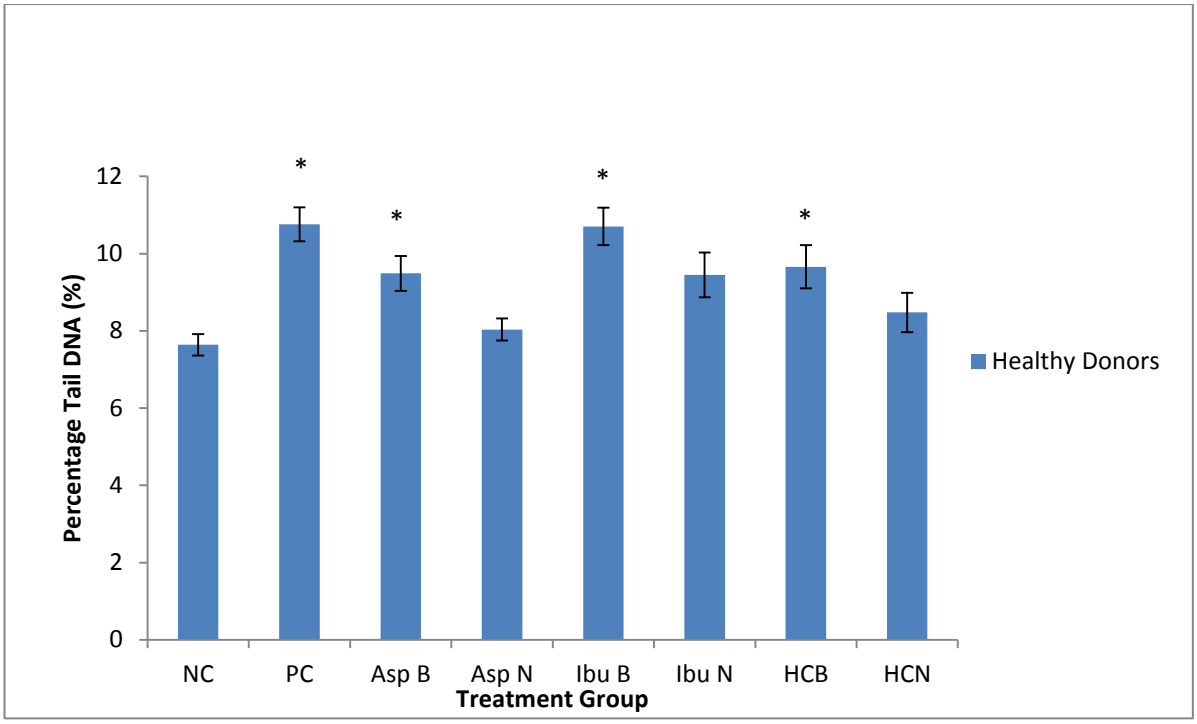

Figure 4-2 Effect of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on lymphocytes from healthy donors using % Tail DNA in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

Table 4-1 Olive Tail Moment and Percentage Tail DNA in Healthy Donors

Treatment	**Olive Tail Moment** **Mean ± SEM**	***p* value**	**% Tail DNA** **Mean ± SEM**	**p value**
Negative Control (NC)	1.0 ± 0.1	-	7.64 ± 0.28	-
Positive Control (PC)	1.86 ± 0.19	< 0.05	10.76 ± 0.44	< 0.05
Aspirin Bulk (ASP B)	1.53 ± 0.17	< 0.05	9.49 ± 0.45	< 0. 05
Aspirin Nano (ASP N)	1.09 ± 0.1	ns	8.03 ± 0.29	ns
Ibuprofen Bulk (Ibu B)	1.81 ± 0.23	< 0.05	10.7 ± 0.49	< 0.05
Ibubrofen Nano (Ibu N)	1.45 ± 0.18	ns	9.45 ± 0.58	ns
Hydrocortisone Bulk (HCB)	1.30 ± 0.15	ns	9.660 ± 0.56	< 0.05
Hydrocortisone Nano (HCN)	1.16 ± 0.11	ns	8.48 ± 0.51	ns

4.4.2 Genotoxicity of aspirin, ibuprofen and hydrocortisone (bulk and nano particles) on peripheral blood lymphocytes from patients with haematological cancers.

The main target of testing these nanotised drugs is to evaluate their genotoxic profile and determine their potential as a cancer treatment option. Previous studies in our laboratory have looked at peripheral lymphocytes from patients with a number of different cancers including lung, prostate and breast cancer however these experiments have not been conducted on cancerous tissue. In this study, we used lymphocytes from haematological cancer patients in order to assess the genotoxicity of these compounds on cancerous cells. The DNA of cancer patients is inherently unstable and therefore their DNA is more susceptible to DNA damaging agents.

Results from the OTM from haematological cancer patients (figure 4-3) showed a significant increase in genetic toxicity of Asp B ($p < 0.05$) and Ibu B ($p < 0.05$), with Ibu B being the most genotoxic agent. In both aspirin and ibuprofen, the nano formulations exhibited reduced genotoxicity when compared to the bulk counterpart, with both nano formulations being marginally increased when compared to the negative control. Interestingly, the HC B showed reduced genotoxicity when compared to the negative control (OTM 1.37 and 1.39 respectively), however this was not significant. The HC N formulation closely matched the HC B, being fractionally higher with an OTM value of 1.42. Results from the percentage of tail DNA (figure 4-4) were similar in that Asp B and Ibu B demonstrated a significant increase in genotoxicity when compared to the negative control ($p < 0.05$ each) with reduced amounts of DNA damage seen in nano forms when compared to bulk. In contrast to OTM results, HCB

demonstrated marginally increased levels of DNA damage when compared to the negative control (8.85 % and 8.43% respectively, table 4-2).

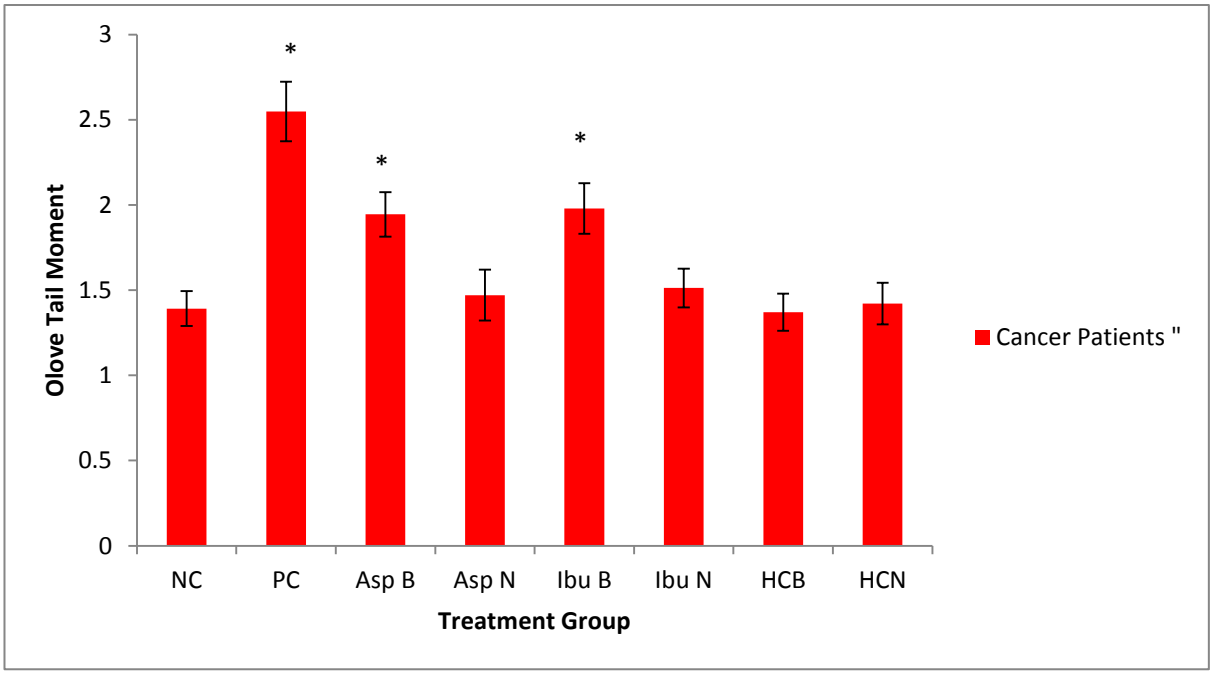

Figure 4-3 Effect of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on lymphocytes from haematological cancer patients using Olive Tail Moment in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

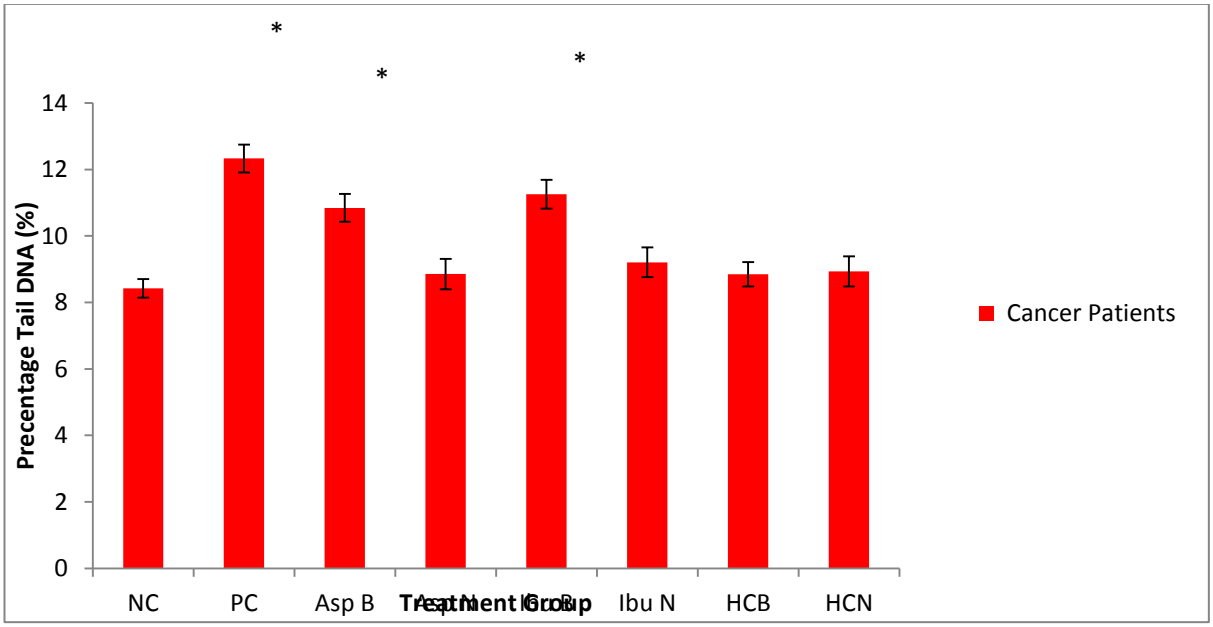

Figure 4-4 Effect of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on lymphocytes from haematological cancer patients using % Tail DNA in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

Table 4-2 Olive Tail Moment and Percentage Tail DNA in Haematological Cancer Patients

Treatment	**Olive Tail Moment** **Mean ± SEM**	***p* value**	**% Tail DNA** **Mean ± SEM**	***p* value**
Negative Control (NC)	1.39 ± 0.1	-	8.43 ± 0.28	-
Positive Control (PC)	2.55 ± 0.17	< 0.05	12.34 ± 0.42	< 0.05
Aspirin Bulk (ASP B)	1.95 ± 0.13	< 0.05	10.85 ± 0.42	< 0.05
Aspirin Nano (ASP N)	1.47 ± 0.15	ns	8.86 ± 0.46	ns
Ibuprofen Bulk (Ibu B)	1.98 ± 0.15	< 0.05	11.25 ± 0.43	< 0.05
Ibubrofen Nano (Ibu N)	1.51 ± 0.11	ns	9.21 ± 0.45	ns
Hydrocortisone Bulk (HCB)	1.37 ± 0.11	ns	8.85 ± 0.36	ns
Hydrocortisone Nano (HCN)	1.42 ± 0.12	ns	8.94 ± 0.45	ns

4.5 Discussion

The comet assay is considered a standard method of evaluating DNA damage and repair in individual cells, being easily performed on various tissue types and relatively inexpensive. It is highly sensitive and therefore is capable of detecting low levels of DNA damage including strand breaks, alkali-labile sites and DNA-DNA/DNA-protein crosslinks. DNA damage and defective repair mechanisms are hallmarks of cancer and therefore the comet assay has the potential to investigate the characteristics of a wide range of cancerous cells in response to DNA damaging agents however, Frötschl (2015) highlighted the limitations of the *in vitro* comet assay as results may not be applicable to *in vivo* conditions. Previous results from our laboratory (unpublished), indicated that the nano versions of aspirin and ibuprofen induced less DNA damage than their bulk counterparts, with aspirin nanoparticles causing reduced migration when

compared to the negative control in PBL of healthy individuals and patients with breast, lung and prostate cancer. It was hypothesised that aspirin nanoparticles conferred a geno-protective effect. We therefore set out to determine the effects of these drugs, as well as hydrocortisone, on actual cancer cells and therefore used the PBL of patients with haematological cancer.

In both the healthy control group and the cancer patient group, a significant increase in DNA damage was observed for the bulk compounds of aspirin and ibuprofen, with hydrocortisone bulk also showing increased levels of DNA damage only in the healthy control group. In each instance, the nano formulations of the drugs did not induce a significant increase in DNA damage. The results for aspirin were somewhat surprising as they did not agree with previous studies in our laboratory or with studies demonstrating the protective effect of aspirin. Aspirin has been shown to confer a geno-protective effect when co-administered with a known genotoxic agent. Obrecht-Pflumio et al. (1996) demonstrated that administration of aspirin to mice before treatment with the carcinogen Ochratoxin A dramatically reduced the amount of DNA adducts seen in the urinary bladder and kidney. A similar effect was seen when aspirin was co-administered with Mitomycin C (MMC) in the somatic mutation and recombination test (SMART) and the DNA repair assay in Drosophila melanogaster (Niikawa et al., 2006, Niikawa and Nagase, 2007).

Ibuprofen bulk induced the highest levels of DNA damage in the comet assay; however the nano formulation did not result in a significant increase. This is in agreement with other studies revealing the genotoxic potential of this formulation in chromosomal aberration assays and micronucleus assays (Tripathi et al., 2012, Ragugnetti et al., 2011), these results however were in

contrast with findings from Philipose et al. (1997) who noted a weakly, but not significant, genotoxic effect in the sister chromatid exchange (SCE) in the bone marrow of mice and no mutagenic effects in the Ames test.

In the healthy control population, the treatment with hydrocortisone bulk resulted in a significant increase in DNA damage, however this was not seen in the cancer patient group. This was surprising as the due to the instability of the DNA of cancer patients, it was predicted that the DNA of cancer patients would be more sensitive to genotoxic agents. More surprisingly was the apparent geno-protective effect seen with hydrocortisone nanoparticles on the PBL of the patients with cancer. The genotoxicity of hydrocortisone has been previously demonstrated (Bali et al., 1990) using micronuclei and sister chromatid exchange analyses with human PBL and mouse bone marrow studies. This clear clastogenic effect was not seen in this experiment. A possible explanation could be due to the single dose of hydrocortisone used and the short incubation time (30 minutes). According to Fahmy et al. (2015), a single dose of hydrocortisone did not have an effect on chromosomal aberrations, however, repeated dosing showed a significant increase in the frequency of sister chromatid exchanges in a dose-dependent manner, in the bone marrow of mice. Another explanation could be the result of DNA cross-linking during the comet assay. DNA-crosslinking is normally suspected when there is reduced migration of DNA when compared to the negative control. This was seen, only marginally, in the presence of hydrocortisone nano. The possibility of DNA cross linking in the experiments involving hydrocortisone will need to be investigated to fully interpret the results. This could be done according to Tice et

al. (2000) and Pfuhler and Wolf (1996) involving the addition of a known genotoxic agent and comparing the migration to that without the agent.

Although the haematological cancer patient group demonstrated slightly higher levels of DNA damage in both the olive tail moment and percentage DNA in the tail when compared to healthy individuals, this was not as considerable as we expected. It has been well established that the basal levels of DNA damage in patients with cancer are significantly higher than healthy individuals and it is based on this finding that many cancer monitoring and indeed diagnostic tests are being conducted (McKenna et al., 2008, Anderson et al., 2014). Inappropriate storage and thaw time could be responsible for these findings. During our assays, we also discovered that the voltage during electrophoresis was not being maintained throughout the tank, which could have adversely affected the migration of DNA.

Chapter 5

Effect of hydrocortisone in a highly oxidising environment in the comet assay

5 Effect of hydrocortisone in a highly oxidising environment in the comet assay

5.1 Introduction

5.1.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive molecules generated by the partial reduction of oxygen and include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO·). They are produced endogenously during mitochondrial oxidative phosphorylation as a by-product of cellular respiration, and may also be introduced exogenously through xenobiotics. When ROS manage to overwhelm the antioxidant mechanisms of the cell, either through an increase in ROS levels or a decrease in antioxidant capacity, oxidative stress occurs. This altered redox status leads to the damage, either direct or indirect, of lipids, proteins and nucleic acids and has been implicated in carcinogenesis (Trachootham et al., 2009), with advanced stage tumours exhibiting multiple genetic mutations and high levels of ROS. Increased levels of ROS have also been associated with an increase in tumour metastasis through gene activation, with ROS scavengers leading to a decrease in metastatic potential in tumours in mice (Ishikawa et al., 2008). ROS are able to modify proteins through the redox regulation of reactive cysteine residues leading to a change in structure and subsequently, function. ROS also have a role in cellular signalling and influence processes such as proliferation, differentiation, inflammation and cell survival through multiple ROS-sensitive signalling pathways, maintaining the cancerous phenotype of the cell (Ray et al., 2012, Storz, 2005). Elevated levels of ROS are also associated with DNA damage leading to lesions in the genome, contributing to cancer initiation, maintenance and progression. Maintaining the balance between antioxidant

systems and ROS generation is vital in cancer treatment and prevention; therefore it is of great importance to assess the antioxidant potential of drugs.

5.2 Experimental aim

In this experiment, we looked at the antioxidant potential of hydrocortisone in its bulk and nano form to establish if the two formulations of the drug were able to reduce the effects of a highly oxidising environment, in the comet assay.

5.3 Materials and Methods

All chemical used in the comet assay are listed in Table 2.1. Methods are as described in section 2.3 and 2.7. To test the effect of hydrocortisone in a highly oxidising environment, 50 µg/ml of hydrocortisone (both bulk and nano) were co-treated with 50µM hydrogen peroxide. Hydrogen peroxide is a potent oxygen radical and served to generate oxidative stress in this experiment.

5.4 Results

In this assay, samples were co-treated with 50µM hydrogen peroxide to assess the effect of hydrocortisone (bulk and nano) in the presence of a highly oxidising environment. As this was the same concentration of hydrogen peroxide as that used in the positive control, the results were compared to this positive control as this reflects the highly oxidising environment without the additional treatment.

5.4.1 Effect of hydrocortisone in a highly oxidising environment in healthy donors

The presence of hydrocortisone nano particles significantly reduced the effects of the hydrogen peroxide when compared to the positive control in both the OTM (1.32) and percentage tail DNA (8.80%) ($p < 0.05$) (Figures 5-1, 5-2 and table 5-1). A reduction in hydrogen peroxide effects were also seen in the bulk

compound, however, these were not significant. Interestingly, both results for HC N were not significantly different from the negative control.

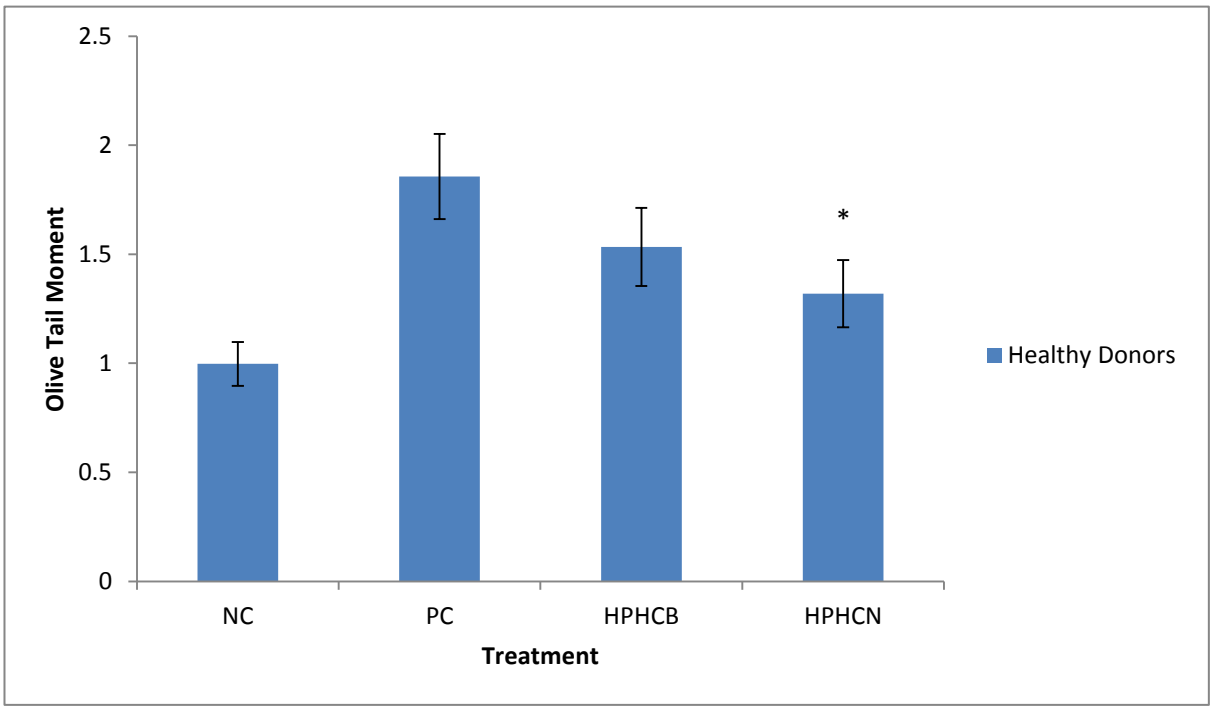

Figure 5-1 Effect of hydrocortisone in a highly oxidising environment in healthy donors using Olive Tail Moment in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

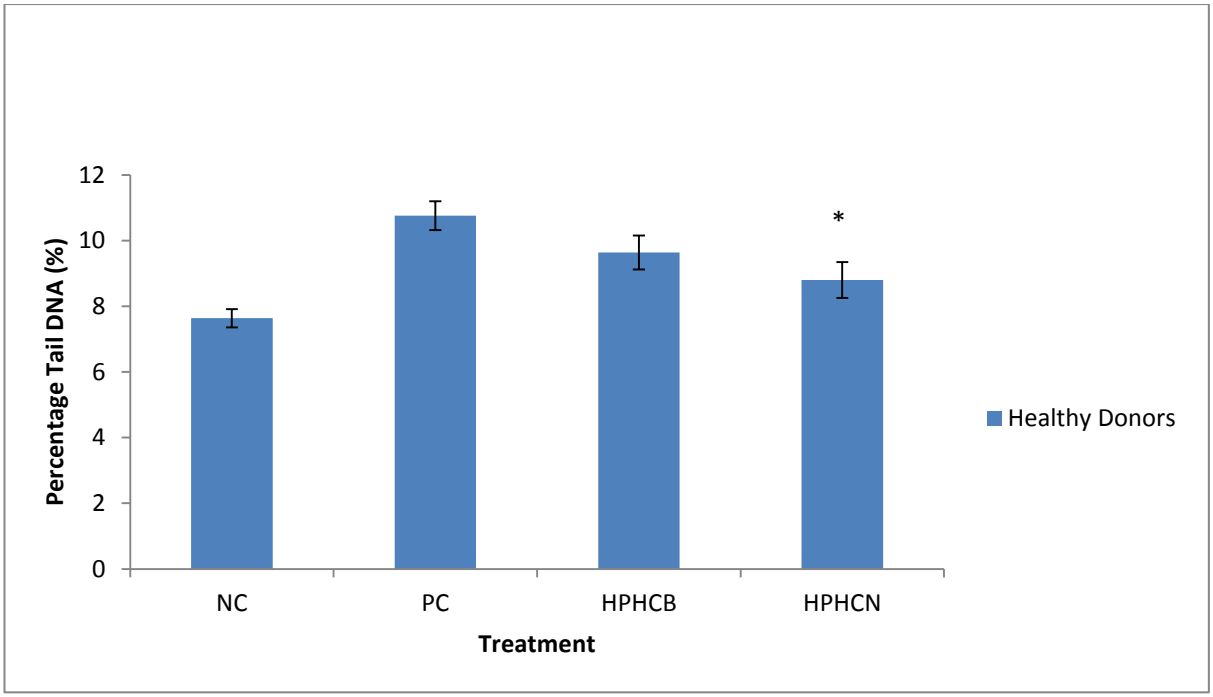

Figure 5-2 Effect of hydrocortisone in a highly oxidising environment in healthy donors using % Tail DNA in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

Table 5-1 Olive Tail Moment and Percentage Tail DNA of healthy donors

Treatment	**Olive Tail Moment** **Mean ± SEM**	***p* value**	**% Tail DNA** **Mean ± SEM**	***p* value**
Negative Control (NC)	1.0 ± 0.1	-	7.64 ± 0.28	-
Positive Control (PC)	1.86 ± 0.19	-	10.76 ± 0.44	-
Hydrogen Peroxide + Hydrocortisone Bulk (HPHCB)	1.53 ± 0.18	ns	9.64 ± 0.51	ns
Hydrogen Peroxide + Hydrocortisone Nano (HPHCN)	1.32 ± 0.15	< 0.05	8.79 ± 0.55	< 0.05

5.4.2 Effect of hydrocortisone in a highly oxidising environment in haematological cancer patients

In lymphocytes from patients with haematological cancers, the presence of hydrocortisone bulk and nano particles significantly reduced the effects of the hydrogen peroxide when compared to the positive control (figures 5-3, 5-4 and table 5-2). An OTM result of HC B was marginally higher than that obtained for HC N, with a similar trend seen in the percentage of tail DNA. As seen in the healthy donors, the HC N (OTM and % Tail DNA) were not significantly different from the negative control. The HC B OTM result was also not significantly different from the negative control.

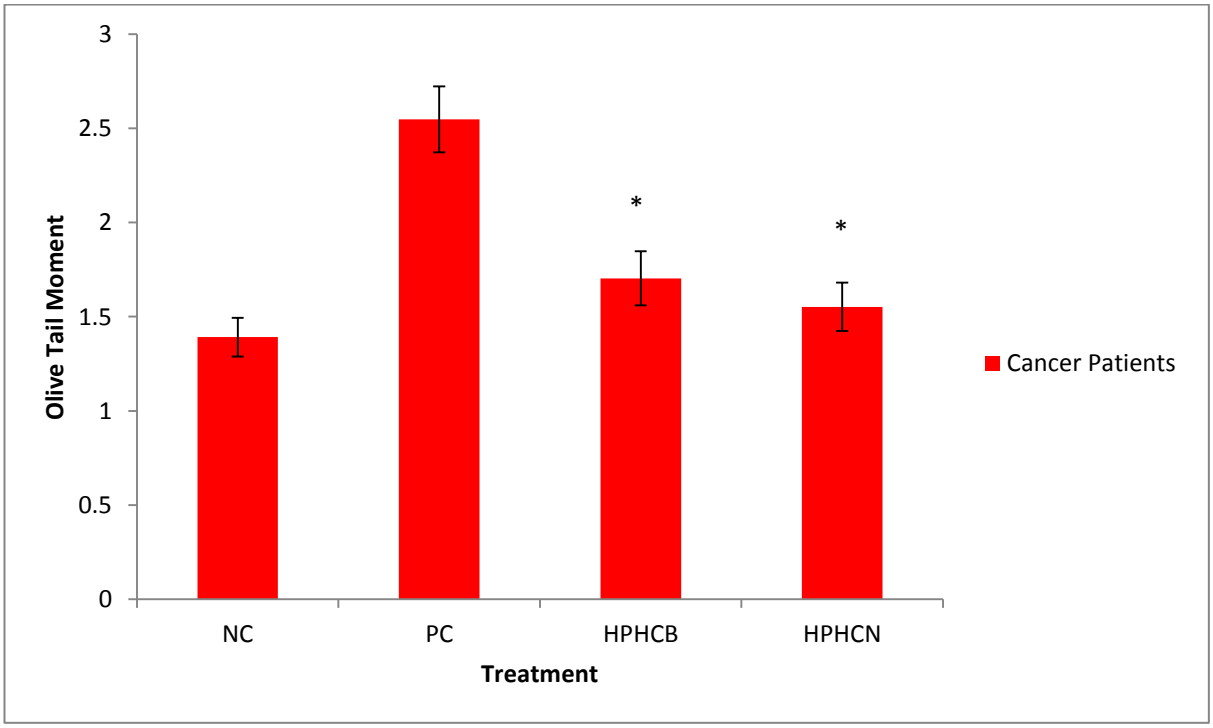

Figure 5-3 Effect of hydrocortisone in a highly oxidising environment in haematological cancer patients using Olive Tail Moment in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

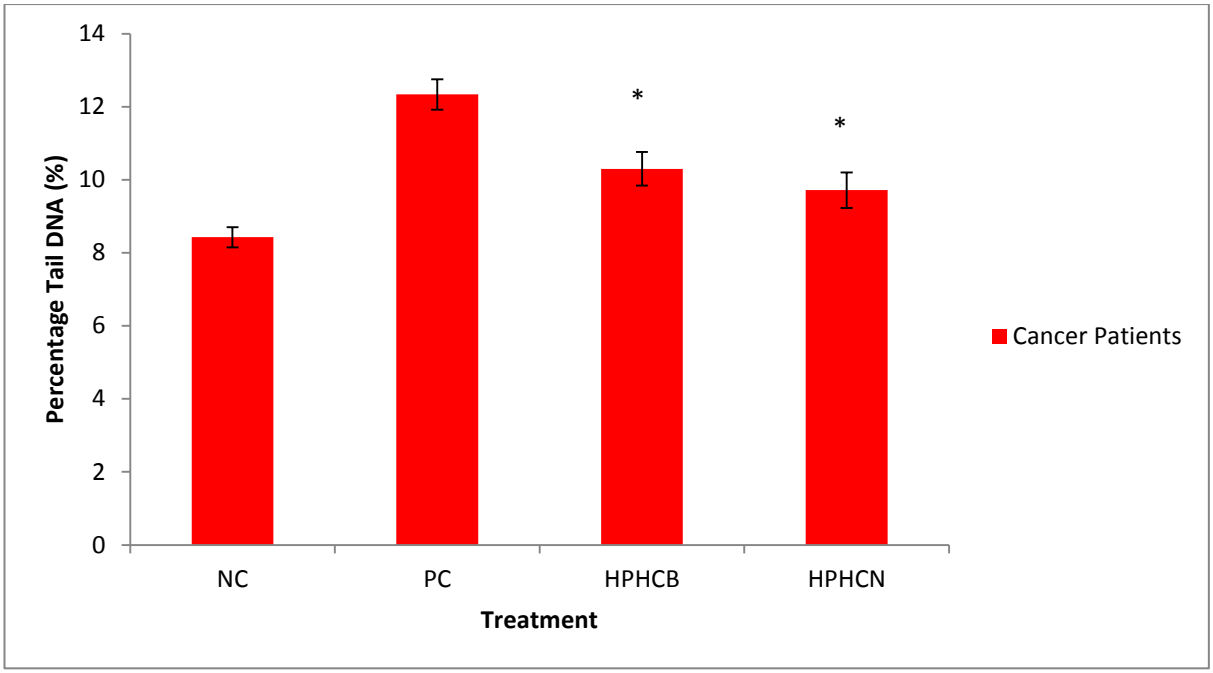

Figure 5-4 Effect of hydrocortisone in a highly oxidising environment in haematological cancer patients using % Tail DNA in the comet assay.

* indicates a significance of $p < 0.05$. Errors bars represent SEM.

Table 5-2 Olive Tail Moment and Percentage Tail DNA of haematological cancer patients

Treatment	**Olive Tail Moment** **Mean ± SEM**	***p* value**	**% Tail DNA** **Mean ± SEM**	***p* value**
Negative Control (NC)	1.39 ± 0.1	-	8.43 ± 0.28	-
Positive Control (PC)	2.55 ± 0.17	-	12.34 ± 0.42	-
Hydrogen Peroxide + Hydrocortisone Bulk (HPHCB)	1.70 ± 0.14	< 0.05	10.30 ± 0.46	< 0.05
Hydrocortisone Nano (HPHCN)	1.55 ± 0.13	< 0.05	9.71 ± 0.48	< 0.05

5.5 Discussion

Oxidative stress occurs when the amount of reactive oxygen species exceeds the antioxidant capacity of the cell, leading to a number of clinical conditions, including cancer (Auten and Davis, 2009). Lee et al. (2013) showed that hydrocortisone had antioxidant effects during ischemia and reperfusion and therefore we set out to determine whether hydrocortisone (bulk and nano particles) displayed antioxidant effects in PBL in the presence of H_2O_2. In our previous experiments involving hydrocortisone nanoparticles, the reduction in particle size was not accompanied by an increase in DNA damage (see chapter 4), however the association between ROS generation and nanoparticle toxicity is well documented (Fu et al., 2014) and therefore we wanted to determine whether hydrocortisone nanoparticles would exacerbate an oxidising environment or alternatively alleviate some of the DNA damage generated by ROS .

The comet assay has previously been used to determine antioxidant properties of various agents in a highly oxidising environment (Anderson et al., 1994). In PBL of both study groups, healthy donors and haematological cancer patients, the presence of hydrocortisone in the highly oxidising environment induced by H_2O_2 treatment resulted in a reduced incidence of DNA damage when compared to controls in the absence of hydrocortisone. The nano version of hydrocortisone had the greatest effect, with results not significantly different from that of the negative control that was not treated with H_2O_2. This indicates that the presence of hydrocortisone could potentially act as a direct antioxidant or is able to influence the generation and capacity of endogenous antioxidant systems. G. Sandal (2013) demonstrated a similar finding when looking at the

influence of hydrocortisone on the antioxidant system in preterm infants with bronchopulmonary dysplasia. They found a significant decrease in the total oxidant status (TOS) and oxidative stress index (OSI) with an increase in total antioxidant capacity (TAC) after treatment with hydrocortisone.

The oxidative effects of H_2O_2 are as a result of its spontaneous conversion to the highly reactive hydroxyl radical (OH·), catalysed by Fe^{2+} in the Fenton reaction (Winterbourn, 1995). Another possibility of the effects of hydrocortisone seen here could be an interference with this Fenton reaction, resulting in reduced levels of the hydroxyl radical being produced. Another possibility is the effect of hydrocortisone on the expression of inflammatory mediators. Reuter et al. (2010) reviewed the relationship between inflammation, oxidative stress and cancer risk, illustrating that they are closely liked.

Oxidative stress is a clear link between environmental toxicity and carcinogenesis. Oxidative lesions resulting from the direct and indirect effects of free radicals have been implicated in the aetiology of cancer (Fuchs-Tarlovsky, 2013). Increased levels of oxidative stress markers and decreased levels of antioxidants seen in different types of cancer highlight the important role ROS play in the pathophysiology of cancer (Badjatia et al., Sharma et al., 2009, Klarod et al., 2011). To mitigate the effects of elevated levels of ROS in cancer development and treatment, the use of antioxidants gained much interest. A number of randomised controlled studies however, were unable to provide evidence that antioxidant supplementation was beneficial in primary cancer prevention (National Cancer Institute, 2014). Studies looking at the use of antioxidants to alleviate treatment toxicities have produced contradictory results (Ladas et al., 2004), with concerns being raised over the ability of antioxidants

to alter or reduce the effectiveness of specific therapies leading to tumour protection and reduced survival (Lawenda et al., 2008). The different mechanisms of antioxidant and cancer therapy action have been implicated in the inconsistencies seen and therefore the combinations need to be matched to ensure the interaction produces a desired effect.

As hydrocortisone is a powerful anti-inflammatory agent, the reduction in inflammatory signal could result in the reduction of oxidative stress. Further work is required in order to determine the exact mechanism of this apparent antioxidant effect of hydrocortisone. It would therefore also be appropriate to determine the effect of hydrocortisone in combination with other cancer therapies to further understand the interactions between the drugs.

Chapter 6

Genotoxic effects of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on peripheral lymphocytes in the cytokinesis block micronucleus assay

6 Genotoxic effects of aspirin, ibuprofen and hydrocortisone bulk and nano preparations on peripheral lymphocytes in the cytokinesis block micronucleus assay.

6.1 Introduction

6.1.1 Micronucleus assay

The micronucleus (MNi) assay is an essential assay in genotoxicity as it enables chromosome mutations that are a direct consequence of DNA damage to be assessed which is an important event in carcinogenesis. Micronuclei are a product of chromosome fragments or entire chromosomes that lag behind at anaphase and are subsequently excluded from the nucleus. The MNi assay allows both chromosome loss and breakage to be measured reliably however this damage can only be expressed as MNi in cells during interphase. The MNi frequency also declines with repeated division and therefore comparison of MNi frequency cannot be established between populations of dividing cells (Fenech, 1997). This lead to a modification of the technique in which cytochalasin-B (CB) was added to block cytokinesis and halt cell division in the binucleated phase. The CBMN assay can be used to measure chromosome breakage, loss, rearrangement, gene amplification and excision repair. It is also used to determine cell division inhibition, necrosis and apoptosis (Fenech, 2006, Fenech et al., 2011) The CBMN is preferred over the MNi as half the numbers of cells require scoring and these are restricted to cells that have divided once and are recognised by their binucleated (BiN) appearance. In addition to MNi visualisation, nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) can be visualised. NPBs are an indication of chromosome rearrangement and this is normally missed as cells proceed through anaphase and telophase rapidly however the inhibition of cytokinesis in CBMN allow BN cells with NPBs to

accumulate and be observed. These NPBs are important as they are an initiating event in breakage-fusion-bridge (BFB) cycles which leads to chromosomal instability. Nuclear buds (NBUD) are an indication of gene amplification. Excess amplified DNA is relocated to the periphery of the nucleus where it buds off as a MN linked to the nucleus by a stalk of nucleoplasmic material. The CBMN assay is a well-established technique however its efficiency is dependent on Cyt-B concentration and therefore the optimum dose needs to be established for each cell type to ensure cells to not escape the cytokinesis block (Surrallés *et al*., 1992).

6.2 Materials and Methods

All chemicals used in the CBMN assay are listed in table 2.1. Methods are as described in section 2.8. Whole blood was collected by venepuncture into lithium heparin Vacuette containers from healthy individuals and patients with haematological cancers and was processed on the same day as collection. 5 samples from each healthy donors and cancer patients were used.

6.3 Results

At the doses for the three drugs selected, neither drug was significantly genotoxic when compared to the negative control, in both the healthy donors and haematological cancer patients; however the frequency of MNi were slightly raised (figure 6-1 and table 6-1). In healthy donors, the frequency of MNi was higher in Asp B when compared to Asp N, which supports data obtained from the comet assay, indicating that a decrease in particle size of aspirin is not associated with an increase in genotoxicity. Surprisingly, the frequency of MNi from cells treated with Ibu B equalled the levels obtained with Ibu N, which is in contrast to the trend seen in the comet assay. In each case of the comet assay,

the nano form of the ibuprofen showed a lower degree of genotoxicity than the bulk compound. HC B showed the highest increase in MNi in all drugs tested, with HC N showing decreased levels when compared to the bulk compound; however it had a higher MNi frequency than Asp and Ibu (bulk and nano). It should be noted that the positive control treated with mitomycin C in one of the healthy donors showed an abnormally high induction of MNi when compared to the other healthy donors. All other results were consistent. This could have been an error in the MMC dosing as the other treatments did not show increased MNi frequencies, therefore making increased susceptibility to DNA damaging agents unlikely. The Nuclear Division Index (NDI) for healthy donors ranged from 1.81-2.05 which was within normal expected limits of 1.3-2.2; (Fenech, 2007) with the frequency of bi-nucleated cells exceeding 50% of the fraction in each instance (figure 6-2). The frequency of NPBs and NBUDs were also within normal limits.

In patients with haematological cancers, Asp N induced a marginally higher level of MNi when compared to Asp B, which was surprising as this is in contrast to other results obtained in healthy donors and that seen in the comet assay. However the MNi frequency for aspirin was considerably less than that seen in ibuprofen, both bulk and nano, which is consistent with data from the comet assay. Both Ibu B and HC B induced higher frequencies of MNi when compared to their nano counterpart. It is clear from figure 6-1 that the difference in MNi frequency is significantly increased in the haematological cancer group when compared to the healthy donors. The NDI for patients with haematological cancers ranged from 1.73 – 2.04, with the lowest NDI lower than healthy individuals however still within normal ranges with a frequency of BN cells of

over 50% (figure 6-3). The frequency of NPBs and NBUDs were marginally higher than that obtained for healthy individuals, especially with regard to NBUDs however all were within normal limits.

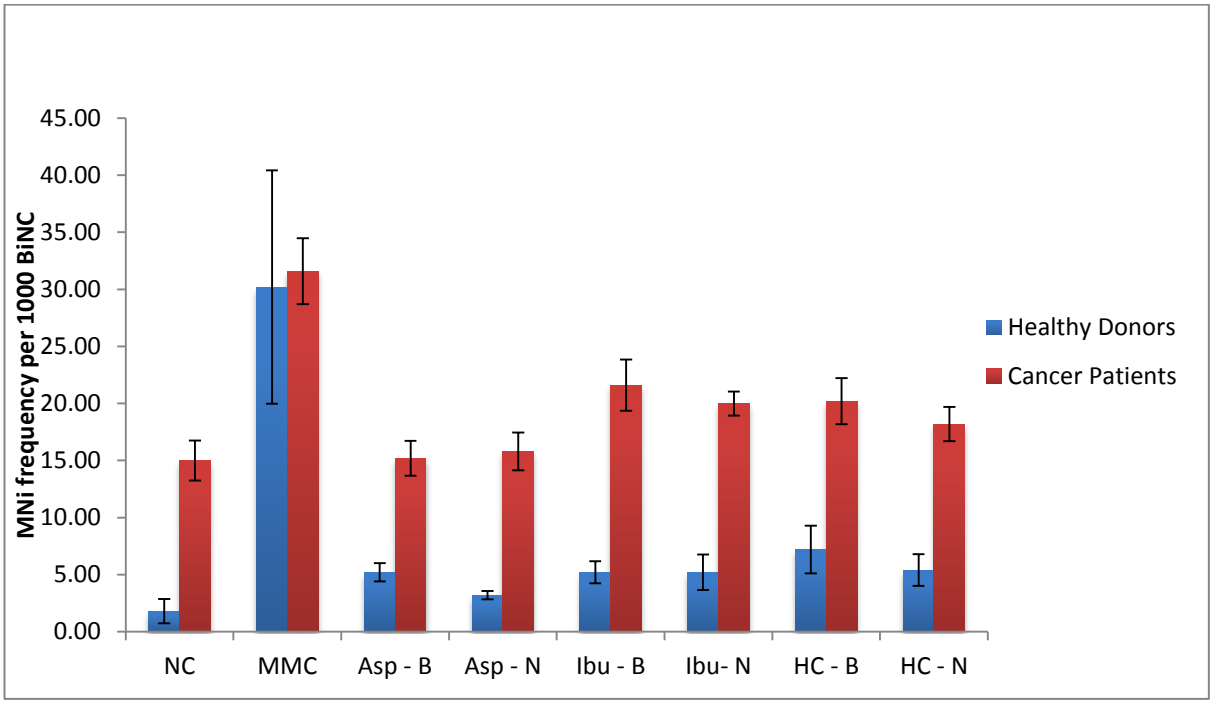

Figure 6-1 Frequency of BiNC with MNi per 1000 cells using the CBMN Assay. Results from healthy donors are in blue and haematological cancer patients in red.

Errors bars represent SEM.

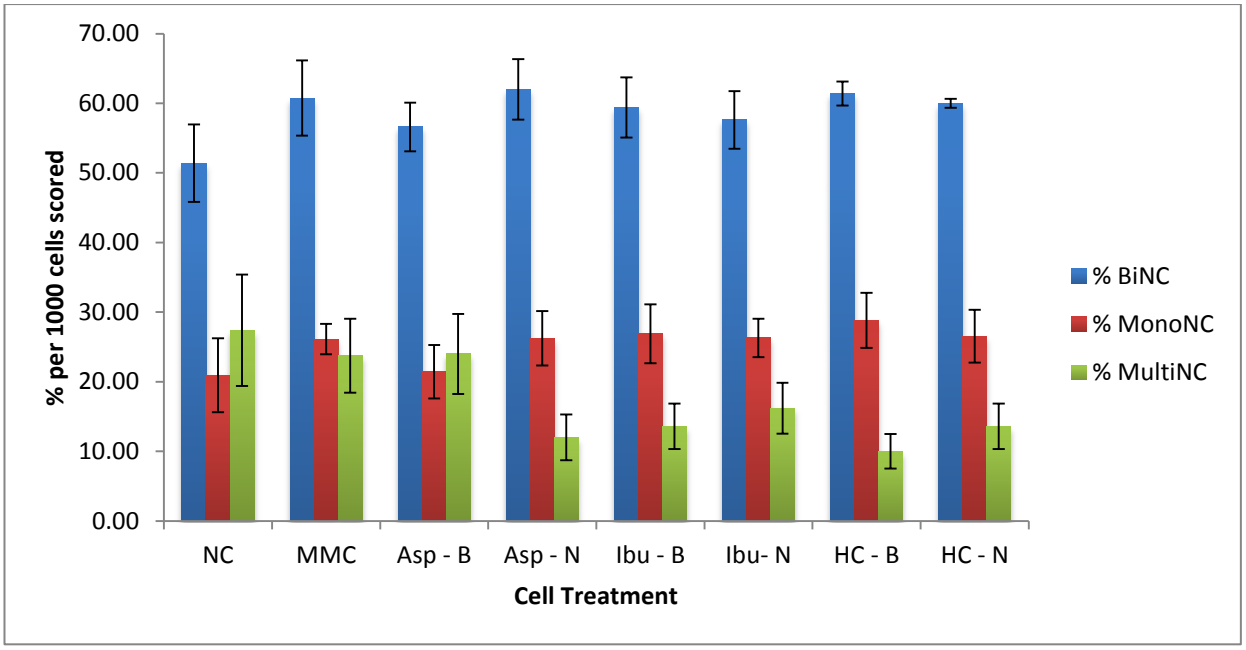

Figure 6-2 Proliferative status of viable cells in Healthy donors. Number of nuclei (%) per 1000 scored cells in the CBMN assay of BiNC (blue), monoNC (red) and multiNC (green).

Error bars represent SEM.

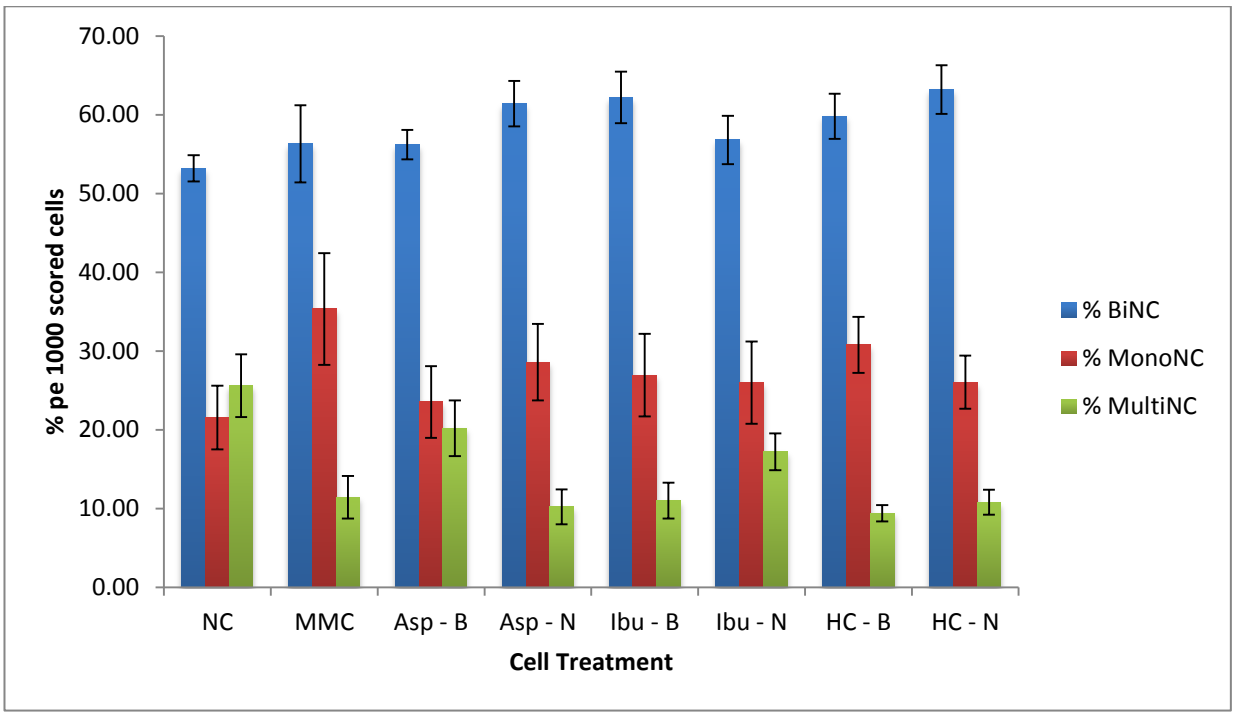

Figure 6-3 Proliferative status of viable cells in patients with haematological cancer. Number of nuclei (%) per 1000 scored cells in the CBMN assay of BiNC (blue), monoNC (red) and multiNC (green).

Error bars represent SEM.

Table 6-1 Micronucleus assay proliferative and genotoxic effects

Subject	Treatment Group	NDI	% BiNC	% MoNC	% Multi-NC	BiNC cells		
						BiMNi	BiNPB	BiBuds
Healthy individuals	NC	2.05 ± 0.12	51.40 ± 5.57	20.94 ± 5.31	27.40 ± 8.00	1.80 ± 1.07	0.00 ± 0.00	0.60 ± 0.40
	MMC	1.87 ± 0.05	60.74 ± 5.40	26.12 ± 2.19	23.74 ± 5.31	30.20 ± 10.23	1.40 ± 1.40	0.00 ± 0.00
	Asp - B	2.03 ± 0.10	56.60 ± 3.49	21.44 ± 3.84	24.00 ± 5.74	5.20 ± 0.80	0.20 ± 0.20	0.80 ± 0.49
	Asp - N	1.86 ± 0.06	62.00 ± 4.34	26.22 ± 3.91	12.00 ± 3.29	3.20 ± 0.37	0.20 ± 0.20	0.20 ± 0.20
	Ibu - B	1.87 ± 0.06	59.40 ± 4.31	26.88 ± 4.24	13.60 ± 3.26	5.20 ± 0.97	0.00 ± 0.00	0.60 ± 0.40
	Ibu- N	1.90 ± 0.05	57.60 ± 4.15	26.28 ± 2.75	16.20 ± 3.65	5.20 ± 1.56	0.20 ± 0.20	0.60 ± 0.40
	HC - B	1.81 ± 0.06	61.40 ± 1.72	28.82 ± 3.94	10.00 ± 2.49	7.20 ± 2.08	0.20 ± 0.20	0.40 ± 0.40
	HC - N	1.87 ± 0.07	60.00 ± 0.63	26.54 ± 3.81	13.60 ± 3.26	5.40 ± 1.40	0.20 ± 0.20	0.20 ± 0.20
Cancer Patients	NC	2.04 ± 0.08	53.20 ± 1.66	21.54 ± 4.05	25.60 ± 3.98	15.00 ± 1.76	0.60 ± 0.40	1.20 ± 0.49
	MMC	1.73 ± 0.10	56.30 ± 4.89	35.34 ± 7.09	11.42 ± 2.71	31.60 ± 2.89	2.40 ± 0.40	1.20 ± 0.37
	Asp - B	1.97 ± 0.08	56.20 ± 1.85	23.52 ± 4.55	20.20 ± 3.54	15.20 ± 1.53	0.40 ± 0.24	1.00 ± 0.45
	Asp - N	1.82 ± 0.07	61.40 ± 2.87	28.58 ± 4.84	10.20 ± 2.20	15.80 ± 1.66	0.80 ± 0.20	1.40 ± 0.51
	Ibu - B	1.86 ± 0.07	62.20 ± 3.26	26.92 ± 5.25	11.00 ± 2.28	21.60 ± 2.25	0.40 ± 0.24	1.00 ± 0.55
	Ibu - N	1.91 ± 0.08	56.80 ± 3.06	25.98 ± 5.24	17.20 ± 2.33	20.00 ± 1.05	0.60 ± 0.24	1.60 ± 0.75
	HC - B	1.78 ± 0.04	59.80 ± 2.85	30.78 ± 3.55	9.40 ± 1.03	20.20 ± 2.03	0.80 ± 0.20	0.80 ± 0.20
	HC - N	1.85 ± 0.04	63.20 ± 3.10	26.04 ± 3.39	10.80 ± 1.59	18.20 ± 1.50	0.60 ± 0.40	1.00 ± 0.32

NC – Negative control, PC- positive control with mytomycin C treatment, Asp-B – aspirin bulk, Asp-N – aspirin nano, Ibu-B – ibuprofen bulk, Ibu-N – ibuprofen nano, HC-B – hydrocortisone bulk, HC-N – hydrocortisone nano, NDI – nuclear division index, BiNC- binucleated cells, MoNC – mononucleated cells, Multi-NC – multinucleated cells, BiMNi – binucleated cells with micronuclei, BiNPB – binucleated cells with nucleoplasmic bridges, BiBuds – binucleated cells with nuclear buds.

6.4 Discussion

The Cytokinesis-block micronucleus assay has become an important test in the screening of potentially genotoxic compounds. It has the ability to detect clastogenic and aneugenic events in multiple cell types. The presence of micronuclei and other abnormalities such as nucleoplasmic bridges and nuclear buds are indicators of genomic instability, a hallmark of cancer. In this study, we looked at the genotoxicity of aspirin, ibuprofen and hydrocortisone in bulk and nano formulations in the CBMN assay. The nuclear division index (NDI) is a marker of cell proliferation and therefore a measure of general cytotoxicity with a large degree of chromosomal damage resulting in a lower NDI. If all viable cells fail to divide, they will be mono-nucleated with a NDI score of 1.0. If all viable cells completed nuclear division, they will be binucleated with an NDI of 2.0. If viable cells divide more than once and appear multi-nucleated, the NDI score will be above 2.0 (Fenech, 2007). In both study groups, healthy donors and haematological patients, the NDI of the negative control was 2.05 and 2.04 respectively, indicating a low level of chromosome damage. The NDI for haematological cancer patients was surprising as a lower NDI, indicative of genomic instability, would have been expected. All treatments in both groups resulted in a decrease of the NDI; however these were all in normal limits. The proportion of apoptotic and necrotic cells however were not scored, limiting the data available to assess the cytotoxic effects of the drugs. The MNi frequency increased in each treatment group when compared to the negative control; however this increase was not significant. Aspirin in its bulk and nano form were the least genotoxic agents in both study groups, with hydrocortisone bulk inducing the highest increase in MNi frequency in healthy controls and

ibuprofen bulk inducing the highest increase in MNi frequency in haematological cancer patients. In general, the study group of patients with haematological cancers had a significantly higher MNi frequency when compared to healthy donors, in all treatment groups as well as the negative control. This was as expected as a higher degree of chromosome damage and genomic instability was expected in this group, making the PBL more susceptible to genetic insult giving rise to increase MNi frequencies (Fenech et al., 1999, El-Zein et al., 2008). Repeated measures were difficult in light of only receiving one blood sample from the patients with haematological cancers. This experiment would produce more robust results if the cohort was expanded and repeated testing was done in addition to establishing a dose-dependent relationship for each drug formulation.

These results are encouraging as this is the first time these drugs have been tested in their nano form directly on cancer cells. Although no geno-protective effect was seen, neither of the drugs induced significantly increased levels of chromosomal abnormalities and therefore the potential for them to be used as an adjuvant in cancer therapy remains plausible.

Chapter 7

General Discussion

7 Discussion

Mutations in DNA can result from a number of sources, including physical, chemical and biological means leading to genomic instability and cancer. Therefore it has become essential to assess genotoxicity as part of the drug validation process. The comet and micronucleus assays have been recognised as a valid test for genotoxicity by the regulatory agencies due to their sensitivity and high statistical power in identifying mutagenicity in the form of DNA and chromosomal damage. As this work is exploratory, the use of the *in vitro* comet and micronucleus assays was employed.

The link between chronic inflammation and cancer is well established. Strong correlations between the presence and persistence of inflammation at a particular site with the development of pre-cancerous lesions exist (Nelson et al., 2004, Otsuki, 2003, Macarthur et al., 2004, Coussens and Werb, 2002) intimating that inflammation facilitates cancer development and progression. During chronic inflammation, pro-inflammatory mediators are upregulated, altering the dynamics of the local microenvironment that promotes the development of cancer through multiple events including increased incidence of DNA damage, increased cellular proliferation and inhibition of apoptosis (Hofseth and Ying, 2006). Due to the numerous pro-tumour effects generated by the presence of inflammation, it has become a key target in cancer treatment and prevention. This report looked at three well known anti-inflammatory drugs, two NSAIDs and one corticosteroid, and their effect on DNA damage in peripheral blood lymphocytes in healthy donors and patients with a variety of different haematological malignancies to determine whether they had geno-protective or genotoxic effects in individual cells.

NSAIDs inhibit COX enzyme activity which reduces the effects of inflammation. It has therefore become a key drug class in the effort to find agents to improve the outcome of certain cancerous states as well as being a preventative measure. A number of studies have demonstrated the potential for aspirin in the prevention of cancer with associations of reduced metastasis, reduced incidence and a reduction in mortality in colorectal cancer, adenocarcinoma, lung, prostate, pancreatic, breast and oesophageal cancer (Flossmann and Rothwell, 2007, Rothwell et al., 2011, Rothwell et al., 2010b, Rothwell et al., 2012, Streicher et al., 2014, Bosetti et al., 2012). Evidence also suggests that aspirin may have geno-protective action, with treatment leading to an increase in DNA repair mechanisms (Dibra et al., 2010). These studies however conflict with reports showing no association between aspirin use and the reduced incidence in cancer (Sturmer et al., 1998, Cook et al., 2005) and an association between increased breast cancer incidence and aspirin use (Friis et al., 2008). Our results, in both the comet assay and the micronucleus assay, did not find a geno-protective effect for aspirin. These results do not agree with other data generated in our laboratory (Najafzadeh et al., 2016) in which aspirin exhibited anti-cancer effects in lung cancer. In this study, however, it was the first time these compounds had been tested directly on cancerous cells and therefore variations were to be expected. Ibuprofen was clearly the most genotoxic of the two NSAIDs, with higher rates of DNA damage and MNi induction. This is in agreement with finding from Tripathi et al. (2012) and Ragugnetti et al. (2011) who demonstrated increased chromosomal aberrations in mouse bone marrow and increased MNi frequency in *Oreochromis niloticus* fish, respectively.

Although results here are different, there is overwhelming evidence that NSAIDs have potential in the treatment of cancer. In their current form, aspirin and ibuprofen both have limitations in their bioavailability. Aspirin is a class III drug and therefore absorption is the limiting step. Ibuprofen, a class II drug, is only slightly soluble in water and therefore the bioavailability is limited by its dissolution rate. In order to increase the bioavailability of these drugs, nano forms are being produced. Our results when comparing the nano forms of the drugs to their bulk forms demonstrate that a decrease in particle size corresponds to a difference in response. In each case, the nano form of the drug was less genotoxic than its bulk counterpart, often not significantly different from the untreated control. This highlights the potential of these drugs in their nano form to be used as therapeutic options, however more testing needs to be done before conclusions can be confidently drawn.

Another anti-inflammatory drug examined was the glucocorticoid hydrocortisone. Hydrocortisone has been used in a co-treatment regime in many haematological malignancies due to its anti-inflammatory and anti-cancer effects. These effects are due to the regulation of the glucocorticoid receptor which reduces the expression of pro-inflammatory genes, negatively regulates survival cytokines and induces the expression of pro-apoptotic genes, leading to a suppression of tumour progression and metastasis (Lin and Wang, 2016). Yano et al. (2006) found that the glucocorticoids dexamethasone and hydrocortisone supressed androgen-independent prostate cancer growth through the inhibition of tumour-associated angiogenesis.

In our studies of the genotoxicity of hydrocortisone in the comet assay, treatment of PBL with hydrocortisone in both the bulk and nano form did not

induce a significant increase in DNA damage in both study groups, with hydrocortisone treatment in the haematological cancer patient group very similar to the untreated control. The genotoxicity of hydrocortisone seen in the micronucleus assay however was higher than that seen in the comet assay, however the induction of MNi was not significant. This is in contrast to the studies by Fahmy et al. (2015), Fahmy (2014) who demonstrated the dose-dependent increase in chromosomal aberrations and sister chromatid exchanges after multiple does of hydrocortisone. They did however find that a single treatment of hydrocortisone at different doses did not induce genotoxicity (Fahmy et al., 2015) which could explain the lack of toxicity found in this work. Although the nano version of hydrocortisone induced less DNA damage than that of its bulk counterpart, especially in anti-oxidant assays, this difference in action of the two formulations was not as profound as that of aspirin and ibuprofen, demonstrating that the reduction in particle size of hydrocortisone did not generate a significantly different response from the bulk form. Our results also demonstrated, at this particular dose, the potential of hydrocortisone as an antioxidant with hydrogen peroxide-induced oxidative stress reduced in co-treatment experiments. A study by Alotaibi et al. (2013) demonstrated that polyphenol compounds can act as both antioxidants and pro-oxidants, depending on the form and dose of administration. It is therefore reasonable to conduct further experiments to determine if hydrocortisone (in either bulk or nano) could act in a similar manner.

Human lymphocytes were chosen to assess genomic sensitivity to potential genotoxins in the alkaline Comet assay and the Micronucleus assay. Peripheral blood lymphocytes are regarded as suitable cells in biomonitoring experiments

(Albertini et al., 2000) as large populations circulate through the entire body, being exposed to different microenvironments including genetic insults (Anderson et al., 2014). The effect of long term cryo-storage on whole blood is said to maintain the viability of cells for up to ten years (Abbruzzese et al., 2013). The effects of cryopreservation on isolated lymphocytes is conflicting with Duthie et al. (2002) reporting that cryopreservation had no detrimental effect on endogenous or induced mutation frequencies in human lymphocytes however the cryopreserved lymphocytes were defective in repair assays when compared to fresh lymphocytes. Fang et al. (2015) however noted that lymphocytes cryopreserved prior to lysis and electrophoresis exhibited significantly increased frequencies of single-strand breaks when compared to fresh lymphocytes. Another factor to be considered is the use of whole blood instead of isolated lymphocytes. Studies indicate that the presence of whole blood induced significant increases in DNA damage in lymphocytes in the comet assay and that DNA damage was elevated 10-fold in lymphocytes analysed in the presence of red blood cells (RBC) compared with isolated lymphocytes (Narayanan et al., 2001). Components in whole blood, including lysis of RBC and activated neutrophils, are capable of causing increased incidence of DNA strand breaks through the release of haemoglobin and ROS generation. This is further supported by Chuang and Hu (2004) who described the inability to use whole blood for in vitro work due to the interference from RBC and recommended RBC lysis and separation by centrifugation prior to use if lymphocytes could not be isolated. The method used in this study involves the use of DMSO as a cryo-preservative and iron chelator, protecting the sample viability from RBC lysis. This method of using whole blood instead of isolated

lymphocytes demonstrated no significant difference between the two methods, with the former being a more rapid method requiring a smaller blood volume (Najafzadeh and Anderson, 2016).

Conclusion

Anti-inflammatory medications have great potential to be used as adjuvants and in combination therapy in cancer treatment and represent a novel, less toxic treatment option. Current use of these agents has been restricted by their side effects; however we have demonstrated increased activity when the particle size is decreased to the nano scale. This offers increased reactivity and bioavailability, without increased genetic toxicity during *in vitro* assays on human PBL.

8 Study limitations

There were many confounding factors between the healthy control group and the haematological cancer patient group which made comparisons between these groups difficult. Many of the samples from the healthy donors group had been previously collected and stored with details released after completion of the study. Some of these details were very limited as questionnaires were not filled out with as much detail as those in the haematological cancer group. As volunteers were recruited from the university campus, bias was introduced in terms of age and ethnicity. Samples from the haematological cancer group had a great degree of bias with majority of patients recruited from the same ethnic background and generally from the older population. In light of these confounding factors, results were compared within a group and not between groups.

9 Future work

Further work in this study would initially involve recruitment of a healthy control group that would enable comparisons to be made between the healthy control group and the haematological cancer group. A broader range of glucocorticoid drugs would be analysed to further investigate the anti-oxidant effects of these drugs in cancer patients. It would be interesting to compare the short acting hydrocortisone to the intermediate acting triamcinolone and the long acting glucocorticoid dexamethasone, which both have superior anti-inflammatory effects with reduced mineral-corticoid effects when compared to hydrocortisone. The Comet Repair assay would be performed on all proposed and previously used drugs in this study to determine the effect of treatment on DNA repair mechanisms in healthy and cancer patients. Performing qPCR using human cell cycle PCR arrays would also enable us to identify the expression profiles of genes involved in positively and negatively regulating the cell cycle.

A more detailed look at the structure of the different nanoparticles using SEM would help in understanding the interactions of nanoparticles with host cells. Further to this, TEM could be used to investigate nano particle entry into cells and cell localisation.

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Appendix 1 : Abbreviations

AP1: activating protein 1

ASP B: Aspirin bulk

ASP N: Aspirin nano

AuNPs: gold nanoparticles

CBMN: Cytokinesis-Block Micronucleus assay

CuO: copper oxide

COX-1: Cyclooxygenase enzyme 1

COX-2: Cyclooxygenase enzyme 2

DUSP1: Dual specificity phosphatase 1

IBU B: Ibuprofen bulk

IBU N: Ibuprofen nano

MMC: Mitomycin C

NSAIDS: Non-steroidal anti-inflammatory drugs

DSB: DNA double strand break

EDTA: Ethylene diamine tetrachloro acetic acid

FBS: Foetal bovine serum

GC: Glucocorticoid

GR: Glucocorticoid receptor

GSH: Glutathione

H_2O_2 : Hydrogen peroxide

HC: hydrocortisone

HC B: hydrocortisone bulk

HC N: hydrocortisone nano

HO: Hydroxyl radical

LMP: Low melting point agarose

MN: Micronucleus

NAC: N-acetylcysteine

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NC: Negative control

NDI: The nuclear division index

NF-kB: nuclear factor kappaB

NMP: Normal melting point agarose

NPs: Nanoparticles

NSAID: Nonsteroidal anti-inflammatory drugs

NAG-1: NSAID-induced gene

OS: Oxidative stress

PBL: Peripheral blood lymphocytes

PC: positive control

PG: Prostaglandin

PHA: Phytohaemagglutinin

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SEM: Standard error of the mean

SGCE: Single cell gel electrophoresis

S1-P: sphingosine-1-phosphate

SSB: single strand break

TiO_2: Titanium oxide

µM: Micro molar

ZnO: Zinc oxide

Appendix 2 : Population Characteristics

Population characteristics of healthy donors

Sample	Gender	Age	Ethnicity	Smoking History	Cigarettes per day
1	M	42	Asian	Non-smoker	-
2	M	63	White Other	Non-smoker	-
3	F		Arab	Non-smoker	-
4	M	32	-	Non-smoker	-
5	M	36	Arab	Smoker	15
6	F	23	White British	Non-smoker	-
7	F	23	White Other	Non-smoker	-
8	M	18	White British	Non-smoker	-
9	F	40	Asian	Non-smoker	-
10	M	29	Black African	Non-smoker	-
11	M	27	White British	Past smoker	3

Population characteristics of haematological cancer patients

Sample	Gender	Age	Ethnicity	Smoking History	Cigarettes per day	Cancer diagnosis	Other medical conditions	Prescribed drug use / other medication
1	F	55	White British	Past Smoker	30	Follicular lymphoma	Previous breast cancer	Anastrozole
2	F	62	White British	Non-smoker	-	Marginal zone lymphoma	Fibromyalgia, COPD	Amitriptyline, tramadol, cetirizine, seretide, ventalin, setraile, ,
3	M	74	White British	Past Smoker	12	Systemic marginal zone lymphoma	None	Blood pressure tablets Vitamin C
4	M	50	White British	Past Smoker	10	Extranodal marginal zone lymphoma	Previous testicular cancer	None
5	M	66	White British	Non-smoker	-	Follicular lymphoma	Rosacea, psoriasis	Oxytetracycline, acitretin , cod liver oil
6	F	54	White British	Smoker	20	Mantle cell lymphoma	Ulcerative colitis	Atorvastatin, bendroflumethiazide, doxazosin, ramipril
7	F	85	White British	Smoker	2	IgG MGUS	COPD, T2 diabetic, ischemic heart disease, polymyalgia	Budesonide, formoterol, Glyceryl trinitrate, omeprazole, simvastatin, calcichew, aspirin,

							rheumatica	paracetamol, tiotropium bromide, atenolol, movicol, alendronic acid
8	M	89	White British	Past Smoker	-	Marginal zone lymphoma	diverticular disease	Lansoprazole, bendroflumethiazide, aspirin, amlodipine
9	M	81	White British	Past Smoker	20	Melanoma and chronic lymphocytic leukaemia	None	Fish oil
10	M	64	White British	Smoker	12	Marginal zone lymphoma	Chronic heart failure	Bisoprolol, forceval, Ramipril, bumetamide, lansoprazole, spirolactone, folic acid, quetiapine, thiamine, vit B
11	F	77	White British	Non-smoker	-	Follicular lymphoma	None	None
12	M	50	White British	Non-smoker	-	Follicular lymphoma	eczema	Flucoxicillin
13	M	81	White British	Past Smoker	10	Follicular lymphoma /composite lymphoma	Heart block - pacemaker, hypertension, asthma, impaired glucose tolerance	ramipril, aspirin, doxazosin, salbutamol spray, felodipine, pravastatin, adcal
14	F	63	White British	Past Smoker	20	Marginal zone	Osteoarthritis,	Ramipril, simvastatin

						lymphoma	type 2 diabetes, chronic kidney disease stage 3/4	
15	F	69	White British	Past Smoker	2	Follicular lymphoma	None	None
16	M	79	White British	Past Smoker	3	Marginal zone lymphoma, prostate cancer	None	Sodium alginate, potassium bicarbonate, tamsulosin, warfarin, felodipine, nitrofurantoin, sildenafril
17	F	83	White British	Non-smoker	-	Follicular lymphoma	None	None
18	M	78	White British	Past Smoker	25-30	Systemic marginal zone lymphoma	demyelinating neuropathy, IgM paraprotein	Adcal d3, alendronic acid
19	M	66	White British	Non-smoker	-	Pre-cancerous CD5 +ve, CD25 –ve lymphoproliferative disorder	Chronic kidney disease, hypertension	Amlodipine, Lisinopril, atorrastatin, clopidogrel

Appendix 3 – Consent form

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

Centre Number:

CONSENT FORM

Title of Project: **Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints**

Reviewed by Leeds East Research Ethics Committee (REC)
(REC reference number: 12/YH/0464)

Names of Researchers: Prof. D Anderson, Dr. Mojgan Najafzadeh, Mr M Salhab

	Please tick box
1. I confirm that I have read and understand the information sheet (version) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	☐
4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet.	☐
5. I agree to take part in the above study.	☐

_____	_____	
Patient number	Date	
_____	_____	_____
Name of Person taking consent	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 4 – Data collection form

UNIVERSITY OF BRADFORD
MAKING KNOWLEDGE WORK

School of Life Sciences

DATA COLLECTION FORM

STUDY TITLE: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints

REVIEWED BY LEEDS East RESEARCH ETHICS COMMITTEE (REC)

(REC REFERENCE NUMBER: 12/YH/0464)

PATIENT NUMBER		DATE OF SAMPLE	
AGE			
SEX (PLEASE TICK)	M / F	CONSENT	Y / N
ETHNIC GROUP		INFORMATION SHEET	Y / N
OCCUPATION			

CURRENT SMOKER Y/N PAST SMOKER Y/N HOW MANY/MUCH PER DAY?
CIGARETTES CIGARS PIPE
ALCOHOL Y/N UNITS PER WEEK

DIET	WESTERN	ASIAN	OMNIVORE	VEGETARIAN	VEGAN

VITAMINS / ANTI-OXIDANTS (PLEASE LIST)	
PRESCRIBED DRUG USE (PLEASE LIST)	
RECREATIONAL DRUG USE	Y/N
IF YES PLEASE LIST	

11 MEDICAL

CANCER
Inflammatory disease

EXTENT		SITE		HISTOLOGY		SURGERY	

CANCER	
Inflammatory disease	
Pre cancerous state	
OTHER MEDICAL CONDITIONS (PLEASE LIST)	
Family history of cancer and Inflammatory disease	
Chemotherapy or radiotherapy	

Appendix 5 – Participant information sheet

Participant Information Sheet for patients

Study title: Genetic and environmental effects in lymphocytes from different cancerous, precancerous and inflammatory conditions using various genetic endpoints

Reviewed by Leeds East Research Ethics Committee (REC)
(REC reference number: 12/YH/0464)
Invitation to the research study
We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish to and you will be allowed around 24hours to consider this.
(Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study).
Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1
In this study white blood cells will be treated in a test tube with chemical solutions or particles or UV radiation to determine if patients with cancerous and inflammatory diseases are more at risk after exposure. A blood sample of around 2 teaspoons (5-7 ml) will be taken. Samples will be stored only for the duration of the study and used for studies of a similar nature or to check original responses. This is for various research programmes involving post doctoral fellows and PhDs.

Why have I been invited?
You have been invited because you have a disease state and we should like to determine if these chemicals or UV irradiation could be more harmful to people with a disease state than those without such a disease.

Do I have to take part?
It is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason.

Part 2

What will happen to me if I take part?

Only a single blood sample will be taken for this research study. A brief questionnaire will need to be completed by the researchers.

Each individual will be given a coded study number so that your clinical data can be linked in an anonymous way with the research results.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on a group basis i.e. collective responses from patients with diseases compared to collective responses from people without diseases. Results could be published in the form of scientific papers. The work will benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, the Consultant and research team will be
prepared to talk to you individually about study results.

People who cannot take part in the study

People who are not well enough to take part will be excluded.

If you have any further questions, you could contact the research team:

Mr Salhab, Bradford Teaching Hospitals NHS Foundation Trust
St Lukes Hospital, BD5 0NA .
Telephone: 01274 36 5063

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