NATURAL PRODUCT RADIOPROTECTION

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By

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Thesis Abstract

This thesis examines select natural dietary compounds for their radioprotective properties in vitro. These compounds were selected on the basis of their use in the treatment of CFIDS (Chronic Fatigue Immune Dysfunction Syndrome). CFIDS in humans encompasses symptoms similar to those in ionizing radiation exposures. Therefore, compounds that address CFIDS symptoms might also have utility in dietary chemical radioprotection. Specifically, this thesis examines the radioprotective effects of plant metabolites –curcumin, andrographolide, d-limonene – and endogenous metabolites – phenylacetate, ursodeoxycholate, tauroursodeoxycholate – in clonogenic in vitro experiments. Compounds were examined for their radioprotective effects in p53 variant human colon carcinoma cells which share similar features to radiosensitive stem cells of the gastrointestinal tract. Experiments examined compounds on the basis of their chemoprotective, bystander inhibitory, radioprotective and radiomitigative properties. The d-limonene demonstrated notable chemoprotective properties as noted by the preferential toxicity to p53 defective cell lines. There were no compounds that exhibited bystander inhibitory effects. In acute high dose experiments, the phenylacetate demonstrated significant radioprotective effects up to 2 Gy with minor radioprotective effects from tauroursodeoxycholate at 0.5 Gy exposures. There were no compounds that demonstrated radiomitigative effects. Ultimately, this thesis demonstrates that natural consumer products in the treatment of IR-like syndromes provide an attractive basis on which to screen compounds the fields of radioprotection.

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Declaration of Academic Acheivement

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Chapter 1: Natural Consumer Products in Dietary Radioprotection

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Abstract

Objective

This review evaluates important hallmarks of dietary radioprotection, namely the antioxidative, immunomodulatory, and anti-carcinogenic qualities of compounds, to support the basis of non toxic natural products as an attractive screening platform to satisfy these criteria in the field of chemical radioprotection. Specifically, the review examines non-essential plant and endogenous metabolites as an alternative to synthetic chemical compounds. This review examines and samples the available literature on various compounds, methods, and endpoints with respect to each of the criteria.

Conclusions

The available sample literature altogether supports the use of natural products in dietary chemical radioprotection. Despite their effectiveness, there are notable issues that studies in this field have to address to increase their impact in terms of radioprotective activity. Therefore, the review concludes with recommendations to the field including the use of combinations of compounds and delivery systems to promote uptake of compounds.

Keywords

Radioprotection; Drug Radiation Interactions; Natural Products

Introduction

Ionizing radiation (IR) is a fundamental component of our physical environment – the result of energetic particles and waves that have sufficient energy to release an electron bound by atomic forces from its respective atom. In biological systems, these interactions disrupt molecular bonds to produce free radicals that propagate and destroy cellular structures - an effect that organisms have adapted to and tolerate to an extent [Lenhert and Iyer 2002]. The multitude of endogenous molecular defenses to free radical mediated damage, namely enzymatic antioxidants and (DNA) repair mechanisms, are highly effective in handling products of oxidative metabolic processes [Sage and Harrison 2011]. However, the quality of free radicals (in terms of chemical species and reactivity) that radioactive exposures produce are dissimilar to that of oxidative phosphorylation (OXPHOS) in aerobic cells. Molecular strategies that solely rely on the use of enzymatic antioxidants are therefore ineffective to the variety and reactivity of free radicals typical of IR exposures [Sage and Harrison 2011]. Additionally, the products of OXPHOS diffuse throughout the cell to produce scattered lesions in DNA (deoxyribonucleic acid). In contrast, the energy deposition events of IR interactions produce clustered DNA damage referred to as ‘complex lesions’ which are particularly difficult to repair [IAEA Radiobiology Syllabus 2014]. These factors evidently have some role in compromising the effectiveness of endogenous defenses to IR exposures. Damage to cellular targets, particularly lesions encompassing unrepaired nuclear DNA (nDNA) and mitochondrial DNA

(mtDNA), might also result in cell killing or otherwise be propagated to the progeny of cells resulting in mutagenesis and genomic instability [Kam and Banati 2013; Desouky et al. 2015]. Release of 'bystander' signals and cytokines from these impacted/ irradiated cells also prompt IR-like effects in neighbouring cells and the induction of pro-inflammatory responses resulting in non-targeted effects [Prise and O'sullivan 2009]. Altogether, adverse effects to IR include tissue/organ damage, induction of inflammatory mediators, fibrosis, chronic immune dysfunction, somatic /germ line mutations, genomic instability, and carcinogenic processes [Hall 2019].

Given that our environment, both natural and artificial, contains IR it is of considerable importance to examine these interactions in humans. This objective is particularly important as the artificial environment of radioactive exposures via medical (diagnostic radiology and radiotherapy), nuclear reactors (power and research), household exposures (radon gas and building products), travel (high altitude flights and space tourism) as well as those of proposed deep space colonization, substantially exceeds that of the natural environment in terms of dose rate and exposure [IAEA Radiobiology Syllabus 2014]. The deleterious free radical mediated effects of artificial IR subject the cellular environment to an additional burden/stressor that might be beyond the effective repair capacity of irradiated cells. These effects are noticeable and exacerbated in radiosensitive individuals with specific predispositions in the genes encompassing DNA repair factors (ex. ATM) and transcription factors (ex. p53) [Hall 2019]. Therefore, a

major field of radiobiology is concerned with the development of chemical radioprotection. IR exposures necessitate the development of interventional strategies /compounds that confer multiple levels of protection at the chemical, cellular, and systemic level from short term lethal and long term chronic injury effects. The application of certain strategies might require combinations of treatments and are context dependent. For instance, apoptosis is a favourable outcome to low dose exposures because it eliminates mutagenic cells from the stem cell pool that might otherwise proliferate to produce malignancies in tissues. Whereas in high dose exposures inhibition of apoptosis is favourable because cells have to survive to resume organ functionality in the organism. The development of compounds that promote the regulation of proper cellular function, halt carcinogenic processes, as well as inhibit apoptosis are examples of effective chemical radioprotection.

Objective

Despite the successful development/research of synthetic radioprotective compounds, their uses are restricted/subject to, toxic side effects, narrow window of administration, unpractical route of administration via either peritoneal or intravenous routes to confer high degrees of radioprotection [Nambiar et al. 2011]. These negative features do not exclude their applicability in certain situations where these compounds might be of high value including acute high

dose total body irradiation (TBI) exposures and within very narrow windows of administration that simulate the controlled exposures of an experiment (ex. clinic). Otherwise, given these issues, their practicality to serve as effective chemical radioprotection to the chronic effects of IR exposure are of low value. To overcome these issues requires the delivery of non toxic products, preferably natural consumer compounds, because of their convenience in terms of oral administration. Therefore, there is particular emphasis in this article on the use of dietary supplants, aside from essential dietary vitamins, as a practical method of radioprotective delivery. The objective of conventional radioprotection is to spare cells from the immediate lethality of acute, high dose IR exposures the basis of which is the antioxidant neutralization of free radicals. These are not per se the only objectives of radioprotection which extend to encompass anti-inflammatory and anti-carcinogenic, effects. This article examines natural compounds that satisfy each of these particular criteria.

Basis of Antioxidant Chemical Radioprotection

The interactions between IR and matter occur throughout the cell with pertinent biological structures including the cellular membrane, cytosol, subcellular organelles and their respective contents. Chemically, these structures consist of organic molecules in solvent medium (i.e water) that permit the energetic reactions of homeostatic cellular/biochemical processes. IR interacts with these

molecular targets to produce ions of varying kinetic reactivities that produce damage inside the cellular environment [Cheeseman and Slater 1993]. Particular attention is paid to those ions that are capable of generating free radicals, that is, produce chemical species that have an unpaired electron. These radicals are considered to be particularly reactive and therefore damaging given their ability to react with important cellular molecules rendering them biologically inert. Of particular concern are those radicals that result from the interaction of IR with water, molecular oxygen, and nitrogen containing molecules such as nitric oxide and molecular nitrogen [von Sonntag 2006]. The result of IR induced reactions with oxygen and nitrogen containing molecules are reactive oxygen species (ROS) and reactive nitrogen species (RNS), respectively, and are in high abundance given the availability/necessity of these physiologically relevant molecules to aerobic organisms [von Sonntag 2006]. IR produced ROS and RNS molecules are spectrally abundant and distinct in composition to those of oxidative phosphorylation (OXPHOS) which consist primarily of hyperoxide and hydrogen peroxide for which there are enzymatic antioxidants (SOD and catalase) to dissociate their respective substrates [Herst et al. 2017]. Additionally, the high localization of OXPHOS products to the mitochondria cristae restricts their resultant impact on the surrounding medium including nDNA and cellular organelles [Kam and Banati 2013].

On the contrary, IR produced ROS and RNS consist of many chemical species including those of OXPHOS as well as hydroxyl, perhydroxyl, and nitroxyl species which are notable for their high kinetic reactivity [von Sonntag 2016]. Unlike OXPHOS byproducts, these chemical products occur ubiquitously throughout the entirety of the cell volume and in turn are able to inflict lesions throughout the cell [Yoshii et al. 2011]. Furthermore, the effects are exacerbated by highly reactive radicals that propagate the damage in chain reactions throughout certain molecules as is the case of lipid peroxidation [Halliwell and Gutteridge 2015]. These reactions are characterized in organic chemistry by the process of initiation, propagation, and termination of radical ions inside the cell. The initiation step involves exposure to the ionizing event; propagation involves reactions with other molecules to produce chain reactions; and termination results in the production of stable molecules via interaction with antioxidants, that is, molecules capable of accepting an additional electron to generate stable products/ molecules. The IR mediated production of radicals oversaturates the capacity of endogenous enzymatic antioxidants and low molecular weight (LMW) antioxidants (ex. vitamin), thereby inhibiting their ability to carry out their homeostatic roles in energy production leading to the pathogeneses of oxidative stress [Rahal et al. 2014]. Oxidative stress is characterized by the impairment of redox regulation in the cell resulting in an excessive burden of proxidants which causes dose dependent toxic effects. Physiologically, the acute effects of high oxidative stress display as tissue injury from cellular destruction whereas chronic

oxidative stress might be precursor to carcinogenesis and immune mediated dysfunction events [Mittal et al. 2014; Kryston et al. 2011]. Therefore, elimination of production of free radicals and oxidative stress represents the initial mechanism and criterion in effective radioprotection.

There are various methods to enact termination of free radicals - these include the mentioned use of enzymatic antioxidants which dissociate/rearrange the radicals to less reactive intermediate substrates and ultimately stable products [Herst et al. 2017]. Examples of these include the mitochondrial enzymatic antioxidant system consisting of SOD enzymes with distinct cofactors such as MnSOD, CuSOD, and ZnSOD which generate hydrogen peroxide from hyperoxide [Herst et al. 2017]. Subsequently, catalase dissociates hydrogen peroxide from products of these SOD reactions to water and oxygen. Similarly, selenium cofactors in glutathione (GSH) enzymes are able to transfer the electrons to the substrates through hydrogen atom donation which detoxify the proxidant environment in the cells [Revesz 1985]. Therefore, GSH, thioredoxin, and similar thiol compounds are another important class of antioxidants that function to eliminate free radicals by modulating intracellular redox reactions. These intracellular antioxidants are the basis of synthetic radioprotective strategies to reduce the burden of oxidative stress in cells. Given the high molecular weight of proteins, attempts to administrate this cargo into cells involve binding enzymes to extracellular receptors promoting engulfment of the package. Similarly, gene therapy approaches employed to promote expression of the antioxidative response elements via Keap-Nrf

expression also have to satisfy this delivery requirement [Suzuki and Yamamoto 2015]. Regardless of approach, effective delivery is cumbersome and despite their moderate efficacy in acute high dose trial experiments, the practicality in chronic exposures is low with only some protection from specific radicals (i.e OXPHOS radicals) [Machtay et al. 2006]. Other endogenous methods of cellular protection from IR include, to an extent, the use of sacrificial molecules, primarily proteins that react with proxidants. These include classes of Heat Shock Protein (HSP) that cover and protect essential biomolecular structures (ex. nDNA), and albumin, each of which terminate free radical reactions through the production of mixed disulfides [Greenberger 2009].

The non enzymatic antioxidants constitute another class of radioprotective compounds. These compounds, notably compounds that include those of the WR series, are essentially modifications of the amino acid cysteine. These particular compounds show demonstrable responses in vitro and in vivo characterized by the Dose Reduction Factor (DRF), which quantifies the degree to which the compounds confer radioprotection. The DRF of these compounds is around 3, which significantly aids in the radioprotective responses to high dose exposures. Despite their effectiveness, these compounds demonstrate high toxicity at radioprotective doses which induces hypotension in vivo therefore are of low practicality in these situations [Andreassen 2003]. The accompanying side effects are also an issue in which utility in low dose chronic IR exposures is of low value. Despite the lack of success among synthetic approaches to take advantage of

intracellular antioxidant systems, exogenous dietary compounds have been reported to increase expression of intracellular antioxidant systems and operate via their own potent antioxidant chemical properties [Prasad et al. 2017].

Natural Compound Antioxidants

The study by Lee et al (2010) demonstrated that the natural antioxidant curcumin, derived from the spice turmeric, increased expression and availability of Heme Oxygenase-1 (HO-1) by 3-18 fold in lung endothelial cells as well as fibroblasts derived from C57BL/6 mice fed 5% (w/w) dietary curcumin prior to single 13.5 Gy thorax irradiation. In vitro, pulmonary microvasulature endothelial cells demonstrated decreases in ROS production, specifically hydrogen peroxide, as shown by fluorescence microscopy with H2DCFDA stain. The ROS alleviation corresponds to decreases of ~ 2 fold from that of controls in response to 25, 50, and 100 micromolar curcumin treatment to 2 Gy irradiation. The treatment with 5% (w/w) curcumin altogether increases the survival of mice over the course of the experiment.

In an in vivo/in situ rat study by Long et al (2018), dark tea extract also alleviated the effects of high dose acute IR injury by significant restoration of expression of SOD, catalase, and glutathione peroxidise (GSHpx) levels in C57BL/6 mice livers treated with 6 Gy TBI doses and 200 mg/kg in vivo compared to controls. This

result is in correlation with decreases of intracellular ROS levels by ~2 fold as measured with DCFDA stain and flow cytometry and increases of ~2 fold of in vitro hematopoetic progenitor cell (HPC) colonies. Another notable polyphenolic tea derived antioxidant is theaflavin. The study by Han et al (2017) demonstrated the radioprotective effect of this compound on 4 Gy TBI exposed Nrf2 wild type and Nrf2 null mice to evaluate the effects on downstream transcriptional activation of HO-1, NOQ-1, and SOD2 enzymatic antioxidants. In Nrf2 wild type mice, the study found significant increases in expression of ~ 2 fold in each of HO-1, SOD2, GSHpx, and 30% in NOQ-1 as shown by Western blots. These are corroborated with corresponding reductions in oxidative stress and accompanying reductions in Double Strand Break (DSB) induction as measured by fluorescence microscopy of ROS and gammaH2AX foci respectively. The Nrf2 null theaflavin exposed mice do not demonstrate similar degrees of radioprotection which suggests that Nrf2 is the central regulator in the radioprotective mechanism. Similarly, in another study by Hu et al (2011), the main bioactive polyphenols of tea, notably epigallocatechin, demonstrated significant alleviation of lipid peroxidation as measured by Malondialdehyde levels in TBI irradiated mice fed diets administered ~ 100 mg/kg daily of individual tea polyphenol content. The polyphenols decreased malondialdehyde by ~1.7 fold to that of irradiated controls and also increased SOD levels by ~ 1.2 in those treatments.

The bioactive component of flaxseed, sicoisolariciresinol diglucoside (SDG) is another demonstrated radioprotective compound. In the in vitro study by

Velalapoulou et al (2016), the administration of 50 micromolar SDG on murine lung epithelial, endothelial, and fibroblast cells conferred radioprotective effects at 2 Gy of gamma radiation exposure as shown by increases in clonogenic viability and expression of HO-1, Glutathione S-Transferase M1 (GSTM1), and , NAD(P)H quinone oxidoreductase 1 (NQO1) cytoprotective enzymes. The protein expression of HO-1 and NQO1 were increased significantly by ~ 2 and ~ 2.3 fold respectively measured by analysis of western blots. The synthetic derivative of SDG, LGM2605, also shows high levels of radioprotection in an ex vivo study of lung tissue (Velalapoulou et al. 2017). The study examined the antioxidant gene expression of HMOX1 and NQO1 on lung tissue irradiated with 4 Gy of protons and treated with LGM2605 4 hr prior to irradiation. Results of qPCR analysis of samples demonstrate significant expression of NQO1 with LGM2605 in response to irradiation from that of control treatments though no effect on HMOX1 expression. The study by Mishr et al (2016), demonstrated that LGM32605 is able to scavenge active chlorine species and reduce chlorinated nucleobases in DNA, acting as an additional level of protection alongside activation of enzymatic antioxidants.

Another potent and recognized natural antioxidant is resveratrol. The study by Zhang H et al (2013), demonstrated the effects of orally administered resveratrol on C57BL6 mice lethality, SOD2, GSHpx expression and enzyme activity. The resveratrol increased survival in mice exposed to 7.2 Gy TBI and demonstrated an approximately ~ 2 and ~ 2.5 fold increase in expression of SOD2 and GSHpx

respectively measured with qt-PCR. The enzyme activity of SOD2 and GSHpx also increased correspondingly by ~ 1.2 fold. The study by Zhang J et al (2016), demonstrated the utility of Vam3 which is a natural resveratrol dimer with antioxidant properties similar to that of resveratrol with similar implications to radioprotection. In this study, total ROS levels were measured with DCFDA in hematopoetic progenitor cells from C57BL/6 mice administered 50 mg/kg and 4 Gy of TBI. Additionally, enzyme activity of SOD and GPX1 were also examined as part of the study on antioxidants. The study demonstrated high reductions in ROS levels by ~ 2 fold to that of irradiated controls and also demonstrated significant increase in enzymatic activity of GPX1 and SOD demonstrating the utility of this radioprotective compound.

The study by Khan et al (2015) examined the antioxidant capacity via ABTS and DPPH tests of sesamol in gastrointestinal crypt cells of C57BL/6 mice irradiated with an LD50/30 dose of 7.5 Gy and found that there is significant an inhibition of lipid peroxidation and alleviation of ROS levels in gastrointestinal tissues. Radioprotective effects of orally administered walnut oligopeptides were examined by Zhu et al (2019), on BALB/c mice exposed to 3.5 Gy and 8 Gy doses of Co-60 gamma rays. The study demonstrated an augmentation of intracellular antioxidant system capacity alongside reductions in intestinal permeability compared to controls. This is particularly important as GI injury is highly dangerous in TBI exposures.

Another potent antioxidant is astaxanthin derived from marine organisms. Exposures to the carotenoid astaxanthin in 4 Gy irradiation of C57BL/6 mice, demonstrated in the study by Xue et al (2017), suggests that there are increases in expression of nuclear Nrf2 in response to compound treatments. Nrf2 is a transcription factor that regulates the cellular responses to oxidative stress via the expression of endogenous antioxidants HO-1and SOD enzymes. The study demonstrated an increase in the Hematopoetic Stem Cell (HSC) enzymatic activities of SOD, catalase, and GSHpx antioxidants by approximately 2, 6, and 2 fold respectively as measured by flow equipment with corresponding decreases in ROS as measured by DCFDA and MitoSOX probes.

The use of ebselen, seleno-organic compounds effects has also shown considerable radioprotection from in vitro and in vivo experiments and is non toxic unlike inorganic selenium given these are bound to organic compounds . The study by Tak and Park (2009) examined the ROS dependent toxicity of IR on U937 cells and mice given 2 Gy treatments and the modulation of these responses with administration of 5 micromolar and 10 mg/kg of compound respectively on these test systems. The study used hematoxylin, eosin and DHE to measure the intracellular and tissue specific levels of ROS each of which demonstrated reductions of ~15% thus show radioprotection.

The administration of endogenous antioxidant metabolites has also shown effective radioprotection of organisms through reduction of intracellular oxidative

stress. In the study by Manda et al (2007), the intraperitoneal administration of 200 mg/kg of alpha-lipoic acid, an important coenzyme of alpha – ketoacid dehydrogenase reactions, to C3H mice conferred significant radioprotection over control treatments receiving 4 and 6 Gy irradiation. The study measured lipid peroxidation, protein carbonyl, non-protein sulfhydryl contents, and ferric reducing ability of plasma in various organs. Results demonstrated decreases in lipid peroxidation of ~25-75% between tissues, decreases of ~25-35% in protein carbonyl content, increases of ~15% in non protein sulfhydryl contents, and similarly increases of ~15-20% in ferric reducing ability. The study by Spotheim-Maurizot (1995) examined the effects of the endogenous polyamine putrescine on radiolysis of plasmid DNA in vitro. Examination of radioprotective parameters in terms of ROS measurements demonstrated significant increase in radioprotection by factor of 2 fold. These are attributable to the scavenging of hydroxyl radicals in the bulk medium as well as at sites of attachment of polyamines.

Natural products are effective in reducing the impacts of IR lesions to cells and organisms, an effect partly attributed to termination of free radicals thus avoiding negative downstream events of exposures. Despite their effectiveness as antioxidants, these compounds have to be in the medium to interact directly with the reactive radicals either pre-irradiation, during, as well as very shortly following irradiation to confer radioprotection otherwise radioprotective effects are marginal in effect. Additionally, high LET sources of IR produce densely

ionizing clusters of free radicals to which antioxidants are predominantly ineffective. Otherwise, these exposures require very high amounts of antioxidants over that which is non toxic to cells. Therefore, to negate the downstream IR effects requires the use of compounds that are able to modulate and promote intracellular regulation.

Basis of Immunomodulatory Radioprotection

The immediate effects of acute high dose IR exposures at the cellular level result in excess loads of ROS and RNS mediated chemical interactions that are responsible in excessive apoptosis, mitotic catastrophe, necrosis, bystander cellular signalling, as well as delayed reproductive effects in the case of genomic instability that result in various organ dependent effects [Pouget et al. 2011]. The etiology of IR pathogenicity primarily arises from excessive damage to the nDNA and mtDNA that enact cellular signalling cascades prompting destruction of cells in tissue and therefore release of intracellular contents. ‘Danger Associated Molecular Patterns’ ‘DAMP’ molecules and cytokines prompt an immune response to the sites of IR exposure which target cellular antigens and recruitment of inflammatory factors resulting in inflammasomes [Burnette and Weichselbaum 2015]. Therefore, the primary mechanism of deleterious action of acute IR exposures is cellular depletion and overt inflammatory responses. In the case of TBI, as well as high dose localized exposures to organs, these effects include,

amongst others, impairment of organ function and fibrotic events [Sohn 2015]. Specifically, injury to stem cells is of particular concern given their role is to replenish the mature functional compartment of tissues. Otherwise, impacts to these cells leads to the dysfunction of tissues/organs that result in compromise of organ function. The stem cells of the hematopoetic and gastrointestinal systems are especially vulnerable given their high turnover rate in tissues. Therefore, repopulation of affected organs/systems with stem cells is the major course of treatment [IAEA Radiobiology Syllabus 2014].

With respect to inflammatory responses, symptomology of IR exposures is similar to that of reactions to immunogenic antigens. Direct irradiation of immune cells occurs/triggers releasing interleukins, notably pro-inflammatory interleukin (IL)-1, to surrounding immune cells [Formenti et al. 2009]. Specifically, granulocytes, such as mast cells, degranulate releasing inflammatory mediators/contents (histamines and leukatrines) that act on surrounding cell populations to elicit responses that recruit inflammatory cells to the site of IR injury thereby provoking inflammatory responses [Hall 2019]. Responses include dilation of intercellular endothelial cellular basements, membrane leading to fluid leakage in tissues, epithelial production of mucus, and inflammasome production resulting systemically in the symptoms typical of acute IR exposures [Stoecklein et al. 2015]. Given the sensitivity of HSC to IR, the hematopoetic system subsides to myelosuppression briefly following inflammatory responses to exposures with drops in erythrocytes, lymphocytes, and thrombocytes eliciting effects such as

hemorrhaging, infection and impairment [Gostner et al. 2013]. Though immune responses are beneficial in instances of tissue repair, overt and acute high dose uncontrolled immune activation is dangerous without treatment.

Therefore, effective radioprotective interventions have to retain immune function in tissues similar to the Toll-Like Receptor (TLR)-5 agonists that inhibit lymphocyte depletion in tissues [Krivokrysenko et al. 2015]. Despite the use of antioxidants to aid in oxidative stress, the surviving pool of cells requires additional support to aid in tissue function, therefore requires a stimulatory mitogenic stimulus to cells in tissues. Typically, such treatments involve the use of growth factors such as Granulocyte Monocyte – Colony Stimulating Factor (GM-CSF), which act on extracellular receptors in haemopoetic stem cells to promote maturation and mitogenic activity [Singh et al. 2015]. These treatments address the immediate deficit in differentiated cells in tissues though with excessive use are prone to receptor downregulation and perhaps carcinogenic phenotypes in cells along with inflammatory responses [Hong 2016]. Use of anti-inflammatory interventions with natural consumer products are worthy of consideration in addressing chronic inflammatory effects. Ultimately, these treatments alter the function of immune cells and their effector cells from that of pro-inflammatory microenvironments to those of anti-inflammatory tissue repair environments.

Natural Immunomodulatory Compounds in Radioprotection

In the study by Kunwar et al (2011), the organoselenium compound- 3,3'-diselenodipropionic acid- derivative of selenocystine showed anti-inflammatory effects at 2 mg/kg prior to mice receiving 7 Gy TBI. The study examined the expression of immune serum markers such as Tumour Necrosis Factor (TNF)-alpha, IL-1, IL-6, inducible Nitric Oxide Synthase (iNOS), and Transforming Growth Factor (TGF)-beta via western blots.Treatments show reductions of ~2 fold in TNF-alpha, and IL-1 with no effect on IL-6, iNOS, and TGF-beta expression. Effects of irradiation on leukocyte infiltration into intestinal tissues were also examined through the expression of ICAM-1 and P-selectin, which are adhesion molecules that mediate the process of leukocyte migration into tissues. Expression of these markers in compound treated mice showed significant reductions from that of IR control cohorts. The effect of irradiation on helper T-cell (Th) Th1 and Th2 populations of immune cells were also measured indirectly via production of IL-2 and IFN-gamma which is dominantly produced by Th1 cells and IL-4 and IL-10 which is produced by Th2 cells. The study demonstrated increases in the Th1/Th2 ratio of cells in IR cohorts demonstrating shifts that promote pro-inflammatory responses whereas the administration of the compound significantly attenuated these shifts.

Thymoquinone is an abundant essential oil derived from niglela sativa seeds with notable anti-inflammatory properties in T-lymphocytes. Therefore, the study by

Guida et al (2016), examined the effects of thymoquinone oral administration to rats receiving 4 Gy TBI over 14 days on T-lymphocyte function via measurements of cytokine production and expression of PD-1 in both CD4 and CD8 T-cell thymus derived populations via flow cytometry. The results demonstrate the significant decrease of ~2 fold in the pro-inflammatory cytokine IL-6 in thymoquinone treated rats with an accompanying downregulation of PD-1 by ~20% from that of controls irradiation treatments. These indicate that thymoquinone protects rats from T-lymphocyte exhaustion. The notable antioxidant resveratrol also shows considerable anti-inflammatory effects. In the study by Kim et al (2014), rats were exposed to a gamma radiation dose of 17.5 Gy in the abdominal area and supplied with a 30% high-protein diet and resveratrol. At day 10 following irradiation the levels of clusters of differentiating CD4 cells, regulatory T cells and serum proinflammatory cytokines in urine were measured. The experimental diet decreased levels of proinflammatory cytokines which led to significant recovery of BMCs from that of IR treatments.

The study by Dobreva et al (2015) examined another compartment of the immune system in response to IR, the specific antibody response mediated by b-lymphocytes on humoural plasma immunoresponsiveness in rabbits treated with Haberlea rhodopensis leaf extract (HRE). The study demonstrated the decrease in antibody production of Immunoglobulin (Ig)G, IgM, IgA antibodies in response to 2 Gy TBI and ~ 2 fold increase with the administration of 1250 mg/kg

compound HRE. The study effectively demonstrates radioprotection in this part of the immune system with the use of this plant product.

In the study Goel et al (2007), treatment of mice with podophyllum hexandrum (200 mg/kg) 2 h prior to TBI increased macrophage survival significantly ($p < 0.05$) to that of irradiated control mice. Compound treatment also countered the decrease in splenocyte proliferation and survival significantly ($p< 0.05$) from that of the irradiated control group via the 3H-TdR method. Compound treatment 2 h prior to TBI also significantly ($p < 0.05$) countered the decrease in the populations of bone marrow cells (BMC) and Cytotoxic T-Cell (CD)4 and CD8 T cells at post irradiation intervals of 24 h and 72 h to unirradiated control. Nitric oxide free radical production was increased significantly ($p < 0.05$) in peritoneal macrophage cultures given 2 Gy dose ex vivo which was reduced by administration of compound 2 hr prior to exposure. TBI (10 Gy) produced reductions to the serum titres of IL-1, IL-3 and various IgG isotypes whereas compound treatment groups countered these radiation induced decreases in the serum titres of IL-1, IL-3 and IgG in mice. Similarly, the extract of Hippophae rhamnoides plant is also reported to have immunostimulatory effects in the study by Prakash et al (2005). Treatment with Hippophae rhamnoides (30mg/kg) 30 min prior to TBI increased viable macrophages significantly ($P<0.05$) from that of unirradiated control and irradiated treatment groups. Compound treatment prior to irradiation reduced total nitrite significantly ($P<0.05$) in peritoneal macrophage cultures given 2 Gy gamma radiation ex-vivo, At 24h following TBI, the CD4/CD8 ratio showed

reductions to 1.5 in comparison with non-irradiated control (1.9), although treatment prior to irradiation restored the ratio to 2.1 in the populations.

Another notable natural compound is beetroot (Beta vulgaris). The study Cho et al (2017) examined the compound with respect to immunostimulatory effects. This compound administered per os to C57BL/6 mice at 400 mg/kg 17 hr prior to 7 Gy TBI stimulated proliferation of BMC colonies via IL-3 production in HPCs with cumulative DRF of 1.1 in mice. The results from another study by Park et al (2008) demonstrate that Elaeocarpus sylvestris extract significantly ($p<0.001$) improved the counts of endogenous colony units in compound treated mice indicating that the compound induced the radioprotection of hematopoietic cells. Treatment also accelerated the recovery and proliferation of granulocytes and lymphocytes similar to those levels in irradiated controls. In another study by Byon et al (2008), the authors examined the effects of the polysaccharide fuciodan extracted from algae on BMCs. The study examined the effects of fucoidan on cytokine production from BMCs and their ability to induce proliferation of allogeneic splenocytes. Results show that fuciodan treatment of BMCs induces an increase in the production of IL-12 and TNF-alpha cytokines. The treatment of BMCs to fuciodan also increases the surface expression of Gr-1 and induces the ability of BMCs to trigger proliferation of responder splenocytes which suggests the upregulation of antigen presenting cell (APC) function in vivo. The function

of APCs is crucial to the immune system effects via appropriate regulation of t-lymphocyte mediated responses.

In the study by Pearce et al (2011), the non-essential amino acid – l-arginine – demonstrated DRFs in HPCs of 1.5 in treated cells. The study examined the enzymatic interaction of l-arginine with nitric oxide synthase, an important component of the immune responses given nitric oxide radicals are used to produce inflammatory responses. The study demonstrates an inhibition of nitric oxide which is responsible in the overt highly pro-inflammatory response to acute IR exposures. The study by Chatterjee et al (2019) evaluated the NOD-like receptor protein 3 (NLRP3) inflammasome activation and Intercellular Adhesion Molecule (ICAM)-1 in response to gamma and proton irradiation and with compound LGM2605 treatment. LGM2605 added 30 min post IR exposure induced robust increases in ICAM-1 ($p<0.0001$) and NLRP3 ($p<0.001$) induction by individual and mix-field exposures which were significantly blunted by LGM2605 administration. Another notable exogenous compound with immunomodulatory effect is arabinoxylan rice bran. In the study by Ghonuem et al (2013), mice given 5 Gy of TBI and fed 40 mg/kg of arabinoxylan rice bran restored the irradiation induced decreases in HPCs significantly over that of control irradiation treatments.

Basis of Anti-Carcinogenic Compounds in Radioprotection

The etiology of chronic injuries/effects of IR, both as the residual effects from acute high dose exposures as well as prolonged low dose chronic exposures, is the result of; stem cell depletion (ex. in the case of necrotic wounds), ROS and cytokine production from surviving irradiated cells that are the result of residual circulating inflammatory cytokines that perpetuate these responses to distant sites (ex. chronic fatigue immune dysfunction, fibrotic events), and altered/defective mutations in survival of exposed and bystander cells (ex. tumours) [Greenberger 2009]. Specifically, in the case of carcinogenesis and organ dysfunction syndromes, defective mutations are the primary concern. The production of ROS and ROS mediated interactions characteristic of IR interactions are responsible in production of 'complex lesions' in nDNA and mtDNA genomes involving clusters of ionizations. These 'complex lesions' consist of multiple lesions encompassing oxidative base damage, Single Strand Breaks (SSBs), abasic sites, nucleobase protein links, and DSBs within base pairs of each other that compromise the effective DNA repair machinery in these affected genomic regions [Friedrich et al. 2018]. The complexity of these lesions are uncharacteristic of those imparted by endogenous oxidative stressors, such as the diffusive products of OXPHOS, rendering DSB repair mechanisms - predominantly NHEJ - ineffective in the protection of genomic material/quality. Ultimately, complex lesions contribute to 'chromothripses' - essentially substantial chromosomal fragmentation of the genome leading to misjoining and

deletion of genomic fragments as result of ineffective repair mechanisms [Liu et al. 2015]. The ineffective recombination and deletion of fragments that contain transcription factors and regulatory elements to regulate the genome altogether contribute to incorrect expression of cellular phenotypes. Therefore, these factors contribute to the production of chromosome aberrations that are responsible in cellular destruction in the case of lethal aberrations as well as in mutations that are precursors to carcinogenesis in exposed tissues. These events are implicated in the production of secondary tumours in acute high dose radiotherapy treatments as well as from chronic environmental exposures [Butof et al. 2013].

Defective mutations in exposed cells might express as alterations in enzymatic stress response elements, transcription factor activity, and mitogenic signals. The impairment of tumour suppressors, cellular repair mechanisms, mitochondrial OXPHOS and up regulation in expression of oncogenes results in anti-apoptotic markers that permit cellular survival within the organism [Kamp et al. 2011]. These cells are able to produce signals that prompt recruitment and alterations in function of surrounding mesenchymal cells and immune cells that support the microenvironment conducive to production of tumours in tissues [Redon et al. 2016]. For instance, irradiated senescent fibroblasts are able to resume functional capacity following irradiation and produce cytokine signals to surrounding tissues [Hall 2019]. Unless efforts are taken to hinder these defective cells and their responses, these effects altogether contribute to the systemic dysfunction of tissues that compromises their ability to function as an organ, therefore causes

impairment of the organism. Chronic effects from tissue dysfunction occur in both rapidly proliferative as well as non-rapidly proliferative cells, except-non rapidly proliferative cells express long latency to their expression of effects as this the case with cardiovascular arthrescleroseis [,Boerma et al. 2016,] and chronic fatigue immune dysfunction syndrome effects [Pall 2008]. Therefore, carcinogenesis is not the only concern from effects of chronic IR exposures. Similar to the acute effects of IR, in chronic effects particular concern are paid to those cells that are part of the stem cell domain as these are responsible for the regeneration of tissues. Specifically, pluripotent stem cells (PSCs) are important as these are responsible in production of multiple progenitor cells that repopulate the tissue to re-establish tissue function from injuries to the cellular environment. There is concern given these cells undergo multiple mitotic events to produce tissue specific cells. Therefore, genomic instability is an issue antimutagenic/anticarcinogenic compounds have to address.
[Wei et al. 2016]

Natural Anti-carcinogenic Compounds in Radioprotection

The study by Elbaz et al (2014), evaluated the ability of epicatechin to stimulate mitochondrial respiration (OXPHOS) in cancer cells as well as to selectively sensitize cancer cells to IR. The study examined the effects of epicatechin on pancreatic and glioblastoma cancer cells in vitro with an oxygen electrode,

Western blot analyses, and clonogenic survival assays. Epicatechin stimulated OXPHOS in Panc-1 cells with no effect on Human normal fibroblasts. Epicatechin sensitized the Panc-1, cells with an average radiation enhancement factor (REF) of 1.7, whereas the compound did not sensitize human normal fibroblast cells to IR with a REF value of 0.9, suggesting cancer cell specific selectivity. Additionally, the compound enhanced p21 induction and Chk2 phosphorylation with IR in cancer, but not normal, cells.

The radioprotective ability of two phenolic acids, chlorogenic acid and quinic acid, on IR-induced nDNA damage was assessed in the study by Cinkilic et al, (2013). The radioprotective effect of the phytochemicals in X-ray induced genomic instability in experiments involving ex vivo human blood lymphocytes was examined using the alkaline comet assay. The results from the alkaline comet assay demonstrate that chlorogenic acid and quinic acid decreased the X-ray irradiation nDNA damage in cells and provided significant radioprotective effects. Chlorogenic acid decreased the irradiation-induced nDNA damage in the range of 4.49–48.15%, and quinic acid in the range of 5.99–53.57% dependent on IR dose and administration dose of compounds. The results show that quinic acid and chlorogenic acid might act as radioprotective compounds. The study by Del Bano (2006) examined the radioprotective effects of carnosol, carnosic acid, and rosmarinic acid with respect to chromosomal damage in irradiated cells through the use of the micronuclei test to evaluate antimutagenic activity. The compounds

demonstrated radioprotective and radiomitigative effects in reduction of micronuclei in irradiated cells treated with compounds to that of irradiation control treatments by ~ 1.75 , 1.5 , 1.67 fold, ($p<0.001$). Therefore, carnosol, carnosic acid, and rosmarinic acid are compounds that show significant effects on antimutagenic activity.

In another study by Gandhi (2013), the authors examined the effects of baicalein plants on human blood cells and mice. Human blood cells irradiated ex vivo with baicalein showed reductions in DNA damage compared to irradiated control. Baicalein administration prior to 4 Gy TBI of mice conferred radioprotection to DNA as measured via blood cells in the alkaline comet assay. Mice exposed to 1.7 Gy TBI showed damage in the BMCs as measured by micronucleation. Baicalein administration prior to irradiation decreased micronucleation in BMCs.

In by Archana et al, (2011), the radioprotective effect of thymol, a monoterpene, on radiation-induced on genotoxicity was analyzed in vitro. In this study, Chinese hamster lung fibroblast (V79) cells were treated with varying amounts of thymol (0-100 μg/mL) for 1 hour prior to 3 Gy exposure and analyzed with single-cell gel electrophoresis (comet assay) and cytokinesis-blocked micronucleus assays to evaluate genotoxicity effects. Treatment of V79 cells with thymol for 1 hour prior to exposure reduced radiation-induced micronucleation as well as the percent tail nDNA and the mean Olive tail moment with notable radioprotective effect

observed at 25 μg/mL ($p<0.01$). The results from this study suggest that thymol suppresses radiation-induced genotoxicity.

The study by Jagetia et al (2006) demonstrated the radioprotective effects of Aphanamixis Polystachya (7.5 mg/kg) administration 1 h prior to 1-5 Gy TBI gamma irradiation in mice with chromosome aberrations studied at 12, 24, and 48 h post irradiation in bone marrow cells. Treatment of mice with Aphanamixis Polystachya prior to 1 – 5 Gy exposure significantly ($p < 0.01$) reduced the frequencies of chromosomal aberrations at each post-irradiation score. Therefore, these treatments reduce the aberrant populations of cells. The study by Harikumar et al (2007) evaluated the radioprotective effect of the plant Phyllanthus amarus in mice, specifically with respect to mouse chromosomal damage. Phyllanthus amarus at 250 and 750 mg/kg conferred protection from the genotoxic effects of IR on mouse chromosomes in terms of reductions in micronuclei and chromosomal aberrations. Therefore Phyllanthus amarus might protect from the clastogenic effects of IR.

In the study by Hsu et al (1997), ursolic acid and oleanolic acid from were evaluated for their ability to inhibit tumor growth and modify hematopoiesis following IR. The in vivo anti-tumor activity of tumor implants by ascitic cells was augmented by the addition of ursolic acid and oleanolic acid in a dose-dependent manner. In separate experiments, the addition of the compounds 30

min prior to 4 Gy TBI showed significant elevations in splenic proliferation to that of irradiation control treatments. Therefore, the beneficial effects of ursolic acid and oleanolic acid on hematopoiesis and immunocompetence suggests they might partially play some role in their anticarcinogenic function. In another study (Hsu et al. 1997) the antitumor efficacy of two iridoid compounds, geniposide and geniposidic acid were examined in mice. The study demonstrated decreases in the growth of implanted tumors by ascitic cells in response to geniposide and geniposidic acid treatments. Similarly, treatments of mice with compounds 30 min prior to TBI promoted splenic proliferation which might have some role in compound anticarcinogenic effects.

The study by Maurya et al (2006) addressed radioprotective effects of the plant derived compound, ferulic acid, throughout in vitro and in vivo experiments examining genotoxicity in peripheral blood leukocytes and tumour cells of mice. Administration of ferulic acid (50 mg/kg) 1 hr prior to 4 Gy TBI in mice bearing fibrosarcoma tumor showed preferential radioprotection of peripheral blood leukocytes and BMCs in comparison to tumor cells as shown by clonogenic assays of extracted cells. The authors showed that this preferential protection in vivo is not attributed to poor vasculature in the tumor cells in an ex vivo study of BMCs and tumor cells that showed only BMCs were protected by ferulic acid.

In another study by Pratheeshkumar et al (2011), the authors examined the administration of Veronia cinerea extract (20 mg/kg) in mice bearing implanted tumour cells. The evaluation of agarose gel electrophoresis of the DNA from BMCs of irradiation control animals showed extensive nDNA damage, but reductions in nDNA damage was seen in animals with administration of Veronia cinerea extract. There was also synergistic action of IR and veronia cinerea in reductions of solid tumours in mice. Additional parameters in the experiment examining cytokine production noted stimulation of GM-CSF and IFN-gamma in response to compound treatment in mice thereby demonstrating some role this mightfactor in the effects. In another study, the radioprotective effect of perillic acid was studied through in vivo experiments (Pratheeshkumar et al. 2010). Similarly, agarose gel electrophoresis of nDNA of mice exposed to IR showed extensive damage that was reduced with perillic acid treatment. Perillic acid treatment also promoted the production of immunostimulatory cytokines such as IFN-gamma and GM-CSF in animals exposed to IR.

The study by Chae et al (2009) examined the effect of the bioactive compound K, from ginseng saponin, on tumour xenografts in athymic BALB/c mice. In the study, compound K (30mg/kg/ day) was injected subcutaneously into the hind legs at the tumor site for 2 days. The mice were exposed locally to 10 Gy and 24 h post irradiation, and another dose of compound K (30 mg/kg/day) was administered at the tumor site. Treatment with compound K and IR separately

resulted in partial tumor xenograft growth regression in mice whereas administration of compound K significantly increased the antitumor effect of IR ($p<0.05$).

In the study by Hosseinimehr et al (2011), the radioprotective effects of hydroalcholic Zataria multiflora plant extract was examined with respect to IR induced genotoxicity in human lymphocytes. Peripheral blood lymphocyte samples collected from human volunteers were incubated with the zataria multiflora extract for 1 h prior to 1.5 Gy irradiation. The treatment of peripheral lymphocytes with zataria multiflora extract extract showed significant decreases in micronuclei binucleated cells to that of irradiation control treatments. The radioprotection and decrease in the frequency of micronuclei at 50 mg/ mL of Zataria extract was significant with 32% micronuclei reduction.

Another notable radioprotective compound that protects from genotoxicity is the metabolite of cysteine, *N*-acetylcysteine (NAC). The study by Demirel (2009) examined the effects of NAC administration in irradiated rat BMCs with respect to chromosomal aberration, micronucleus test, mitotic index and the cellular ratio of polychromatic erythrocytes to normochromatic erythrocytes in irradiated rat femoral BMCs. The rats received 1000 mg/kg NAC prior to 6 Gy TBI exposure. The irradiation controls showed higher frequency of micronuclei and lower

mitotic index and polychromatic erythrocyte/normochromatic erythrocytes to those of irradiation control treatments ($p<o.o05$).

Discussion

The basis of the effects of IR exposures are the primary oxidative interactions that take place within the cell environment. The extent of this damage is dependent on the quality of radiation (ie. LET), dose rate and total dose. Targets of IR primarily comprise subcellular organelles which in turn compromise functionality of cellular processes and, importantly, compromise the integrity of the cellular nDNA contained within the nucleus and mtDNA within the mitochondria. The nDNA and mtDNA are responsible in homeostatic biochemical processes including mitotic events and energy production. Despite endogenous molecular defenses to IR, extensive unrepaired damage causes cell destruction as well as cell mutations within the irradiation field. IR effects therefore persist beyond the initial exposure event to produce genomic instability within cells. Additionally, 'danger' signals propagate outside the irradiation field to produce bystander effects and inflammatory responses. Radionuclides might also be incorporated into tissues and therefore require removal to effectively eliminate the chronic dose delivery to tissue sites. These mechanisms of IR damage translate to cellular and systemic endpoints that are modifiable via the introduction of radioprotective compounds.

Conventional IR interventional strategies target short term IR management of effects from nuclear emergencies and high dose clinical exposures to promote cell survival regardless of the long term implications of chronic inflammatory effects and carcinogenesis as this is the primary priority, that is, of tissue function. These short term interventions are only requirements over the course of duration of exposures whereas long term management extends beyond exposure to resolve residual effects. Notable examples of short term management include the application of synthetic high dose antioxidants. Despite their effectiveness, short term management is not applicable with regards to long term management given the toxicity of treatments and practical route of administration. Additionally, specific experimental strategies that target up regulation of certain enzymatic antioxidants as well as down regulation/ stimulation of receptors encounter feedback mechanisms that impair their ability to function on the long term effects. To negate these effects requires applications of natural consumer products that aid in these short term interventions as well as provide long term care.

The available literature on natural compound radioprotection demonstrates that these substances provide non toxic antioxidant, anti-inflammatory, and anti-carcinogenic qualities chemicals and therefore are a valid platform to screen for radioprotective compounds. There are several issues that natural radioprotection have to address though to increase their impact in the field. Despite their effectiveness with respect to individual radioprotection criteria there are a lack of compounds that overlap each of these criteria and altogether address the

requirements of effective radioprotection. There are compounds that also lack high solubility to be effective in vivo. Therefore, to address each of these hallmarks requires the delivery of mixtures of compounds from each criteria with high solubility to be effective and administer. To confer robust responses to IR requires high availability and absorption by target cells. Therefore, intravenous administration of high doses of compounds is preferable as this promotes the pharmacokinetic distribution of the compound and also the pharmacodynamic action of the compounds. Unfortunately, this lacks the practicality of convenient administration. Therefore, natural radioprotection might benefit from the use of technologies and delivery systems such as liposome encapsulation to increase organism uptake to confer high radioprotection alongside ease of administration. These liposomes also permit the packaging of several compounds together which permits experiments to combine mixtures of compounds and administer (). Given compound administration is via the oral gastrointestinal route, another important addition to the field of natural radioprotection is to incorporate studies that screen compounds to promote the gut microbiota as part of the radioprotective response. This might be an important criterion of radioprotection as well.

In some systems, adaptive responses produce hormetic effects to stimuli (Calabrese 2010). In toxicology, hormetics is the phenomenon by which 'toxic' stressors produce stimulatory effects at low doses with inhibitory effects at high dose exposures. These stressors are shown in the literature to include pro oxidative compounds, heat, as well as IR amongst others (Birringer 2011). The

hormetic responses to these stressors include the induction of cellular repair mechanisms, upregulation of ROS redox responses, mitoprotective effects as well as bystander effects that are 'protective' within this region of exposure.

IR aside, there are similar hormetic responses to natural consumer products. Some compounds exert 'para-hormetic' responses in which the compound mode of action is to induce mild oxidative stresses to cells by which the system responds with an overcompensation of adaptive effects. These effects include the induction of detoxification enzymes which activate the intracellular production of thiols to alleviate ROS in cells (Calabrese 2010). These compound effects are often attributed to their chemical anti oxidative properties which is an indirect result of their oxidative influence on the system. In other examples in which compounds do not elicit ROS production, the compounds interact directly with components in the cell to upregulate these response mechanisms (Calabrese 2010). Therefore, these effects do not require elimination from biochemical systems as these disrupt their protective effects in vivo. There has to be an examination of the compatibility between IR with these compounds at low doses to demonstrate their effectiveness in the field of radioprotection in case the stressors have synergistic effects.

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Chapter 2: Cytotoxic Profiling of Plant Secondary Metabolites on p53 Variant Human Colon Carcinoma Cell Lines

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Declaration of Conflicting Interests

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Abstract

Keywords: chemoprevention, phytochemicals, p53, cytotoxicity, dose response

Chemoprevention strategies employ the use of compounds to inhibit the initiation, promotion, and progression phases of carcinogenesis. The successful chemopreventative candidate must therefore (1) selectively inhibit growth of transformed cells and (2) be administered on a frequent basis to confer maximal protection. Phytochemicals are a subclass of bioactive plant secondary metabolites that exhibit anti-oxidative,anti-carcinogenic and anti-inflammatory properties contributing to proper cell function. To assess the effectiveness of these compounds warrants an understanding of their cytotoxic mode of action. In this study p53 variant human colon carcinoma cell lines were chronically exposed to varying concentrations of the phytochemicals - curcumin, andrographolide, and d-limonene - to determine the role of p53 induced cytotoxicity, with p53 mutant and deficient cell lines representing precancerous lesions. Cytotoxicity was assessed using clonogenic assays and macroscopic colony counts were used to quantify cell survival. The results demonstrate that each phytochemical exhibits selective cytotoxicity towards non functional p53 cell lines suggesting a p53 mediated role in inhibition of cell clonogenicity and potential chemopreventative properties. Though each compound displays this described effect, only the d-limonene

demonstrates considerable chemoprotection suggesting it might have practical implications in vivo.

Introduction

Chemoprevention is an anticancer strategy that involves the use of compounds to inhibit the initiation of carcinogenesis and delay its promotion and progression. Mutations in the genome of cells are caused by the introduction of various carcinogens into the cellular environment (i.e radiation) that are associated with increases in oxidative stress and chronic inflammatory responses that exacerbate the effect. [1,2] Therefore, the successful chemopreventative candidate must have potent anti-oxidative, immunomodulatory, and tumoricidal properties that counteract the carcinogen at the source as well as modify the development of accompanying systemic effects. As carcinogenic processes constitute a long latency period, the candidate must be administered on a frequent basis to confer maximal chemoprotection. Unlike chemotherapeutic compounds, the candidate must exert low cytotoxicity towards non malignant cells while exerting high specificity and maximal lethality towards transformed cells. Specifically, these compounds aim to target deregulation of the cell cycle and target only cells that delineate from proper cell function. [3,4] Cellular deregulation might include alteration of tumour suppressor function via p53 transcription pathway and RAS

oncogene activation. These criteria warrant a cytotoxic examination of these compounds to ensure demonstrable high tissue tolerance and efficacy.

Despite synthetic analogues in the fields of oncology and chemotherapy, chemopreventative strategies usually rely on the use of dietary natural products in exerting their effects on cellular targets. [5,6] Plant secondary metabolites represent a class of natural compounds that have recognized anti-oxidative, anti-inflammatory, and growth inhibitory anti-carcinogenic properties in vivo. [7-9] Phytochemicals refer specifically to those plant derived compounds that exhibit bioactive anti-oxidative capabilities. Subclasses of these phytochemicals include polyphenolic, terpene, and diterpene compounds. [10] Although there are thousands of such phytochemical compounds, not each is compatible with consumption given their toxicity profiles in vivo. Therefore, only a few have been given notable attention with regards to their observed tumoricidal effects. [11]

Aims

Cytotoxic profiles of phytochemicals on isogenic cell lines might provide important information on the mechanism of these substances to determine the targets of growth inhibition. Specific compounds might interfere in certain pathways that are conducive to carcinogenic processes and therefore act to inhibit these processes. Additionally, these mechanisms might provide us with information on modifications that enhance the specific activity of the substances. This study investigates the interaction of the phytochemicals – curcumin,

andrographolide, and d-limonene - with p53 variant human colon carcinoma cell lines – HCT116 p53 wt, HT29, and HCT116 p53 null – where HT29 and HCT116 p53 null cell lines represent precancerous lesions given their non functional p53 status. The dose response of these interactions provides a basic mechanistic effect in terms of phytochemical p53 mediated cytotoxicity and chemoprevention, whereby our criteria of chemoprevention is selective cytotoxic effects towards non functional p53 cell lines.

Materials and methods

Subculture

HCT116 p53 wt, HT29, and HCT116 p53 null cell lines were cultured in T75 flasks (Falcon) containing RPMI 1640 medium (Gibco), FBS (Gibco), 1000 mM penicillinstreptomycin solution (Gibco), and 2mM L-glutamine (Gibco). Cells were maintained in an incubator at 37 ^{o}C with 95% air and 5% CO_2. Subculture was routinely performed when cells were 80-100% confluent using a solution of 0.25 % Trypsin (Gibco), DPBS (Gibco), and 1mM EDTA (Versene) (Gibco) at 37 ^{o}C.

Compounds

Curcumin, andrographolide, and d-limonene are lipophilic compounds and were thus dissolved in the organic solvent, ethanol, as an intermediate prior to application into clonogenic flasks to increase solubility and therefore cell bioavailability. Stock containers of curcumin (> 99.9% purity), andrographolide (> 99.9% purity), and d-limonene (> 99.9% purity) were purchased from Millipore-Sigma. Working concentrations of curcumin were made in a ratio of 1 mg/mL 100% ethanol, andrographolide in 1 mg/mL 100% ethanol and d-limonene was miscible in a 10:1 ratio of 100% ethanol.

Clonogenic Assay

Clonogenic technique by Puck and Marcus was used for cell survival analysis. Briefly, compounds were generated as per the dose optimization section and administered into T25 flasks (Falcon). Cells were detached from stock T75 flasks (Falcon) and resuspended in medium to generate a single cell suspension. Sample aliquot of the cell suspension was counted with the Z2 Cell Counter (Beckman Coulter) to generate values of viable cells. Following administration of compounds into flasks containing varying concentrations of each compound, cells were plated into each T25 flask (Falcon). Cell cultures were incubated for their respective clonogenic period, approximately 9 days for HCT116 p53 wt and HCT116 p53 null containing flasks and 11 days for HT29 cells. Cells were stained following their clonogenic period of incubation with 25% carbol fuschin

in water where macroscopic colonies equal to and over 50 cells satisfy the criteria of reproductive cell survival. Total of three independent experiments were conducted with three replicates per experiment (n=3).

Dose Function Analysis

Exposure of cells to growth inhibitory compounds comprises of two characteristic values in a dose response curve, the non minimal inhibitory concentration (NIC) and the minimal inhibitory concentration (MIC). The NIC value refers to the minimal dose at which the compound begins to inhibit cell growth, whereas the MIC refers to the minimal dose that exerts maximal growth inhibition.

In this study, cytotoxicity curves and corresponding NIC and MIC values were constructed using data from the clonogenic assay cell survival criteria. The inhibitory concentration for each compound was determined using the statistical software analysis package GraphPad Prism 7. The NIC and MIC software interpolated values were determined utilizing a Gompertz function dose response curve based on the Lambert and Pearson method of determining antibiotic susceptibility in bacterial strains. The function assigns NIC and MIC values corresponding to intersections of the slope of the inflection point with the upper and lower asymptote respectively. The 3 parameter Gompertz function is a type of sigmoidal logistic growth and decay function that describes cell survival by the

equation f(x) = a*exp.[-b*exp.(-c*x). Values of a < 0, b > 0, and c > 0 are representative of decreases in cell survival and therefore the characteristic cytotoxicity curve that describes situations of inhibitory growth applicable to administration of anti-tumorigenic compounds to cancerous cells.

Statistical Analysis

The One-Way Analysis of Variance (ANOVA) between groups with follow-up Tukey's multiple comparisons test was conducted using the statistical software package GraphPad Prism 7 to examine the effects of phytochemical exposure on varying p53 function between each cell. The NIC and MIC values define a measured and computed value that characterizes each curve in terms of the biological effect endpoint. The input of each test therefore consist of the NIC and MIC values of each curve along with their associated Standard Deviation (SD) and Degrees of Freedom (dF) to assess differences between groups. The Tukey's multiple comparisons test reports multiplicity adjusted P values that pool the SD of each group and therefore report conservative levels of significance. The confidence interval of 95% and multiplicity adjusted P-value < 0.05 was taken to be significant.

Results

Figures 1-3 represent the dose cytotoxicity profiles of each phytochemical between p53 variant human colon carcinoma cell lines and graphically describe the derivation of Gompertz NIC and MIC best-fit values. The tabulated results in Table 1 put into perspective the relative cytotoxcity of each compound and p53 mediated effects between cell lines. From Table 1, the curcumin had the lowest NIC and MIC values in each of the cell lines, followed by andrographolide and d-limonene. The calculated NIC and MIC values of each of the phytochemicals were lower for the HT29 and HCT116 p53 null cell lines than the HCT116 p53 wt, with the exception of andrographolide where the MIC values were higher for the HT29 and HCT116 p53 null cell lines. Table 2 characterizes the steepness of the response via the NIC/MIC ratio value. The HCT116 p53 wt cell line exhibited steep survival on exposure to each of the compounds, whereas the broad profile of the curves of HT29 and HCT116 p53 null cell lines suggests a less pronounced and effective response per dose of the compounds. The differential effects between the function and non functional p53 cell lines suggest that there might be a p53 mediated mechanism of cell cytotoxicity.

Evaluation of the statistical significance in Table 3 of the best fit values in the curcumin treatments showed there was significance in the comparison between the NIC and MIC values of the HCT116 p53 wt vs. HT29 and HCT116 p53 wt vs. HCT116 p53 null cell lines and the MIC values of HT29 vs. HCT116 p53 null cell lines. Cytotoxicity comparisons in the andrographolide treatments

demonstrate significance between the NIC values of HCT116 p53 wt vs. HT29 and MIC values of HCT116 p53 wt vs. HCT116 p53 null and HT29 vs. HCT116 p53 null cell lines. The d-limonene demonstrated high significance between both the NIC and MIC values of HCT116 p53 wt vs. HT29, HCT116 p53 wt vs. HCT116 p53 null, and the NIC value of HT29 vs. HCT116 p53 null cell lines. Table 3 therefore suggests that there is a role for p53 mediated effects in cell survival at low dose phytochemical exposure, otherwise we might anticipate that there are no differential effects between each of the cell lines especially the HCT116 p53 wt and HCT116 p53 null variant cell lines. The observations of chemopreventative effects as per the experimental criteria are mostly in line with the d-limonene as it demonstrates high selective cytotoxicity towards the p53 mutant HT29 and p53 deficient HCT116 p53 null cell lines.

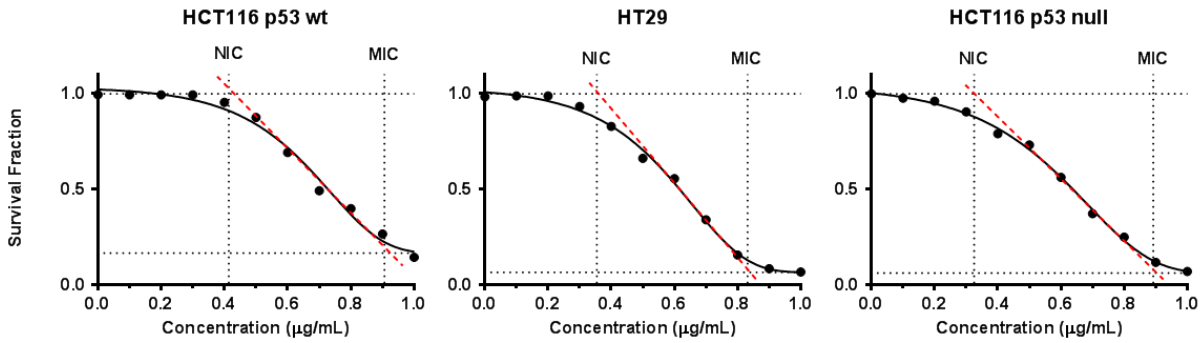

Figure 1: Curcumin cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz

function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

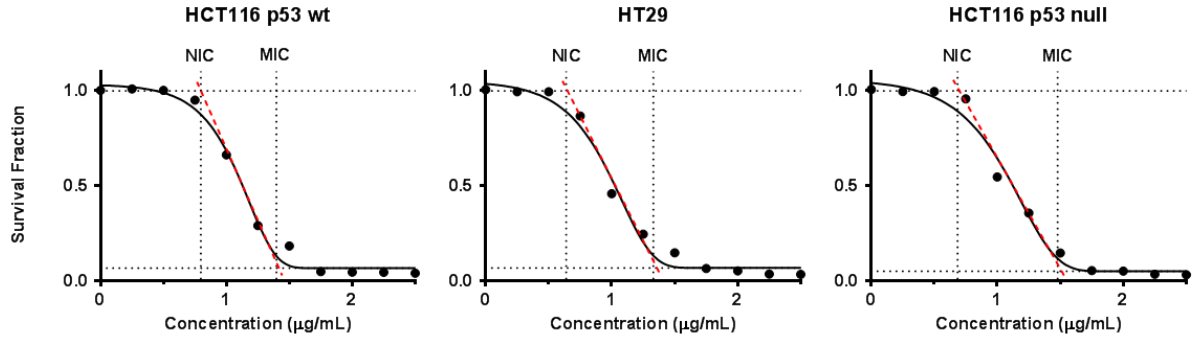

Figure 2: Andrographolide cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

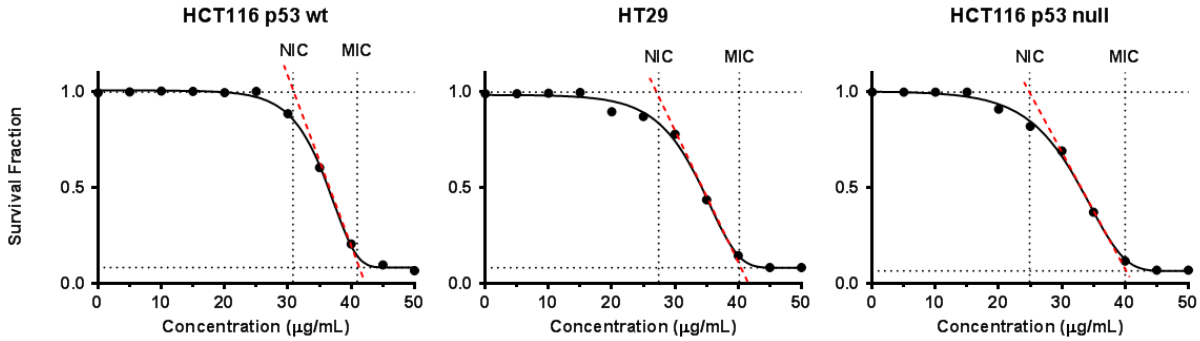

Figure 3: D-limonene cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three

independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

Table 1

	HCT116 p53 wt		**HT29**		**HCT116 p53 null**	
	NIC	MIC	NIC	MIC	NIC	MIC
Curcumin	0.4144	0.9066	0.3548	0.8319	0.3254	0.8926
Andrographolide	0.7986	1.398	0.6394	1.331	0.6849	1.48
D-Limonene	30.83	40.97	27.44	40.17	24.9	40

* Values are expressed in μg/mL

Table 2

	HCT116 p53 wt	**HT29**	**HCT116 p53 null**
	NIC/MIC	NIC/MIC	NIC/MIC
Curcumin	0.457092	0.426494	0.364553
Andrographolide	0.571245	0.480391	0.462770
D-Limonene	0.752502	0.683097	0.622500

Table 3

	HCT116 p53 wt vs. **HT29**		**HCT116 p53 wt** vs. **HCT116 p53 null**		**HT29** vs. **HCT116 p53 null**	
	NIC	MIC	NIC	MIC	NIC	MIC
Curcumin	P = 0.0035	P = 0.0002	P < 0.0001	P = 0.6279	P = 0.1835	P = 0.0017
	**	***	****	ns	ns	**
Andrographolide	P = 0.0092	P = 0.1063	P = 0.0697	P = 0.0410	P = 0.6200	P = 0.0003
	**	ns	ns	*	ns	***
D-Limonene	P < 0.0001	P = 0.0218	P < 0.0001	P = 0.0054	P < 0.0001	P = 0.8117
	****	*	****	**	****	ns

P-Value Chart displays the results of p53 mediated phytochemical interactions and summarizes the One-Way ANOVA analysis with subsequent Tukey's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

Discussion

The results demonstrate that there is likely a p53 mediated role in the induction of growth inhibition and cytotoxicity in response to curcumin, andrographolide, and d-limonene including their respective metabolites on the HCT116 p53 wt, HT29, and HCT116 p53 null colon carcinoma cell lines. Functional p53 is known to operate via binary response mechanisms, that is, at low doses to DNA damaging agents the cell undergoes G1 Cell Cycle arrest allowing for the cell to undergo critical repair mechanisms prior to progression to S phase of the cell cycle. [12] At high doses, the cell accumulates p53 and causes apoptosis in response to sufficient damage to the cells. [12] Chemopreventative strategies targeting cancerous cells require activation of p53 and restoration of its ability to induce apoptosis, a strategy which is not an option in p53 deficient cell lines. Otherwise, it requires a bypass of this mechanism and uses an alternative p53 independent pathway to perform this function. The differential selection between the HCT116 p53 wt, HT29, and HCT116 p53 null cell lines are representative of the latter case. These observations are in line with the finding that the HCT116 p53 wt cells generally exhibit higher NIC and MIC values than HT29 and HCT116 p53 null cells that

lack functional p53 transcription factors. At low doses, the compounds might initiate cell pathways that require p53 mediated cell cycle arrest to bypass the cell mechanism, which might be representative of slight xenohormetic basis for these compounds. It is noteworthy that phytochemicals serve as insecticides in plants. [13] Alternatively, the compound might enhance the activity of p53 and allow it to bypass this mechanism via an alternate pathway. Therefore, these compounds must operate in a p53 dependent way that selectively targets cells that do not have functional p53 and that might require functional p53 operation to inhibit other pathways. At the NIC dose of each compound the HCT116 p53 wt exhibits a steep decline in cell lines. Therefore, at high doses, the compound might activate the p53 dependent apoptotic mechanism that explains the steep portion of the graph representative of the NIC/MIC ratio. Therefore only those cells that lack functional p53 undergo premature cell senescence effects.

In terms of oral dosing implications, the NIC value is the relevant value as the MIC value of non-functional p53 cell lines HT29 and HCT116 p53 null coincides to a significant extent with that of the HCT116 p53 wt cell lines and is therefore likely to cause toxic effects in otherwise non malignant cells. The successful chemopreventive candidate therefore has to have practical dose discrepancies between the NIC values of wt and mutant p53 cell lines to demonstrate in vivo effects. In this respect, the d-limonene satisfies this criterion more so than curcumin and andrographolide as the dose discrepancy between the NIC values of the HCT116 p53 wt and HT29 and HCT116 p53 wt and HCT116 p53 null cell

lines are 3.39 microgrms/mL and 5.93 microgrms/mL respectively. Although the cytotoxicity curves of the curcumin and andrographolide also demonstrate significant difference of NIC values between cell lines, the discrepancy between these values are very low, therefore selective dosing might not be clinically practical. From the literature, d-limonene is known to bind to the RAS and downplay the cell hyperproliferation, which is an additional attribute to its cell regulatory mechanism. [14] Although this study does not address the role RAS, it uses both the HCT116 p53 wt and HCT116 p53 null cell lines each of which contain the RAS mutations, therefore attributed effects must be independent of the mechanism of RAS inhibition. [15]

Conclusion

The use of phytochemicals in chemoprevention might have an important role in targeting various precancerous cell lines through enhancing cell regulatory mechanisms. Their appeal involves their non invasive and practical route of administration via oral consumption. The compounds require frequent intake to confer maximal protective effects, in this regard, cytotoxic profiling of chronic long term exposures to these compounds and their metabolites is important as it serves to provide guidance in this field. In this study, chronic exposures to curcumin, andrgorapholide, and d-limonene on p53 variant cell lines demonstrated that each of the compounds had some level of chemopreventative effect. The most pronounced effect though was observed in the d-limonene as

demonstrated by the NIC dose discrepancies required to inhibit growth of non functional p53 cell lines. Therefore, further investigation might be warranted in the molecular pathways in which d-limonene inhibit these effects.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the research, authorship and/or publication of this article.

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Chapter 3: Cytotoxic Profiling of Endogenous Metabolites Relevant to Chronic Fatigue Immune Dysfunction Syndrome (CFIDS) on p53 Variant Human Colon Carcinoma Cell Lines

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Declaration of Conflicting Interests

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Abstract

Keywords: chemoprophylactic, carcinogenesis, endogenous metabolites, p53, cytotoxicity, dose response

Chemoprophylatic strategies against development of multifactorial diseases utilize compounds to block the multistep events in chronic inflammation and carcinogenesis. The successful chemopreventative candidate therefore must selectively inhibit growth of transformed cells and be administered frequently to confer maximal protection with minimal side effects. In addition to synthetic and exogenous natural compounds, endogenous metabolites represent another class of compounds that exhibit anti-carcinogenic and anti-inflammatory properties contributing to proper cell function. To assess the effectiveness of these compounds warrants an understanding of their cytotoxic mode of action. In this study p53 variant human colon carcinoma cell lines were chronically exposed to varying concentrations of the endogenous metabolites – phenyl acetate, ursodeoxycholate, and tauroursodeoxycholate - to determine the role of p53 induced cytotoxicity, with p53 mutant and deficient cell lines representing precancerous lesions. Cytotoxicity was assessed using clonogenic assays, and macroscopic colony counts were used to quantify cell survival. The results demonstrate that the bile acids, ursodeoxycholate and tauroursodeoxycholate, exhibit selective cytotoxicity towards non functional p53 cell lines suggesting a p53 mediated role in inhibition of cell clonogenicity and potential

chemopreventative properties. Though each compound displays this described effect, the tauroursodeoxycholate demonstrates high significance suggesting it might have practical uses in vivo.

Introduction

Chemoprophylactic strategies in cancer and inflammatory diseases involve the use of compounds aimed to block/inhibit the multistep events in the initiation, progression and promotion phases of these diseases. These multistep events consist of chronic exposures to stressors and carcinogens (including ionizing radiation) that act to produce mutations or inflammatory responses in cells which lead to mutator phenotypes in precancerous cells as well as exacerbate systemic immune dysfunction.[1] The compounds therefore function to modify the intracellular environment to protect from these injuries directly, for instance as antioxidants, as well as inhibit the phenotypic modifications that occur downstream through assisting proper regulation of cell function and promotion of cell apoptosis.[2,3] Alternatively, the compound might also alter the extracellular environment to promote immunostimulatory activity as well as block other surrounding paracrine cellular signalling components that contribute to tumour proliferative processes.[4,5] Cells naturally regulate these carcinogenic events without exogenous compounds via production of endogenous proteins, however, mutations in the genes that code for these products might impair the capability of

these regulatory processes, for instance suppressing tumours in the case of p53 transcription factor and antioxidant activity of catalase enzymes.[6] Therefore, defects/deficiencies in these pathways represent impairments in regulatory processes where the introduction of additional supplementary endogenous compounds might be useful and represent targets in compound screening tests.

Endogenous metabolites represent both intermediates of cellular metabolism that aid in the proper functioning of the cell and by-products that play roles in regulating cellular processes.[7] These compounds are not direct protein products of transcription although enzymatic conversion and therefore enzymes are important in their production.[7,8] Though exogenous compounds represent a worthy lead of research, endogenous metabolites represent another class of non-synthetic alternative compounds that might satisfy the criteria of chemoprevention. Like exogenous compounds, their appeal is administration via oral consumption by in vivo endogenous metabolite supplementation to target cells. Unlike exogenous compounds, these compounds demonstrate utility by supporting regulation and function in many different pathways, for instance phenyl acetate participates in cellular signalling cascades pathways.[9]

Aims

Cytotoxic profiles of endogenous metabolites implicated in Chronic Fatigue Immune Dysfunction Syndrome (CFIDS) on isogenic cell lines might provide

vital data on the mechanistic basis of these substances in vitro to determine the targets of cellular growth inhibition. Specific substances might interfere in certain cellular pathways that are conducive to carcinogenic processes and therefore act to inhibit these processes. Given these compounds exist naturally in vivo they typically exhibit high tolerance levels in the organism in comparison to exogenous compounds because they do not inhibit cellular processes. However, at high doses, feedback mechanisms might impair the efficacy of such treatments by lowering the production of the naturally occurring compounds, therefore appropriate dosing must be accomplished by optimizing the compound cytotoxicity. Additionally, these mechanisms might provide us with information on modifications that enhance the specific activity of the substances. This study investigates the interaction of the endogenous metabolites – phenyl acetate, ursodeoxycholate, and tauroursodeoxycholate - with p53 variant human colon carcinoma cell lines – HCT116 p53 wild-type (wt), HT29, and HCT116 p53 null – where HT29 and HCT116 p53 null cell lines represent precancerous lesions given their non functional p53 status. The dose response of these interactions provides a basic mechanism in terms of endogenous metabolite p53 mediated cytotoxicity and chemoprevention, whereby our criteria of chemoprevention is selective cytotoxic effects towards non functional p53 cell lines.

Materials and methods

Subculture

HCT116 p53 wt, HT29, and HCT116 p53 null cell lines were cultured in T75 flasks (Falcon) containing RPMI 1640 medium (Gibco), FBS (Gibco), 1000 mM penicillinstreptomycin solution (Gibco), and 2mM L-glutamine (Gibco). Cells were maintained in an incubator at 37 ^{o}C with 95% air and 5% CO_2. Subculture was routinely performed when cells were 80-100% confluent using a solution of 0.25 % Trypsin (Gibco), DPBS (Gibco), and 1mM EDTA (Versene) (Gibco) at 37 ^{o}C.

Cell Lines

The HCT116 p53 wt cells and HCT116 p53 null cells are p53 isogenic cell lines of an adherent epithelial human colorectal carcinoma cell line. The HCT116 p53 wt cell line is positive for p53 wildtype expression whereas the HCT116 p53 null cell line lacks p53 expression. The HT29 cells are an adherent epithelial human colorectal adenocarcinoma cell line that possesses G -> A substitution in codon 273 of the p53 gene that results in the conversion of Arg -> His in the protein product, therefore the cell line expresses aberrant non wt expression of the p53 transcription factor.

Compounds

Phenyl acetate, ursodeoxycholate, and tauroursodeoxycholate are ambiphilic compounds and were dissolved in ethanol. Stock containers of phenyl acetate (> 99.9% purity), ursodeoxycholate (> 99.9% purity), and tauroursodeoxycholate (> 99.9% purity) were purchased from Millipore-Sigma. Working concentrations of ursodeoxycholate were made in a ratio of 10 mg/mL of 100% ethanol, taurousodeoxycholate in 10 mg/mL of 100% ethanol and phenyl acetate was made in a 10:1 ratio of 100 % ethanol.

Clonogenic Assay

Clonogenic technique by Puck and Marcus[10] was used for cell survival analysis. Briefly, compounds were generated as per the dose optimization section and administered into T25 flasks (Falcon). Cells were detached from stock T75 flasks (Falcon) and resuspended in medium to generate a single cell suspension. Sample aliquot of the cell suspension was counted with the Z2 Cell Counter (Beckman Coulter) to generate values of viable cells. Following administration of compounds into flasks containing varying concentrations of each compound, cells were plated into each T25 flask (Falcon). Cell cultures were incubated for their respective clonogenic period, approximately 9 days for HCT116 p53 wt and HCT116 p53 null containing flasks and 11 days for HT29 cells. Cells were stained following their clonogenic period of incubation with 25% carbol fuschin

in water where macroscopic colonies equal to and over 50 cells satisfy the criteria of reproductive cell survival. Total of three independent experiments were conducted with three replicates per experiment (n=3).

Dose Function Analysis

Exposure of cells to growth inhibitory compounds comprises of two characteristic values in a dose response curve, the non minimal inhibitory concentration (NIC) and the minimal inhibitory concentration (MIC). The NIC value refers to the minimal dose at which the compound begins to inhibit cell growth, whereas the MIC refers to the minimal dose that exerts maximal growth inhibition.

In this study, cytotoxicity curves and corresponding NIC and MIC values were constructed using data from the clonogenic assay cell survival criteria. The inhibitory concentration for each compound was determined using the statistical software analysis package GraphPad Prism 7. The NIC and MIC software interpolated values were determined utilizing a Gompertz function dose response curve based on the Lambert and Pearson method of determining antibiotic susceptibility in bacterial strains[11]. The function assigns NIC and MIC values corresponding to intersections of the slope of the inflection point with the upper and lower asymptote respectively. The 3 parameter Gompertz function is a type of sigmoidal logistic growth and decay function that describes cell survival by the

equation f(x) = a*exp.[-b*exp.(-c*x). Values of a < 0, b > 0, and c > 0 are representative of decreases in cell survival and therefore the characteristic cytotoxicity curve that describes situations of inhibitory growth applicable to administration of anti-tumorigenic compounds to cancerous cells.

Statistical Analysis

The One-Way Analysis of Variance (ANOVA) between groups with follow-up Tukey's multiple comparisons test was conducted using the statistical software package GraphPad Prism 7 to examine the effects of endogenous metabolite exposure on varying p53 function between each cell. The NIC and MIC values define a measured and computed value that characterizes each curve in terms of the biological effect endpoint. The input of each test therefore consist of the NIC and MIC values of each curve along with their associated Standard Deviation (SD) and Degrees of Freedom (dF) to assess differences between groups. The Tukey's multiple comparisons test reports multiplicity adjusted P values that pool the SD of each group and therefore report conservative levels of significance. The confidence interval of 95% and multiplicity adjusted P-value < 0.05 was taken to be significant.

Results

Figures 1-3 represent the dose cytotoxicity profiles of each endogenous metabolite between p53 variant human colon carcinoma cell lines and graphically describe the derivation of Gompertz NIC and MIC best-fit values. The tabulated results in Table 1 put into perspective the relative cytotoxcity of each compound and p53 mediated effects between cell lines. From Table 1, the phenyl acetate had the lowest NIC and MIC values in each of the cell lines, followed by ursodeoxycholate and tauroursodeoxycholate. The calculated NIC and MIC values for the phenyl acetate were lower for the HCT116 p53 wt than the HT29 and HCT116 p53 null cell lines, whereas treatments of the ursodeoxycholate demonstrated higher NIC and MIC valuesfor the HCT116 p53 wt than the HT29 and HCT116 p53 null cell lines and treatments of tauroursodeoxycholate demonstrated higher NIC values with lower MIC values in the HCT116 p53 wt than both the HT29 and HCT116 p53 null cell lines. Table 2 characterizes the steepness of the response via the NIC/MIC ratio value. The HCT116 p53 wt cell line exhibited steep survival on exposure to the ursodeoxycholate and tauroursodeoxycholate in comparison to the HCT116 p53 null cell line, whereas the opposing observation is evident in the phenyl acetate exposure.

Evaluation of the statistical significance in Table 3 of the best fit values in the phenyl acetate treatments demonstrated there was high significance in the comparison between the NIC and MIC values of the HCT116 p53 wt vs. HT29 and HCT116 p53 wt vs. HCT116 p53 null cell lines, however no significance

between the NIC and MIC values of HT29 vs. HCT116 p53 null cell lines. Cytotoxicity comparisons in the ursodeoxycholate treatments showed slight significance only between the NIC values of HCT116 p53 wt vs. HCT116 p53 null cell lines. The tauroursodeoxycholate demonstrated slight significance between both the NIC and MIC values of HCT116 p53 wt vs. HCT116 p53 null, and the MIC value of HT29 vs. HCT116 p53 null cell lines. Table 3 therefore suggests that there might be a role for p53 mediated effects in cell survival in phenyl acetate exposures, whereas the low significance in the ursodeoxycholate and tauroursodeoxycholate treatments suggests less pronounced intervention of p53 mediated responses. Though the phenyl acetate treatments demonstrate considerable significance in treatment exposures, the NIC and MIC doses of compounds required to achieve growth inhibition are less effective on the HT29 and HCT116 p53 null cell lines in comparison to the HCT116 p53 wt cell line. Therefore, the observations of chemopreventative effects as per the experimental criteria in this study are mostly in line with the tauroursodeoxycholate as it satisfies selective cytotoxicity towards the p53 mutant HT29 and p53 deficient HCT116 p53 null cell lines.

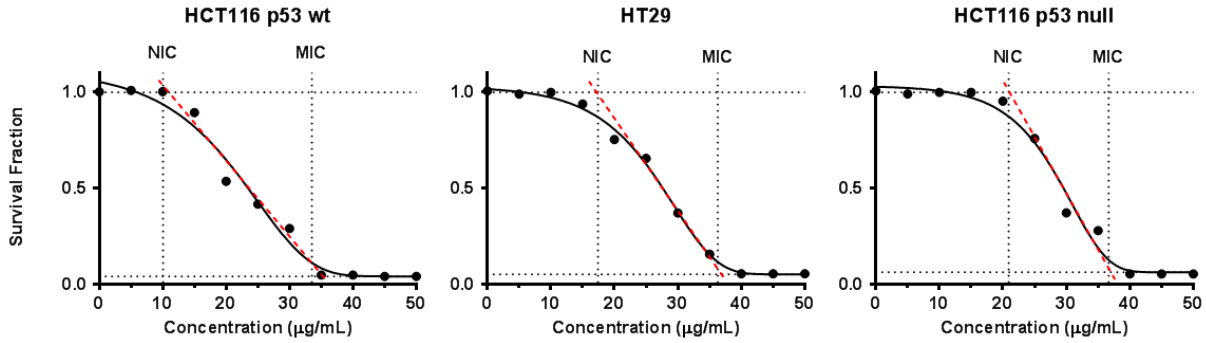

Figure 1: Phenyl acetate cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

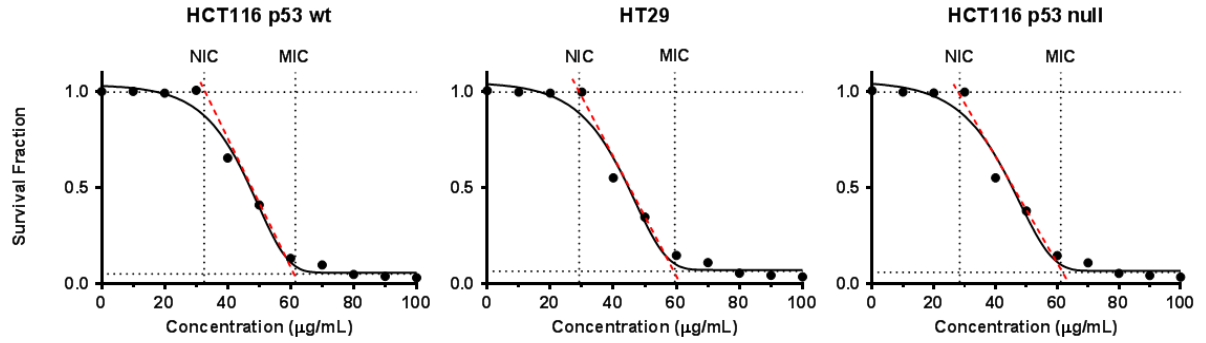

Figure 2: Ursodeoxycholate cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz

function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

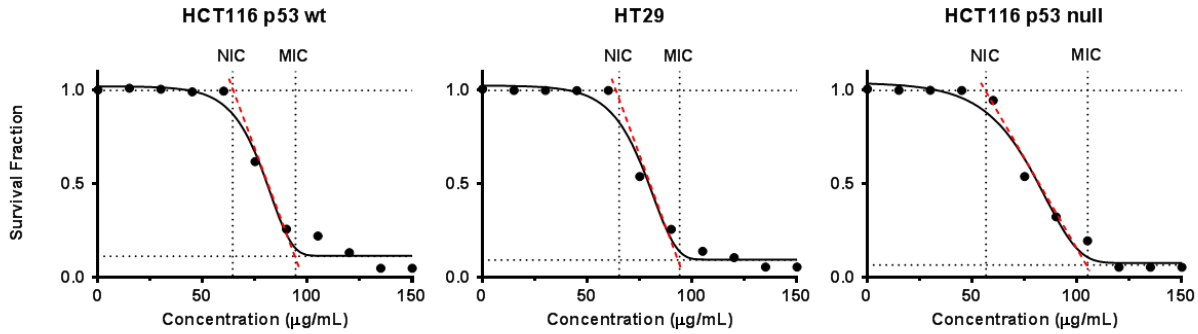

Figure 3: Tauroursodeoxycholate cytotoxicity data on three p53 variant human colon carcinoma cell lines and are representative of triplicate experiments. Values shown on each graph are expressed as the mean survival fraction of three independent experiments (n=3). The solid line corresponds to the best-fit Gompertz function of the data. The dash line represents the slope of the inflection point of the Gompertz function. NIC refers to 'Non-minimal Inhibitory Concentration'. MIC refers to 'Minimal Inhibitory Concentration'. Intersections of the inflection point with the upper and lower asymptote of the Gompertz function represent the NIC and MIC respectively. Concentration data are expressed in micrograms per millilitre.

Table 1

	HCT116 p53 wt		**HT29**		**HCT116 p53 null**	
	NIC	MIC	NIC	MIC	NIC	MIC
Phenyl Acetate	10.08	33.53	17.44	36.29	20.92	36.68
Ursodeoxycholate	32.56	61.52	29.2	59.94	28.43	61.2
Tauroursodeoxycholate	64.39	93.34	62.54	94	56.65	105.1

* Values are expressed in µg/mL

Table 2

	HCT116 p53 wt	**HT29**	**HCT116 p53 null**
	NIC/MIC	NIC/MIC	NIC/MIC
Phenyl Acetate	0.300626	0.480573	0.570338
Ursodeoxycholate	0.529259	0.487154	0.464542
Tauroursodeoxycholate	0.689844	0.665319	0.539010

Table 3

	HCT116 p53 wt vs. **HT29**		**HCT116 p53 wt** vs. **HCT116 p53 null**		**HT29** vs. **HCT116 p53 null**	
	NIC	MIC	NIC	MIC	NIC	MIC
Phenyl Acetate	P < 0.0001	P = 0.0066	P < 0.0001	P = 0.0021	P = 0.0502	P = 0.8783
	****	**	****	**	ns	ns
Ursodeoxycholate	P = 0.1855	P = 0.7668	P = 0.0317	P = 0.9890	P = 0.9383	P = 0.8439
	ns	ns	*	ns	ns	ns
Tauoursodeoxycholate	P = 0.7588	P = 0.9937	P = 0.0187	P = 0.0161	P = 0.0527	P = 0.0394
	ns	ns	*	*	ns	*

P-Value Chart displays the results of p53 mediated endogenous metabolite interactions and summarizes the One-Way ANOVA analysis with subsequent Tukey's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

Discussion

The results demonstrate that there is a p53 mediated role in the induction of growth inhibition and cytotoxicity of phenyl acetate and to a lesser extent the bile acids, ursodeoxycholate and taurousodeoxycholate, including their respective in vitro metabolites. The p53 response pathway functions on the basis of the detection of cellular DNA damage in a dose dependent manner.[12,13] Therefore, at low doses to cellular stressors, the p53 arrests the cell in G1 phase to repair the damage whereas at high doses the cell undergoes apoptosis to the exposure.[14] The inactivation of p53 might impair the ability to promote cellular repair responses, thus contribute to aberrant cell function.[15,16] Chemoprotection strategies therefore require the restoration of p53 capacity with interventional compounds to assist in proper cellular regulation of responses.[17] Given p53 deficient cell lines lack p53 to act as an appropriate target of therapeutic intervention, the compound must also act to target some other aspect of cellular susceptibility in these cell lines. In the case of isogenic p53 variant cell lines, such as the HCT116 p53 wt and HCT116 p53 null, no difference in clonogenicity on exposure to the compound is indicative of p53 independent mediated responses. In cases where clonogenicity between isogenic cell lines is favourable towards p53 null and p53 mutant cells as is the case of phenyl acetate, the compound might promote cytotoxicity via p53 dependent mechanism such that it triggers apoptosis in p53 wt cells. In the cases where there is significance between the isogenic cell lines in favour of clonogenic survival of p53 wt cells at low doses, as is the case of the compounds

ursodeoxycholate and tauoursodeoxycholate, the p53 might inhibit alternative cytotoxic responses. Whereas, at high doses, it promotes cytotoxicity which explains the significance in favour of p53 null of the tauroursodeoxycholate at high dose exposures. Therefore, on the basis of these study criteria, the findings support that the tauroursodeoxycholate is chemoprotective to some extent.

In terms of oral compound dosing, the NIC value represents the relevant value with respect to chemoprotection as the MIC value of the non-functional p53 cell lines, HT29 and HCT116 p53 null, coincides significantly to that of the HCT116 p53 wt cell line and is thus likely to cause adverse effects in otherwise non malignant cells. Therefore, the successful chemopreventive candidate has to have practical dose discrepancies between the NIC values of wt and null p53 cell lines to demonstrate in vivo effects. Given the experiment criteria, only the ursodeoxycholate and the tauroursodeoxycholate demonstrate significance between the NIC values of HCT116 p53 wt and HCT116 p53 null cell lines though not between HCT116 p53 wt and HT29 mutant p53 cell lines. In this respect, the tauroursodeoxycholate satisfies this criterion more so than the ursodeoxycholate as the dose discrepancy between the NIC values of ursodeoxycholate and tauroursodeoxycholate on the HCT116 p53 wt and HCT116 p53 null cell lines are 4.13 microgrms/mL and 7.74 microgrms/mL respectively. Although the cytotoxicity curves of the phenyl acetate also demonstrate significant difference of NIC values between cell lines, the discrepancy between these values are in favour of the HCT116 p53 null and HT29 cell lines.

Conclusion

The supplementation of endogenous metabolites in vivo might serve as an effective chemopreventative approach to targeting precancerous cells by promotion of cell regulatory mechanisms. This chemoprohylactic strategy is particularly practical given the basic administration via oral consumption of compounds. These compounds represent intermediate metabolites which undergo feedback mechanisms in vivo therefore cytotoxic profiling of chronic administration of these compounds is important to confer maximal beneficial effects. In this study, chronic exposures to the compounds – phenyl acetate, ursodeoxycholate, and tauroursodeoxycholate, - demonstrated that only the tauroursodeoxycholate had some level of chemopreventative effect. The appeal of this particular compound is the low overall toxicity with high dose discrepancies between the NIC values that render it suitable to in vivo studies. Therefore, investigation of the pharmacodynamics of the compound warrants study of into the molecular interactions of the tauroursodeoxycholate compound with cellular components.

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the research, authorship and/or publication of this article.

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Chapter 4: An Investigation of Consumer Phytochemicals – Curcumin, Andrographolide, and D-Limonene – as Candidates in Dietary Radioprotection

This manuscript is under consideration for publication.

Title:

An Investigation of Consumer Phytochemicals – Curcumin, Andrographolide, and D-Limonene – as Candidates in Dietary Radioprotection

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Declaration of Conflicting Interests

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Abstract

Ionizing radiation is an artificially and environmentally relevant physical stressor to human health that necessitates the use of dietary compounds as an interventional strategy to reduce the impacts from exposure. Dietary radioprotection candidates have to possess practical qualities including low cytotoxicity, consumer appeal, antioxidant chemical properties, cell regulatory and bystander inhibitory effects to rescue targeted and non-targeted cells. These criteria appear to align well with those of phytochemicals which are non nutrient plant metabolites. The aim of this study was to characterize the radioprotective, radiomitigative, and radiation induced bystander effect inhibiting properties of commercially available consumer phytochemicals – curcumin, andrographolide, and d-limonene - through administration pre- and post-irradiation and in donor and recipient flasks to assess whether these might represent suitable dietary compounds to treat radiation exposures in the range of 0.5 – 5 Gy doses. Clonogenic assays were therefore used to characterize the radiation responses of phytochemical treatments whereby cellular responses to irradiation are quantified with macroscopic colony counts to measure cell survival in terms of reproductive capacity. The results demonstrate that the phytochemicals do not demonstrate statistical significance in radioprotective, radiomitigative, and bystander inhibiting experiments with the exception of d-limonene which, on the contrary,

demonstrates radiosensitization post irradiation administration to cells. The phytochemicals – curcumin, andrographolide, and d-limonene – are insufficient in radioprotective capability to protect from acute, high dose exposures to ionizing radiation. Despite these results, cited aspects of these compounds including their anti-carcinogenic and anti-inflammatory properties might have some role in management of chronic effects from exposures which warrants examination.

Keywords

Phytochemical, radioprotective, radiosensitive, dose-response, clonogenic viability, stem cells

Introduction

Radioprotectors are compounds that confer protection from radiation-chemical interactions within the cell - primarily production of free radicals that react with organelles and molecules to impact their biological function (Mikkelsen and Wardman 2003; Brizel 2007; Leach et al. 2001). These compounds scavenge free radicals in the cellular medium during irradiation to reduce the radiochemical effects of exposure. Radioprotective compounds have to possess sufficient antioxidant activity to initiate these neutralization reactions thereby depleting the excess free radical species, mainly reactive oxygen species (ROS), that produce oxidative stress within cells (Nair et al. 2001; Yamini and Gopal 2010; Shao et al. 2011). Despite the elucidation of synthetic cysteamine compounds that possess free radical scavenging abilities, the toxicity of these radioprotective compounds and intravenous administration in vivo render them highly impractical for use in chemical radioprotection (Andreassen et al. 2003). Therefore, screening and utilization of radioprotective candidates require the compounds be highly practical in terms of toxicity and administration. The use of natural product supplements satisfies these criteria because of their oral route of consumption and high tolerable doses (Lee et al. 2008). Additionally, although conventional radioprotective compounds address and reduce the immediate biochemical effects of radiation to an extent, important residual effects of radiation require frequent

administration of non-toxic compounds post irradiation exposure. These residual effects include Chronic Fatigue Immune Dysfunction Syndrome (CFIDS) as a direct outcome of systemic stem cell dysfunction and depletion (Wang et al. 2010), inflammatory responses from bystander signals that exacerbate the injury (Shan et al. 2007), as well as carcinogenesis from the resultant mutations in tissues (Morgan 2003). Therefore, anti-oxidative compounds require additional mechanisms to regulate cellular responses involving signal transduction and bystander signalling that contribute to successful post irradiation intervention. Compounds that intervene in radiation responses post exposure are deemed radiomitigators. The combined mechanisms of radioprotectors and radiomitigators are important in aiding cell survival, and in turn, proliferative capacity that is necessary to repopulate cell depleted tissues. Radiotherapeutic proposals therefore have suggested the antioxidant and cell regulatory capabilities of plant based compounds to rectify and restore function to irradiated cells. Of particular interest are phytochemicals – bioactive, non-nutrient plant secondary metabolites. Phytochemicals demonstrate potent anti-oxidative and anti-inflammatory effects with low cytotoxicity suggesting utility in responses to acute and chronic irradiations (Yamini and Gopal 2010). Additionally, they are easily incorporated as part of a dietary intake. Altogether these qualities render them particularly suitable and attractive screening compounds for examination in the study of dietary radioprotection. Specifically, this study examines the radioprotective and radiomitigative qualities of the phytochemicals – curcumin, andrographolide, and

d-limonene – given their commercial availability and consumer appeal as natural products.

Curcumin, a common phytochemical used mostly in cuisine, is the main medicinal component of the rhizome turmeric and has demonstrated radioprotective effects. Polyphenolic in structure, curcumin bears ketone groups that neutralize free radicals including ROS (Ak and Gülçin 2008) and exposure to curcumin extracts in vitro increased levels of endogenous glutathione in alveolar epithelial cells (Biswas et al. 2005). In multiple studies, curcumin was shown to alleviate the effects of radiation induced cutaneous toxicity from in vivo exposures to clinical doses of γ-rays (Garg et al. 2005). Curcumin and its metabolites are also versatile with demonstrated ability to quell the inflammatory response via downregulation of inflammatory and fibrogenic cytokines (Okunieff et al. 2006) and inhibit carcinogenesis via cell cycle arrest (Chen et al. 1999) in radioactively exposed cells. Like curcumin, andrographolide is another notable medicinal phytochemical that is recognized for its anti-oxidative properties. In the study by Woo et al., andrographolide upregulated glutathione antioxidant activity in cardiomyocytes in response to hypoxic stressors (Woo et al. 2008). The antioxidant capability of andrographolide was demonstrated in vivo via ROS induced modulation of hydrogen peroxide exposures in rat liver cells (Tripathi and Kamat 2007). D-Limonene, another notable and commercial phytochemical, is derived from citrus rinds with applications as an aromatic flavour ingredient in consumables and industrial detergents. Similar to both curcumin and

andrographolide, d- limonene has shown to possess anti oxidative (Grassmann 2005), and anti inflammatory qualities (Evans et al. 1987) that might have utility in radioprotective responses. Notably, the administration of d-limonene to murine lymphocytes in the experiment by Roberto et al. stimulated enzymatic antioxidant activities of catalase in response to hydrogen peroxide treatment and reduced apoptotic responses in cells (Roberto et al. 2010). In another study by Hamada et al, d-limonene conferred immunostimulatory effects in alveolar macrophages in rats (Hamada et al 2002). Therefore, the versatility of cellular and systemic properties of these compounds might contribute properties essential to dietary radioprotection.

The aim of this study is to investigate and characterize the ionizing radiation responses of cells treated with commercially available natural products that might be of value in treatment of radiotherapy side effects or in nuclear emergency situations where populations are exposed to radiation. Specifically, this study is to characterize the radiotherapeutic ratio of phytochemicals – curcumin, andrographolide, and d-limonene – on in vitro cell survival curves. This study utilizes the clonogenic HCT116 p53 wt colon carcinoma cell line because it possesses stem cell-like properties to simulate responses typical of stem cells in tissues. The clonogenic assay is useful in high throughput screens of compounds as it provides the overall effect of compound on cell survival. Therefore, this

study employs the use of clonogenic assays to examine compound effectiveness in radioprotective, radiomitigative, and 'bystander' situations.

Methods

Subculture

HCT116 p53 wt cells were cultured in T75 stock flasks (Falcon, Durham, NC) containing Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Fetal Bovine Serum (FBS), 1000 mM penicillinstreptomycin solution, and 2mM L-glutamine. Cells were maintained in an incubator at 37 ^{o}C with 95% air and 5% CO2. Subculture was routinely performed when cells were 80-100% confluent using a solution of 0.25% Trypsin, Dulbecco's Phosphate Buffered Saline (DPBS), and 1mM Ethylenediaminetetraacetic acid (EDTA) at 37 ^{o}C. Reagents were obtained from Gibco Life Technologies, Grand Island, NY.

Compounds

Curcumin, andrographolide, and d-limonene are lipophilic compounds, therefore each were dissolved in the organic solvent, ethanol, as an intermediate prior to

application into clonogenic flasks to increase solubility and therefore cell bioavailability. Stock containers of curcumin (> 99.9% purity), andrographolide (> 99.9% purity), and d-limonene (>99.9% purity) were purchased from Millipore-Sigma. Working concentrations of curcumin were made in a ratio of 1 mg/mL of 100% ethanol, andrographolide in 1 mg/mL of 100% ethanol, d-limonene miscible in a 10:1 ratio of 100% ethanol. Non-Minimal Inhibitory Concentration (NIC) values – the minimal amount of compound that does not inhibit cell clonogenicity - of the ethanol solvent control and treatments, curcumin, andrographolide, and d-limonene, were calculated at 0.66% volume, 0.4144 μg/mL, 0.7986 μg/mL, 30.83 μg/mL, respectively (Vukmirovic et al. 2017). The final % volumes of ethanol in the curcumin, andrographolide, and d-limonene correspond to values of 0.04%, 0.08%, and 0.31%, which are below that of the NIC value of the ethanol, therefore solvent is not inhibitory to the compounds effects.

Clonogenic Assay

Clonogenic technique used by Puck and Marcus was used for clonogenic survival analysis (Puck and Marcus 1956). Briefly, cells were detached from the flask and were resuspended in medium. Then an aliquot of the cell suspension was counted using a T20 Automated Cell Counter (Bio-Rad Laboratories, Missisauga, ON,

Canada) to determine number of viable cells. After that, 500 cells were plated into each T25 flask (Falcon, Durham, NC) containing cell culture medium. Flasks for radioprotective experiments were incubated immediately with compound NIC values and given 12 hrs incubation prior to radiation exposure. Flasks for radiomitigative experiments were given 12 hrs incubation prior to radiation exposure with administration of compound NIC values at 90 min post irradiation. Flask controls in both radioprotective and radiomitigative experiments were given 'mock injection' of cell culture media to represent administration of compound treatments. Cell cultures were incubated for their clonogenic period of 8 days following irradiation exposures. Cells were stained following the clonogenic period with 25% Carbol Fuschin (RICCA Chemical Company, Arlington, TX) in water and colonies with 50 cells and more were counted.

Radiation Exposure

Cells were exposed to a 32 TeraBequerel Cesium-137 source (Taylor Source, McMaster University, Canada) at a dose rate of 0.250 Gray (Gy)/min at a source to flask distance of 27.5 cm.

Bystander Effect

Procedural details for the methods of this section are adapted from previous publication by our group. Detailed methods for medium transfer experiments are

found in Mothersill and Seymour (Mothersill and Seymour 1997). In brief, cells were detached, resuspended in medium and counted as described for the clonogenic assay. Donor and recipient T25 flasks were seeded with approximately 300,000 cells and 500 cells in 5 ml of cell culture media, respectively, 24 hours prior to irradiation of donor flasks. Cell culture medium from donor flasks were harvested 90 minutes post irradiation and passed through a 0.22 μm Acrodisc® HT Tuffryn membrane sterile filter (Pall Corporation, Ville St. Laurent, Canada) to remove cells and debris from transferred medium. The transferred medium is commonly referred to as irradiated cell condition medium (ICCM). Recipient cell medium was dumped and replaced with ICCM from the respective treatment group donor flasks. Control donor groups were sham irradiated and medium was transferred to recipient cells, serving as a medium transfer control level of survival. Experiments were performed to determine whether compounds in donor and/or recipient flasks modulate bystander signal production. Medium transfer bystander assays were performed with two distinct arrays of bystander experimental schemes: donor cells plated without compounds in medium and the ICCM transferred to recipient cells plated with compounds in media, and donor cells plated with compounds in standard media and the ICCM transferred to recipient cells plated without compounds in media. Donor cells were irradiated at doses of 0.5 Gy, 2 Gy and 5 Gy to generate the ICCM and subsequently transferred to recipient flasks as described. Recipient cell survival receiving ICCM were normalized to control recipient flasks undergoing medium transfers,

however, their respective donor cells were not irradiated. Colonies were stained and counted 8 days following media transfer, using methods as previously described per clonogenic assay section. Cell survival was determined for n = 3 flasks per treatment group.

Statistical Analysis

Direct Irradiation

Values shown on graphs are expressed in ±Standard Deviation (SD). Data are representative of triplicate experiments. Total of three independent experiments were conducted with three replicates per experiment (n=3). Values are normalized to absolute non-solvent controls. Derivation of the two parameters, α and β, represent the results of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation Y=exp[-1*(α*X + β*X^2)], where α and β represent the linear and quadratic portions of the curve respectively. The linear portion of the curve corresponds to reductions in cell survival from single interactions of radiation with pertinent cellular targets, whereas the quadratic portion corresponds to multiple radiation interactions with pertinent cellular targets. The ratio α/β represents the dose at which the contribution to reductions in cell survival from both the linear and quadratic components are equal in effect. A Two-Way Analysis of Variance (ANOVA) and Dunnett's Multiple Comparisons Test are used to consider significant value for

treatment effectiveness. A confidence interval of 95% and p-value of < 0.05 was selected to be statistically significant. Results were obtained using GraphPad Prism 7 software package.

Bystander Effect

Values shown on graphs are expressed in ±SD. Data are representative of triplicate experiments. Total of three independent experiments were conducted with three replicates per experiment (n=3). Values are normalized to absolute non-solvent controls. A One-Way Analysis of Variance (ANOVA) and Fisher's Least Significant Difference (LSD) Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Results were obtained using GraphPad Prism 7 software package.

Results

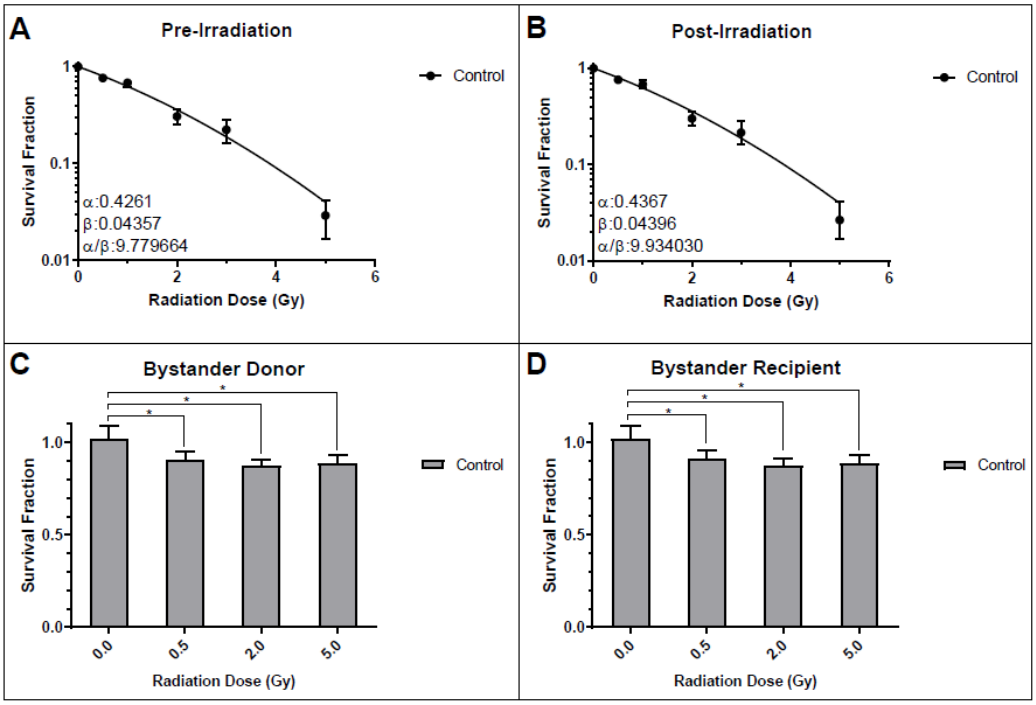

Fig. 1 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Control HCT116 p53 wt survival data on pre-irradiation ‘mock injection’ of medium control treatment on direct irradiation Cesium-137

exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Control HCT116 p53 wt survival data on post-irradiation 'mock injection' of medium control treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Control HCT116 p53 wt survival data on compound 'mock injection' donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Control HCT116 p53 wt survival data on compound 'mock injection' recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. * Units of dose are in Gray (Gy).

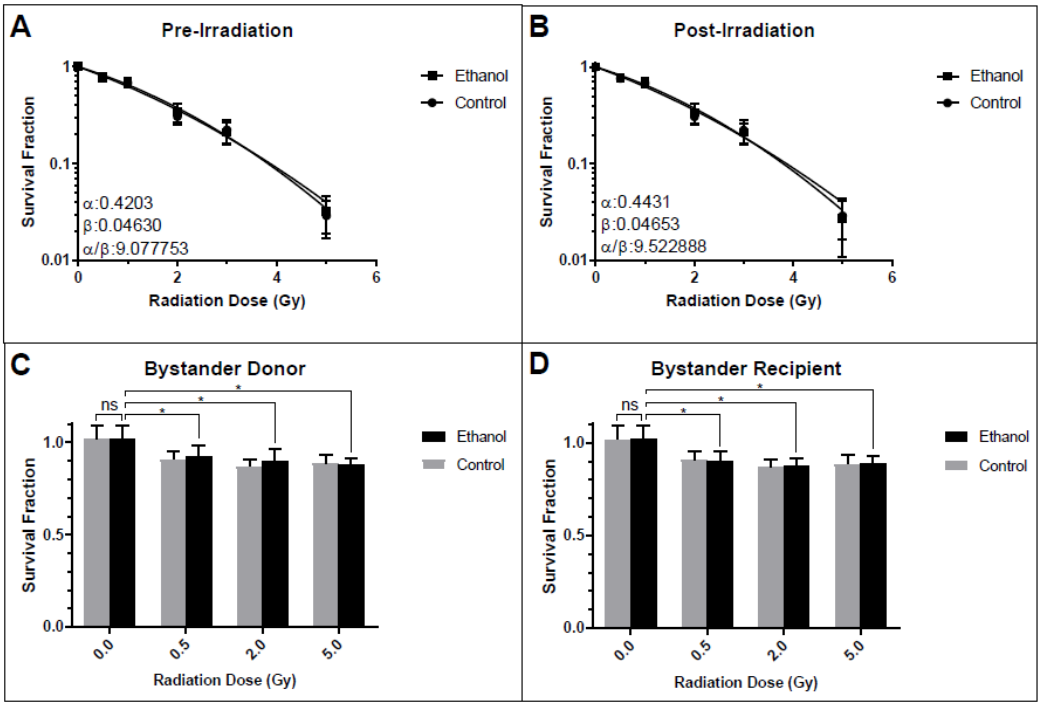

Fig. 2 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC solvent (ethanol) treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC solvent (ethanol) treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell

survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC solvent (ethanol) treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC solvent (ethanol) treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * Units of dose are in Gray (Gy).

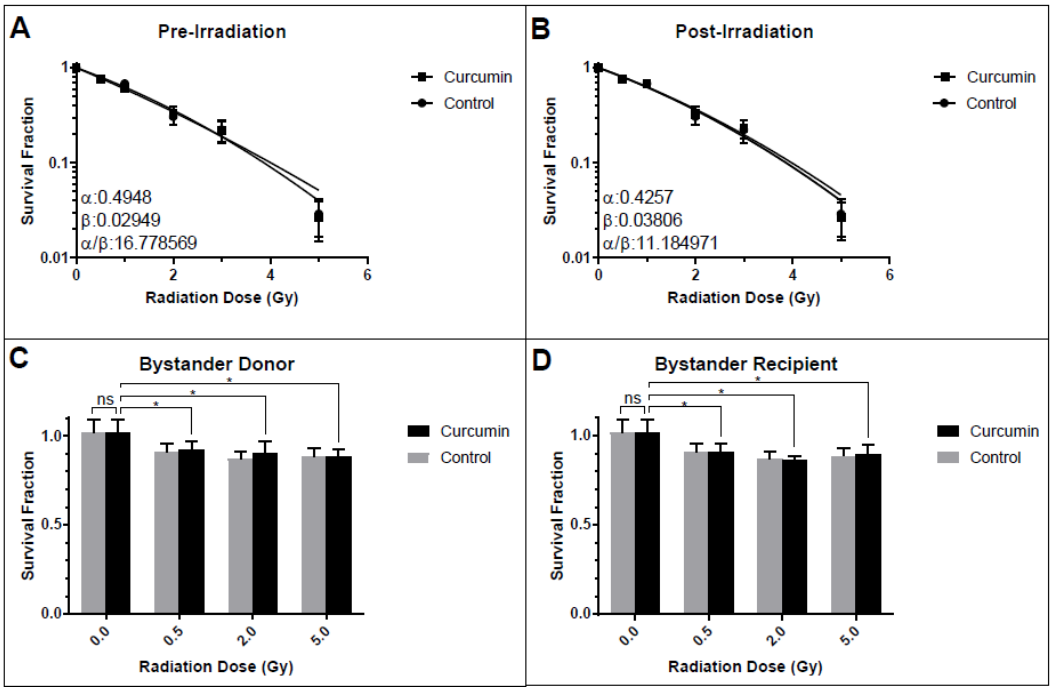

Fig. 3 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC curcumin treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC curcumin treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation Y=exp[-1*(α*X + β*X^2)]. (C) Comparison of

HCT116 p53 wt survival data between control and NIC curcumin treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC curcumin treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * Units of dose are in Gray (Gy).

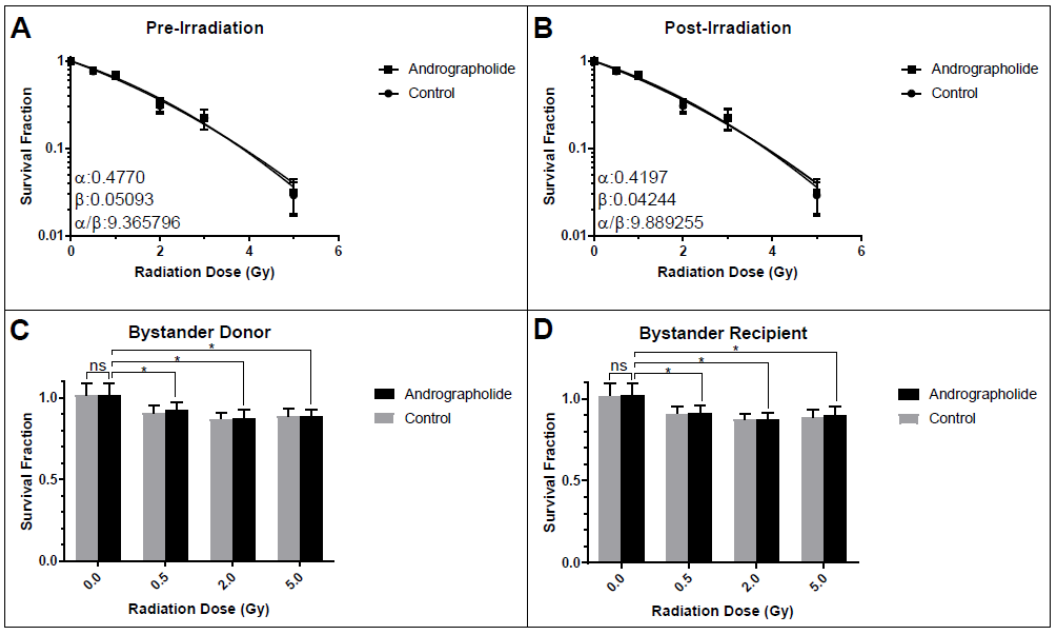

Fig. 4 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC andrographolide treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC andrographolide treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC andrographolide treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC andrographolide treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. *

'NIC' refers to 'Non-minimal Inhibitory Concentration'. * Units of dose are in Gray (Gy).

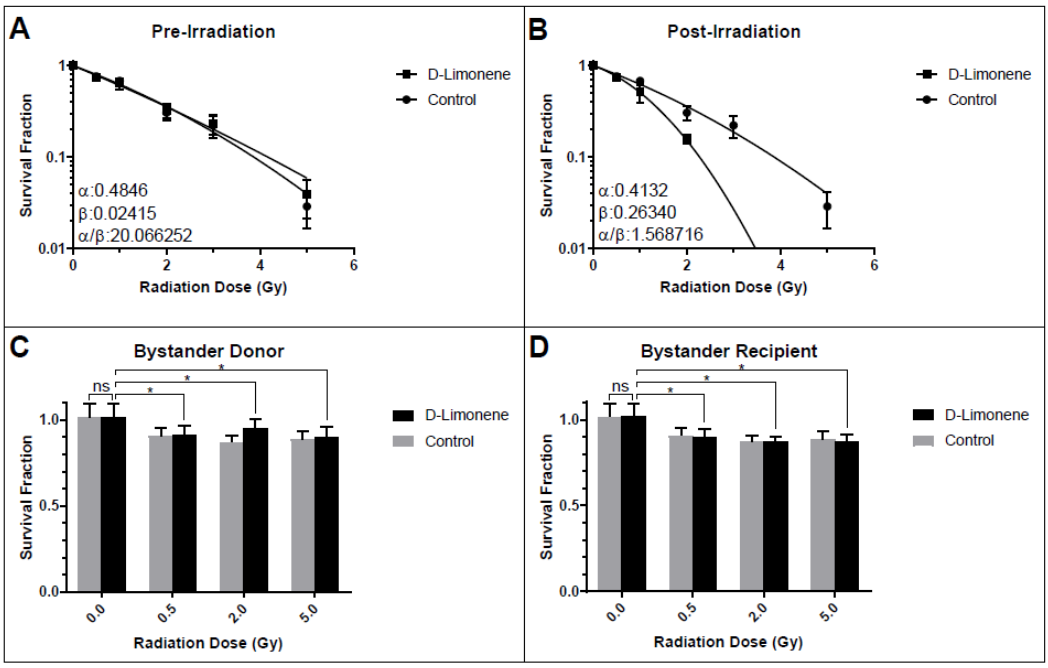

Fig. 5 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC d-limonene treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC d-limonene treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear

regression analysis of the data fitted to the linear quadratic function of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC d-limonene treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC d-limonene treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * Units of dose are in Gray (Gy).

Figure 1 Placement

Figure 2 Placement

Figure 3 Placement

Figure 4 Placement

Figure 5 Placement

Figures 1-5 represent the dose response profiles of the control, ethanol solvent control and treatment groups consisting of the phytochemicals - curcumin, andrographolide, and d-limonene - on the HCT116 p53 wt cell line and graphically describe the derivation of the tabulated results. The tabulated results in Tables 1-8 display the significance of the results in accordance with the figures to analyze the effectiveness of the solvent ethanol control and treatment compounds. From Fig. 2 and Tables 1, 2, 7, and 8, the NIC of ethanol solvent control demonstrates no significant effect on the radioprotective, radiomitigative, and bystander modulatory effects of the experiment therefore excluding its involvement in the radioresponsiveness of the cells in the compound treatments. Table 3 alongside Figures 3(A), 4(A), and 5(A), shows the radioprotective effectiveness of the compounds demonstrating that curcumin, andrographolide, and d-limonene display no significant radioprotection from the compounds at external beam doses of 0.5 Gy, 1 Gy, 2 Gy, 3 Gy and 5 Gy of dose. Despite the lack of radioprotective effect, the curcumin and d-limonene demonstrate notable increases in the α/β ratio of approximately ~ 1.71 fold and ~ 2 fold respectively, therefore demonstrating shifts in the contribution of the quadratic to the linear portion of the cell survival curves. From Table 4 alongside Figures 3(B), 4(B), and 5(B) it is evident that there are no radiomitigative effects from administration of the treatments at 0.5 Gy, 1 Gy, 2 Gy, 3 Gy, and 5 Gy doses. In contrast, d-limonene possesses high significance in radiosensitization effects post irradiation at 1 Gy, 2Gy, 3 Gy, and 5 Gy doses with notable reduction of approximately ~6

fold in the α/β ratio value. The curcumin does produce minor increase of approximately ~1.12 fold on the α/β ratio suggesting that there is modulation in the contribution of the quadratic to the linear portion of the radiomitigative cell survival curve though no significant effects in comparison to control treatments. Similarly, Tables 7 and 8 alongside the corresponding Figures 3(CD), 4(CD), and 5(CD) demonstrate that the compounds do not intervene in the processes of bystander signal production in the donor flasks despite radioprotective effects in direct irradiations, as well as inhibitory receipt of the signal in the recipient flasks at each dose exposure.

Table 1 Placement

Table 2 Placement

Table 3 Placement

Table 4 Placement

Table 5 Placement

Table 6 Placement

Table 7 Placement

Table 8 Placement

Discussion

Exposures to acute high dose radioactivity cause pathogenic oxidative stress in cells that contribute to acute radiation injury as well as chronic systemic issues that include: exacerbation of inflammatory responses (Gough et al. 2013), genomic instability, and tissue dysfunction from depletion of viable cells (Morgan, W 2003). Therefore, radioprotective compounds have to satisfy criteria such as low toxicity, frequent intake and practical route of administration to protect cells from the acute and chronic effects of exposures (Lee et al. 2008). Unlike conventional radioprotective cysteamine compounds, these criteria align well with natural phytochemical compound supplements which might serve as effective chemical radioprotection to directly irradiated and 'bystander' cells. In this study, treatments of curcumin, andrographolide, and d-limonene were applied to the clonogenic HCT116 p53 wt cell line to screen compounds to assess cell viability as an indicator of radioprotective, radiomitigative, and inhibitory bystander effects. The results of the radioprotective assessment of the curcumin, andrographolide, and d-limonene, on the basis of cell clonogenicity, demonstrate that the compounds do not confer radioprotective qualities at acute high dose exposures (≥0.5 Gy). Though the curcumin and d-limonene slightly alter the linear and quadratic parameters of the pre-irradiation non-linear regression analysis via an increase in the α/β ratio suggesting some radioprotection, these

compounds do not confer statistically significant pre-irradiation, post-irradiation and bystander inhibitory radioprotection from exposure. Contrary to radioprotective effects, the d-limonene significantly radiosensitizes the cells post irradiation throughout the dose range of exposures with an accompanying reduction in the α/β ratio value. Therefore, in the context of this experiment, despite reports of antioxidant activity (Ak and Gülçin 2008; Garg et al. 2005; Grassmann 2005), the phytochemicals do not possess sufficient radioprotection to aid cell survival at acute high dose exposures in terms of cell clonogenicity. Despite contrary reports of radiosensitization of cancerous cells to curcumin and andrographolide compounds (Garg et al. 2005; Nantajit et al. 2017), neither of these demonstrates radiomodulatory in vitro effects on this particular carcinoma stem cell-like line. The lack of radiomodulatory effects of the curcumin and andrographolide on the cells might be in part from the low NIC values of the compounds which hinders sufficient dose dependent activity in chemical reactions.

The compounds also demonstrate no interventional response in the radiation induced bystander effect - this suggests no modulatory effect of the compounds to bystander cell signalling in the radiation response. Despite this outcome, the beneficial as well as negative contribution of the bystander effect to the cellular response is context dependent to the exposure (Mothersill and Seymour 2005). Therefore it might be difficult to ascertain whether an important criterion of radioprotection is inhibition of bystander responses. This is because cellular

communication via radiation induced bystander signalling might be beneficial to recovery responses in exposures to low doses and it might also be negative in that overt localized high doses of radiation elicit inflammatory responses that might necessitate interventional compounds (Shan et al. 2007). In the case of the negative impacts, the direct radioprotective effect of these compounds might suffice to quell these negative responses therefore not require an intervention/inhibition of bsytander effects. Otherwise, the use of compounds to modulate cellular signalling requires very specific targets to promote certain responses. The responses from bystander effects might have multitudes of targets important to cell function; therefore these are susceptible to exacerbation of issues from interventional compounds. Thus, in terms of the natural phytochemical radioprotector, an important requirement might be that it has no effect on bystander mechanisms in order to be effective.

Despite these results, the d-limonene might have utility in the field of radiotherapy wherein the use of radiosensitizors to target cancerous cells is of use. The administration of the d-limonene at the NIC compound dose might be clinically effective as it is non-toxic to cells except to those that undergo high dose irradiation impacting only those cells that require elimination from the exposure. Unlike other radiosensitizors, the d-limonene treatment of cells does not demonstrate sensitivity pre-irradiation; therefore it is not pro-oxidative in effect which promotes the production of harmful ROS within the cells. The chemical basis of d-limonene sensitization rather suggests the compound might serve to

promote an apoptotic response in directly irradiated cells following exposure. Given the HCT116 p53 wt cell line is p53 competent, the cell line is able to undergo an apoptotic response to stressors including ionizing radiation. Coinciding with its use as an industrial detergent, the chemical mechanism of radiosensitization might suggest that d-limonene chemically promotes permeation of the cell membrane and subcellular organelles that aid in the release of caspases in an apoptotic response (Rabi and Bishayee 2009). Additionally, d-limonene is an effective anticarcinogenic compound that inhibits the expression of RAS oncogene in malignant cells (Karlson et al. 1996). Given this cell line possesses mutation in RAS the d-limonene might act in part to inhibit the proliferative effect of this mitogenic protein in these cells. However, this study addresses only the basic screening of radioprotective candidate compounds on the single HCT116 p53 wt cell line. Therefore, the clinical utility of the phytochemicals – curcumin, andrographolide, and d-limonene - in various aspects of radioprotection and radiosensitization warrants an additional extensive elucidation of these molecular mechanisms with respect to various cell lines.

Conclusion

Chemical radioprotection from ionizing radiation requires practical oral route of administration, low toxicity and frequent dietary intake to confer persistent protection to cells. Therefore, natural plant derived compounds are particularly suitable as radioprotective candidates that satisfy these criteria. Despite the antioxidant and anti-inflammatory capabilities of an abundance of natural compounds, only few possess enough kinetic reactivity to act as potent free radical scavengers in radiation exposures to mitigate chronic oxidative stress in directly irradiated and 'bystander' cells. In this study, the notable phytochemicals – curcumin, andrographolide, d-limonene - were tested for their radioprotective, radiomitigative and bystander effect inhibiting abilities on the clonogenic survival of the HCT116 p53 wt cell line. The phytochemicals do not confer chemical radioprotective, radiomitigative and bystander effect inhibitory capabilities on clonogenic cell survival. Therefore, interventional administration at the cellular level is insufficient to protect from acute exposures suggesting that in vivo administration is of low utility. Contrary to radioprotection, d-limonene demonstrates radiosensitization which might have use in targeted radiotherapy of carcinoma cells otherwise is not applicable to radioprotection of non-carcinoma cells. Despite these results, additional anti-inflammatory and anti-carcinogenic

properties of these compounds warrant research given these qualities possess some utility in dietary radioprotection.

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Disclosure Statement

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Tables

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Ethanol	P = 0.9996	P = 0.8920	P = 0.9979	P = 0.9496	P = 0.9941	P = 0.9998
	ns	ns	ns	ns	ns	ns

Table 1: P-Value Chart displays the results of solvent control administration pre-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Ethanol	P = 0.9999	P = 0.9980	P = 0.9795	P = 0.9695	P = 0.9856	P = 0.9987
	ns	ns	ns	ns	ns	ns

Table 2: P-Value Chart displays the results of solvent control administration post-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Curcumin	P = 0.9999	P = 0.9953	P = 0.4067	P = 0.9643	P = 0.9999	P = 0.9999
	ns	ns	ns	ns	ns	ns
Andrographolide	P = 0.9994	P = 0.9774	P = 0.8442	P = 0.9943	P = 0.9999	P = 0.9999
	ns	ns	ns	ns	ns	ns
D-Limonene	P = 0.9998	P = 0.9898	P = 0.3016	P = 0.9146	P = 0.9953	P = 0.9972
	ns	ns	ns	ns	ns	ns

Table 3: P-Value Chart displays the results of compound administration pre-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Curcumin	P = 0.9989	P = 0.9965	P = 0.9629	P = 0.9899	P = 0.9999	P = 0.9999
	ns	ns	ns	ns	ns	ns
Andrographolide	P = 0.9998	P = 0.9999	P = 0.9799	P = 0.9959	P = 0.9999	P = 0.9999
	ns	ns	ns	ns	ns	ns
D-Limonene	P = 0.9999	P = 0.8975	P = 0.0157	P < 0.0001	P < 0.0001	P < 0.0001
	ns	ns	*	****	****	****

Table 4: P-Value Chart displays the results of compound administration post-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect		
	0.5	2	5
Control	P = 0.0150	P = 0.0127	P = 0.0190
	*	*	*

Table 5: P-Value Chart displays the results of medium control administration in donor bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect		
	0.5	2	5
Control	P = 0.0179	P = 0.0159	P = 0.0157
	*	*	*

Table 6: P-Value Chart displays the results of medium control administration in recipient bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effects			
	0	0.5	2	5
Ethanol	P = 0.9999	P = 0.0396	P = 0.0379	P = 0.0173
	ns	*	*	*
Curcumin	P = 0.9999	P = 0.0180	P = 0.0449	P = 0.0112
	ns	*	*	*
Andrographolide	P = 0.9995	P = 0.0360	P = 0.0176	P = 0.0197
	ns	*	*	*
D-Limonene	P = 0.9998	P = 0.0197	P = 0.0327	P = 0.0153
	ns	*	*	*

Table 7: P-Value Chart displays the results of solvent control and compound administration in donor bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effects			
	0	0.5	2	5
Ethanol	P = 0.9997	P = 0.0150	P = 0.0379	P = 0.0173
	ns	*	*	*
Curcumin	P = 0.9989	P = 0.0198	P = 0.0167	P = 0.0199
	ns	*	*	*
Andrographolide	P = 0.9998	P = 0.0190	P = 0.0179	P = 0.0197
	ns	*	*	*
D-Limonene	P = 0.9999	P = 0.0177	P = 0.0117	P = 0.0119
	ns	*	*	*

Table 8: P-Value Chart displays the results of solvent control and compound administration in recipient bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

Chapter 5: Characterization of Radioprotective, Radiomitigative and Bystander Signaling Modulating Effects of Endogenous Metabolites – Phenylacetate, Ursodeoxycholate and Tauroursodeoxycholate – on HCT116 Human Colon Carcinoma Cell Line

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Characterization of Radioprotective, Radiomitigative and Bystander Signalling Modulating Effects of Endogenous Metabolites – Phenylacetate, Ursodeoxycholate, and Tauroursodeoxycholate – on HCT116 Human Colon Carcinoma Cell Line

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Exposures to ionizing radiation are able to deplete stem cell reservoirs as well as lead to chronic injury processes that exacerbate carcinogenic and inflammatory responses. Therefore, radioprotective measures that protect from both the acute and chronic biological effects of radiation require frequent intake and practical oral route of delivery of non toxic natural products. The aim of this study was to

characterize the radioprotective, radiomitigative, and radiation induced bystander effect inhibiting properties of endogenous metabolites – phenylacetate, ursodeoxycholate, and tauroursodeoxycholate. This was accomplished through compound administration pre- and post-irradiation as well as in donor and recipient bystander flasks to analyze whether these might represent suitable compounds to confer adequate protection from injury as well as facilitate recovery from the exposures. The clonogenic HCT116 p53 wt cancer cell line in this study shares characteristics of stem cells, such as high reproductive viability, that is an effective marker to demonstrate compound effectiveness. Clonogenic assays were therefore used to characterize radioprotective, radiomitigative, and bystander inhibiting properties of treatment compounds whereby cellular responses to irradiation were quantified with macroscopic colony counts to measure cell survival of flasks. The results demonstrate that the phenylacetate and tauroursodeoxycholate demonstrate statistical significance in administration pre-irradiation that confers radioprotection up to 2 Gy of dose, whereas administration post-irradiation and in bystander experiments does not confer radioprotection in vitro. Therefore, phenylacetate and tauroursodeoxycholate might be effective radioprotectors though offer no radiomitigative properties.

INTRODUCTION

Exposures to acute high dose ionizing radiation depletes stem cell reservoirs within organs and therefore impairs the ability of these organ systems to recover via repopulation of clonogenic cells that are vital to proper tissue function [*1,2*]. These acute damages only represent the immediate effects of radiation as there are important residual effects from exposures that contribute to the development of systemic issues post irradiation [*3,4,5*]**.** The inability of these clonogens, particularly those of the hematopoietic and gastrointestinal system, to re-establish tissue function is the basis of effects of several post-irradiation syndromes such as Chronic Fatigue Immune Dysfunction Syndrome (CFIDS) whereby the dysfunction of the tissue causes systemic inflammatory issues throughout the affected individual [*6,7,8*]. The mutagenic chronic effects of radioactive exposures also promote carcinogenesis within repopulating clonogens that are responsible in tissue-specific and systemic function [*9*]. Though these side effects are attributable mostly to acute high dose exposures in clinical radiotherapy and nuclear emergencies, chronic exposures to low dose radioisotopes might also exacerbate similar responses [*10*]. Therefore, radioprotection from these various exposure scenarios necessitates development of radioprotectors that confer persistent protection from multiple aspects of radiation damage including those

from immediate direct effects of exposures (i.e cell survival) as well as those that are consequent to the exposures (i.e carcinogenesis and inflammation).

Radioprotectors are compounds that confer protection from the radiation-chemical interactions within the cell medium, primarily free radical production, which are the primary injury to organelles and cellular molecules [*11, 12, 13*]. These compounds must be in the cellular medium at the course of irradiation to effectively scavenge free radicals and, warranted the compounds possess sufficient kinetic reactivity to initiate these neutralization reactions, deplete the excess reactive oxygen species (ROS) that might cause oxidative stress within cells [*14, 15, 16*]. Despite the elucidation of synthetic radioprotective compounds that possess these free radical scavenging abilities, these compounds show toxic side effects that only demonstrate effectiveness via intravenous administration in vivo which render them highly impractical to radioprotection [*17*]. Therefore, development of compounds that address this issue require that the compound be highly practical in terms of toxicity and administration. The use of natural endogenous product supplements addresses these issues because of lack of side effects as well as oral route of consumption. In this regard, natural compound radioprotectors are practical in route of administration without significant side effects that might otherwise cause issues in the administration of the compound as is the case of conventional cysteamine radioprotective compounds [*18*].

Though the primary target of radioprotection is chemical intervention of free radicals, compounds might also facilitate recovery of cells, therefore mediate cellular processes that inhibit injury as well as mitigate the damage of exposures [*19*]. Thus, these compounds might also act as radiomitigators, which are compounds that are useful in post irradiation scenarios. Radioprotective candidates also preferably facilitate proper regulation of 'bystander cells', that is - cells that are not directly in the radiation field though contribute to the responses via cellular communication - to allow for proper cell function in the radioactive environment to mitigate negative effects of bystander signalling, carcinogenesis as well as inflammatory processes that occur over the course of as well as consequent to the exposure [*20*]. Radioprotection requires consideration of the acute and chronic aspects of radiation exposures, therefore the use of anti-oxidative, anti-carcinogenic, and anti-inflammatory natural products is subject to intense study given their ability to satisfy these criteria. The aim of this study is to investigate the radioprotective, radiomitigative as well interventional bystander effect responses of cells to the endogenous metabolite compounds – phenylacetate, ursodeoxycholate, tauroursodeoxycholate - that might be of some use to the field. The selection of the compounds is centred on the basis that these specific chemicals are antioxidants and are able to alleviate the symptoms of radiation induced CFIDS cases [*21*]. These experiments also extend on the notion of dietary radioprotection as prior studies in our lab suggest the ability of these

compounds to function as anti-carcinogenic compounds which are also of utility in radiation exposures [*21*].

The emphasis of the current study is on dietary radioprotection, therefore, intestinal cell lines are suitable to this particular study so as to demonstrate applicability on in vivo natural clonogenic cells. This study utilizes the HCT116 p53 wild-type (wt) intestinal cell line because it possesses stem cell properties such as high reproductive viability that render it suitable in this particular experiment. Human intestinal cell lines that have a normal diploid karyotype, bear a naturally stable wild-type TP53 gene and p53-mediated signalling cascades, have high cloning efficiencies, and demonstrate properties of stem cells, are therefore preferable. However, human normal intestinal epithelial cell lines have very low cloning efficiencies, become heteroploidy, acquire mutations in the TP53 gene with impaired p53-mediated functions and acquire tumorigenic characteristics [*22,23,24*]. In contrast, despite having been originated from a primary colorectal carcinoma sample, the HCT116 p53 wt meets the desirable criteria described above: it has a normal diploid karyotype, has a naturally stable wild-type p53, has key functional p53-mediated pathways, has a high cloning efficiency, and exhibit properties of stem-like cells [*23,25,26,27*]. In fact, HCT116 p53 wt lacks CDX1 expression, a marker of intestinal epithelial

differentiation [*28*]. Taken together, the use of HCT116 p53 wt was appropriate in our present study.

MATERIALS AND METHODS

Subculture

HCT116 p53 wt cells were cultured in T75 stock flasks (Falcon, Durham, NC) containing Roswell Park Memorial Institute 1640 (RPMI 1640) medium, Fetal Bovine Serum (FBS), 1000 mM penicillinstreptomycin solution, and 2mM L-glutamine. Cells were maintained in an incubator at 37 ^{o}C with 95% air and 5% CO2. Subculture was routinely performed when cells were 80-100% confluent using a solution of 0.25% Trypsin, Dulbecco's Phosphate Buffered Saline (DPBS), and 1mM Ethylenediaminetetraacetic acid (EDTA) at 37 ^{o}C. Reagents were obtained from Gibco Life Technologies, Grand Island, NY.

Compounds

Phenylacetate, ursodeoxycholate and tauroursodeoxycholate are ambiphilic compounds, therefore each were dissolved in the organic solvent, ethanol, as an intermediate prior to application into clonogenic flasks to increase solubility and therefore cell bioavailability. There are reports in the literature of solvents, such as ethanol, that might demonstrate some radioprotective effects at certain solvent doses [29]. Therefore, to dismiss this confounding factor in our experiments requires the test of an additional ethanol solvent control that demonstrates no significant effects on our cell lines. Stock containers of phenylacetate (> 99.9% purity), ursodeoxycholate (> 99.9% purity), and tauroursodeoxycholate (>99.9% purity), were purchased from Millipore-Sigma. Working concentrations of phenylacetate were made in a 10:1 ratio of 100% ethanol, tauroursodeoxycholate in a ratio of 10 mg/mL of 100% ethanol, and ursodeoxycholate in 10 mg/mL of 100% ethanol. Non-Minimal Inhibitory Concentration (NIC) values – the minimal amount of compound that does not inhibit cell clonogenicity - of the ethanol solvent control and treatments, phenylacetate, ursodeoxycholate and tauroursodeoxycholate, were calculated at 0.66% volume, 10.08 μg/mL, 32.56 μg/mL, and 64.39 μg/mL respectively [21]. The final % volumes of ethanol in the phenylacetate, ursodeoxycholate, and tauroursodeoxycholate treatments correspond to values of 0.11%, 0.33%, and 0.64%, which are below that of the NIC value of the ethanol, therefore solvent is not inhibitory to the effects of the compounds.

Clonogenic Assay

Clonogenic technique used by Puck and Marcus was used for clonogenic survival analysis [30]. Briefly, cells were detached from the flask and were resuspended in medium. Then an aliquot of the cell suspension was counted using a T20 Automated Cell Counter (Bio-Rad Laboratories, Missisauga, ON, Canada) to determine number of viable cells. After that, 500 cells were plated into each T25 flask (Falcon, Durham, NC) containing cell culture medium. Flasks for radioprotective experiments were incubated immediately with compound NIC values and given 12 hrs incubation prior to radiation exposure. Flasks for radiomitigative experiments were given 12 hrs incubation prior to radiation exposure with administration of compound NIC values at 90 min post irradiation. Flask controls in both radioprotective and radiomitigative experiments were given 'mock injection' of cell culture media to represent administration of compound treatments. Cell cultures were incubated for their clonogenic period of 8 days following irradiation exposures. Cells were stained following the clonogenic period with 25% Carbol Fuschin (RICCA Chemical Company, Arlington, TX) in water and colonies with 50 cells and more were counted.

Radiation Exposure

Cells were exposed to a 32 TeraBequerel Cesium-137 source (Taylor Source, McMaster University, Canada) at a dose rate of 0.250 Gray (Gy)/min at a source to flask distance of 27.5 cm.

Bystander Effect

Procedural details for the methods of this section are adapted from previous publication by our group. Detailed methods for medium transfer experiments are found in Mothersill and Seymour (31). In brief, cells were detached, resuspended in medium and counted as described for the clonogenic assay. Donor and recipient T25 flasks were seeded with approximately 300,000 cells and 500 cells in 5 ml of cell culture media, respectively, 24 hours prior to irradiation of donor flasks. Cell culture medium from donor flasks were harvested 90 minutes post irradiation and passed through a 0.22 μm Acrodisc® HT Tuffryn membrane sterile filter (Pall Corporation, Ville St. Laurent, Canada) to remove cells and debris from transferred medium. The transferred medium is commonly referred to as irradiated cell condition medium (ICCM). Recipient cell medium was dumped and replaced with ICCM from the respective treatment group donor flasks. Control donor groups were sham irradiated and medium was transferred to recipient cells, serving as a medium transfer control level of survival. Experiments were performed to determine whether compounds in donor and/or recipient flasks

modulate bystander signal production. Medium transfer bystander assays were performed with two distinct arrays of bystander experimental schemes: donor cells plated without compounds in medium and the ICCM transferred to recipient cells plated with compounds in media, and donor cells plated with compounds in standard media and the ICCM transferred to recipient cells plated without compounds in media. Donor cells were irradiated at doses of 0.5 Gy, 2 Gy and 5 Gy to generate the ICCM and subsequently transferred to recipient flasks as described. Recipient cell survival receiving ICCM were normalized to control recipient flasks undergoing medium transfers, however, their respective donor cells were not irradiated. Colonies were stained and counted 8 days following media transfer, using methods as previously described per clonogenic assay section. Cell survival was determined for n = 3 flasks per treatment group.

Statistical Analysis

Direct Irradiation

Values shown on graphs are expressed in ±Standard Deviation (SD). Data are representative of triplicate experiments. Total of three independent experiments were conducted with three replicates per experiment (n=3). Values are normalized to absolute non-solvent controls. Derivation of the two parameters, α

and β, represent the results of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation Y=exp[-1*(α*X + β*X^2)], where α and β represent the linear and quadratic portions of the curve respectively. The linear portion of the curve corresponds to reductions in cell survival from single interactions of radiation with pertinent cellular targets, whereas the quadratic portion corresponds to multiple radiation interactions with pertinent cellular targets. The ratio α/β represents the dose at which the contribution to reductions in cell survival from both the linear and quadratic components are equal in effect. A Two-Way Analysis of Variance (ANOVA) and Dunnett's Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 95% and p-value of < 0.05 was selected to be statistically significant. Results were obtained using GraphPad Prism 7 software package.

Bystander Effect

Values shown on graphs are expressed in ±SD. Data are representative of triplicate experiments. Total of three independent experiments were conducted with three replicates per experiment (n=3). Values are normalized to absolute non-solvent controls. A One-Way Analysis of Variance (ANOVA) and Fisher's Least Significant Difference (LSD) Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of

90% and p-value of < 0.05 was selected to be statistically significant. Results were obtained using GraphPad Prism 7 software package.

RESULTS

Figures 1-5 represent the dose response profiles of the control, ethanol solvent control and treatment groups consisting of the endogenous metabolites - phenylacetate, ursodeoxycholate, and tauroursodeoxycholate - on the HCT116 p53 wt cell line and graphically describe the derivation of the tabulated results. The tabulated results in Tables 1-8 display the significance of the results in accordance with the figures to analyze the effectiveness of the ethanol solvent control and treatment compounds. From Fig. 2 and Tables 1, 2, 7, and 8, the NIC of ethanol solvent control demonstrates no significant effect on the radioprotective, radiomitigative, and bystander modulatory effects of the experiment therefore excluding its involvement in the radioresponsiveness of the cells in the compound treatments. Table 3 alongside Figures 3(A), 4(A), and 5(A), shows the radioprotective effectiveness of the compounds demonstrating that phenylacetate displays high significance up to 2 Gy of external dose, ursodeoxycholate demonstrates no significance, and the tauroursodeoxycholate demonstrates low significance at 2 Gy of external dose with no significant radioprotection from the compounds at 3 Gy and 5 Gy of dose. Additionally, the phenylacetate and tauroursodeoxycholate demonstrate notable reductions in the

α/β ratio of approximately ~12 fold and ~2 fold respectively, therefore demonstrating shifts in the contribution of the linear to the quadratic portion of the cell survival curves. From Table 4 alongside Figures 3(B), 4(B), and 5(B) it is evident that there are no radiomitigative effects from administration of the phenylacetate, ursodeoxycholate and tauroursodeoxycholate treatments at 0.5 Gy, 1 Gy, 2 Gy, 3 Gy, and 5 Gy doses. The tauroursodeoxycholate does produce minor increase of approximately ~1.26 fold on the α/β ratio suggesting that there is modulation in the contribution of the quadratic to the linear portion of the radiomitigative cell survival curve though no significant effects in comparison to control treatments. Similarly, Tables 7 and 8 alongside the corresponding Figures 3(CD), 4(CD), and 5(CD) demonstrate that the compounds do not intervene in the processes of bystander signal production in the donor flasks despite radioprotective effects in direct irradiations, as well as inhibitory receipt of the signal in the recipient flasks at each dose exposure.

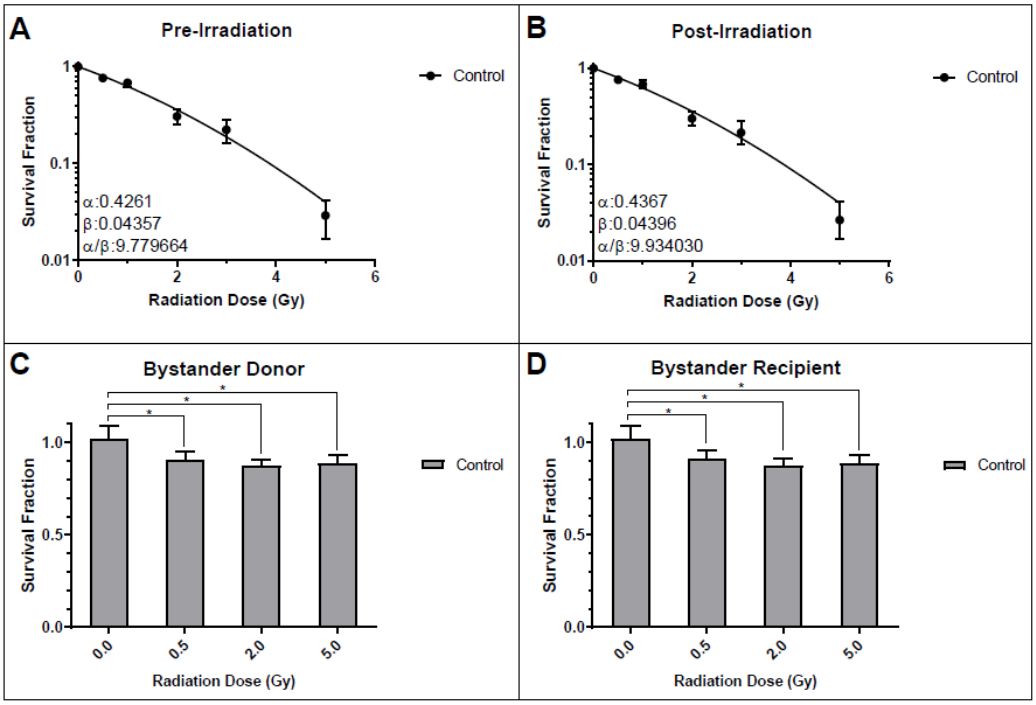

Fig. 1 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Control HCT116 p53 wt survival data on pre-irradiation 'mock injection' of medium control treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Control HCT116 p53 wt survival data on post-irradiation 'mock injection' of medium control treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell

survival according to equation Y=exp[-1*(α*X + β*X^2)]. (C) Control HCT116 p53 wt survival data on compound 'mock injection' donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Control HCT116 p53 wt survival data on compound 'mock injection' recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. * Units of dose are in Gray (Gy).

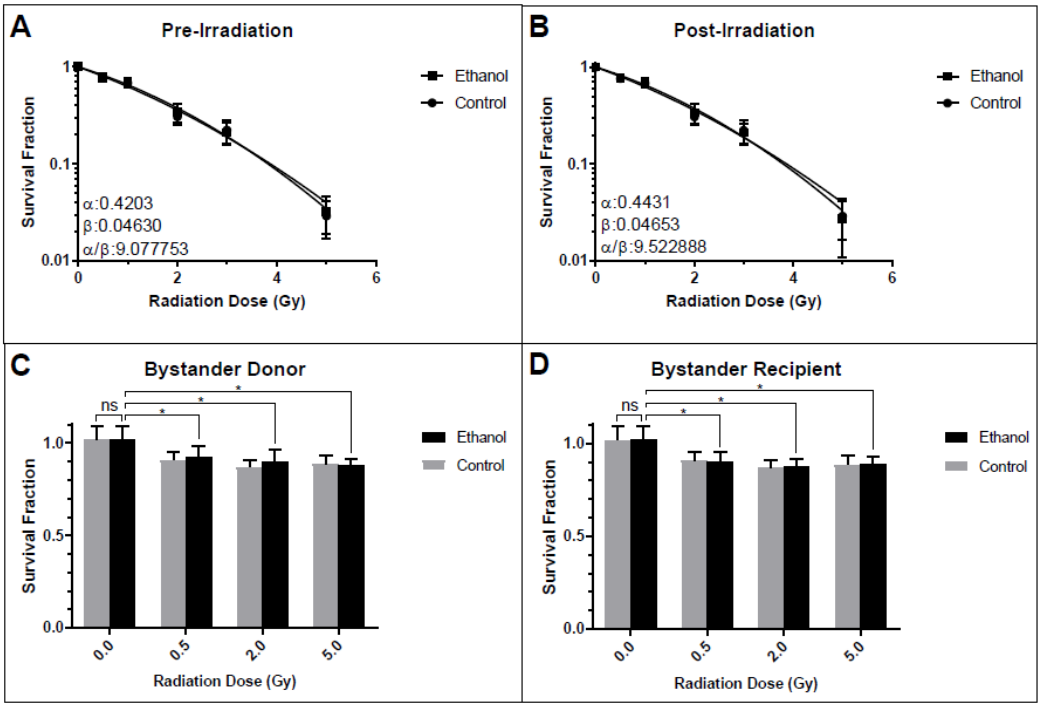

Fig. 2 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC solvent (ethanol) treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC solvent (ethanol) treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC solvent (ethanol) treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC solvent (ethanol) treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. *

'NIC' refers to 'Non-minimal Inhibitory Concentration'. * Units of dose are in Gray (Gy).

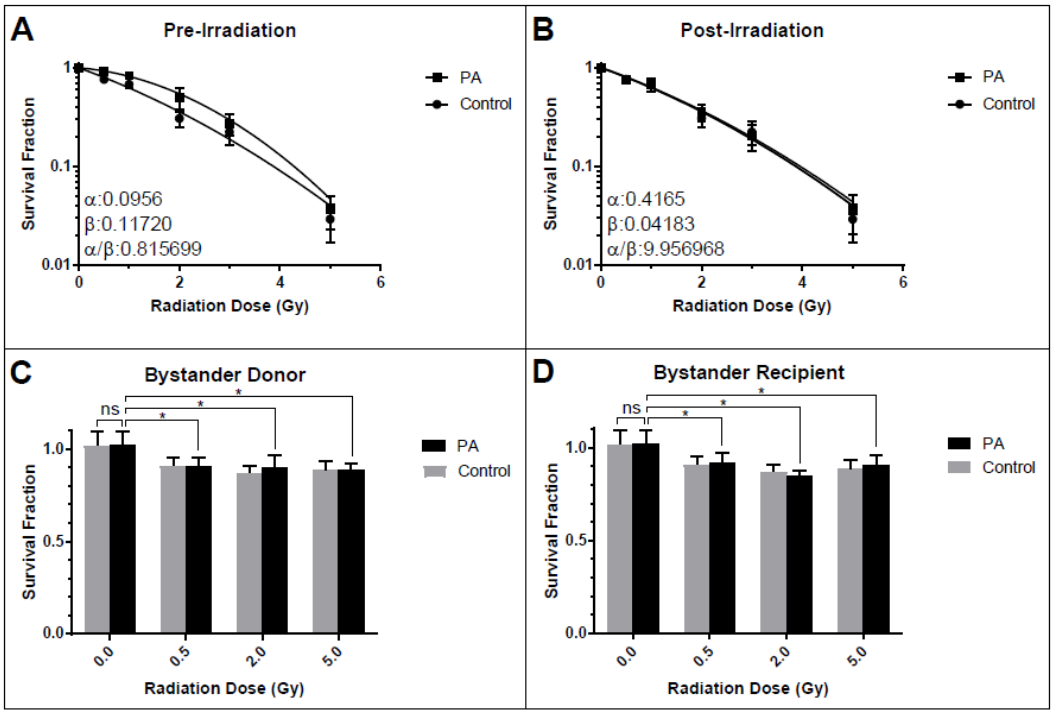

Fig. 3 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC phenyacetate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC phenylacetate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs

(A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC phenylacetate treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC phenylacetate treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * 'PA' refers to 'phenylacetate'. * Units of dose are in Gray (Gy).

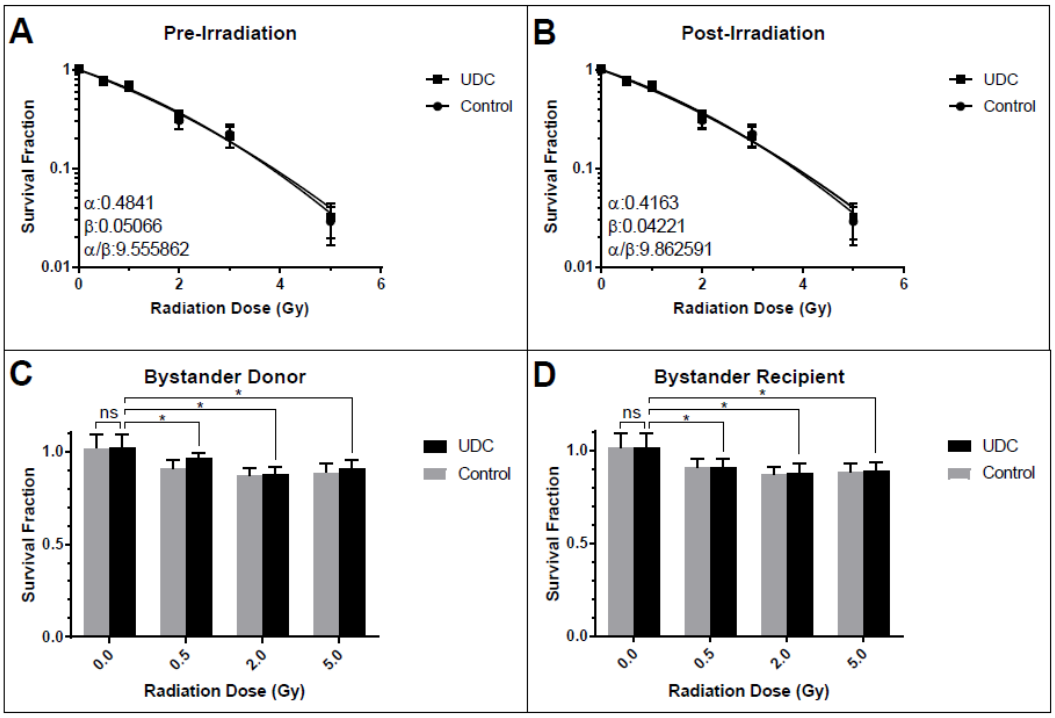

Fig. 4 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC ursodeoxycholate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC ursodeoxycholate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function

of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC ursodeoxycholate treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC ursodeoxycholate treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * 'UDC' refers to 'ursodeoxycholate'. * Units of dose are in Gray (Gy).

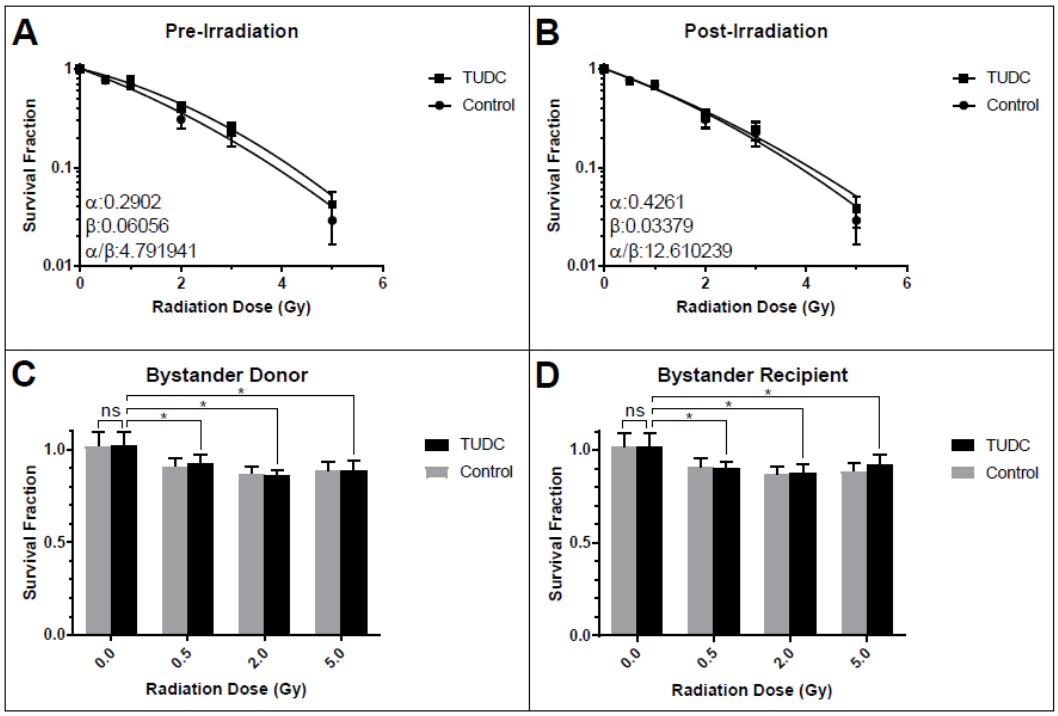

Fig. 5 Caption

Data are representative of three independent triplicate (n=3) experiments and values shown on each graph are representative of the mean survival fraction normalized to the cell plating efficiency with error bars expressed in ±Standard Deviation. (A) Comparison of HCT116 p53 wt survival data between control and pre-irradiation NIC tauroursodeoxycholate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. (B) Comparison of HCT116 p53 wt survival data between control and post-irradiation NIC tauroursodeoxycholate treatment on direct irradiation Cesium-137 exposures of 0.5, 1, 2, 3 and 5 Gy doses. For graphs (A) and (B), α and β values represent the resultant parameters of the non linear regression analysis of the data fitted to the linear quadratic function

of cell survival according to equation $Y=\exp[-1*(\alpha*X + \beta*X^2)]$. (C) Comparison of HCT116 p53 wt survival data between control and NIC tauroursodeoxycholate treatment on donor bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. (D) Comparison of HCT116 p53 wt survival data between control and NIC tauroursodeoxycholate treatment on recipient bystander effect medium transfer treatments of Cesium-137 exposures of 0.5, 2 and 5 Gy doses. For graphs (C) and (D), a One-Way Analysis of Variance (ANOVA) and Fisher's LSD Multiple Comparisons Test are used to consider significant value for treatment effectiveness. A confidence interval of 90% and p-value of < 0.05 was selected to be statistically significant. Control values of 0.5, 2 and 5 Gy bystander effects without NIC compound treatment are shown for comparison. * 'NIC' refers to 'Non-minimal Inhibitory Concentration'. * 'TUDC' refers to 'tauroursodeoxycholate'. * Units of dose are in Gray (Gy).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Ethanol	P = 0.9996	P = 0.8920	P = 0.9979	P = 0.9496	P = 0.9941	P = 0.9998
	ns	ns	ns	ns	ns	ns

Table 1: P-Value Chart displays the results of solvent control administration pre-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Ethanol	P = 0.9999	P = 0.9980	P = 0.9795	P = 0.9695	P = 0.9856	P = 0.9987
	ns	ns	ns	ns	ns	ns

Table 2: P-Value Chart displays the results of solvent control administration post-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Phenylacetate	P = 0.9999	P < 0.0001	P < 0.0001	P < 0.0001	P = 0.1749	P = 0.9995
	ns	****	****	****	ns	ns
Ursodeoxycholate	P = 0.9944	P = 0.9994	P = 0.9146	P = 0.9898	P = 0.9995	P = 0.9998
	ns	ns	ns	ns	ns	ns
Tauroursodeoxycholate	P = 0.9999	P = 0.0001	P = 0.0011	P = 0.0175	P = 0.7307	P = 0.9901
	ns	***	**	*	ns	ns

Table 3: P-Value Chart displays the results of compound administration pre-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Direct Irradiation					
	0	0.5	1	2	3	5
Phenylacetate	P = 0.9999	P = 0.9979	P = 0.9998	P = 0.9999	P = 0.9999	P = 0.9917
	ns	ns	ns	ns	ns	ns
Ursodeoxycholate	P = 0.9999	P = 0.9899	P = 0.9978	P = 0.9999	P = 0.9957	P = 0.9999
	ns	ns	ns	ns	ns	ns
Tauroursodeoxycholate	P = 0.9999	P = 0.9997	P = 0.9955	P = 0.9897	P = 0.9999	P = 0.9999
	ns	ns	ns	ns	ns	ns

Table 4: P-Value Chart displays the results of compound administration post-irradiation exposure and summarizes the Two-Way ANOVA analysis with subsequent Dunnett's multiple comparisons test. P-values are multiplicity adjusted P-values with 95% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect		
	0.5	2	5
Control	P = 0.0150	P = 0.0127	P = 0.0190
	*	*	*

Table 5: P-Value Chart displays the results of medium control administration in donor bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect		
	0.5	2	5
Control	P = 0.0179	P = 0.0159	P = 0.0157
	*	*	*

Table 6: P-Value Chart displays the results of medium control administration in recipient bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect			
	0	0.5	2	5
Ethanol	P = 0.9999	P = 0.0396	P = 0.0379	P = 0.0173
	ns	*	*	*
Phenylacetate	P = 0.9999	P = 0.0156	P = 0.0236	P = 0.0110
	ns	*	*	*
Ursodeoxycholate	P = 0.9999	P = 0.0379	P = 0.0177	P = 0.0179
	ns	*	*	*
Tauroursodeoxycholate	P = 0.9999	P = 0.0266	P = 0.0147	P = 0.0153
	ns	*	*	*

Table 7: P-Value Chart displays the results of solvent control and compound administration in donor bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

	External Beam Dose (Gy) – Bystander Effect			
	0	0.5	2	5
Ethanol	P = 0.9997	P = 0.0150	P = 0.0379	P = 0.0173
	ns	*	*	*
Phenylacetate	P = 0.9999	P = 0.0175	P = 0.0100	P = 0.0195
	ns	*	*	*
Ursodeoxycholate	P = 0.9974	P = 0.0197	P = 0.0186	P = 0.0189
	ns	*	*	*
Tauroursodeoxycholate	P = 0.9999	P = 0.0175	P = 0.0171	P = 0.0191
	ns	*	*	*

Table 8: P-Value Chart displays the results of solvent control and compound administration in recipient bystander flasks and summarizes the One-Way ANOVA analysis with subsequent Fisher's LSD multiple comparisons test. P-values are reported to within 90% confidence interval. * represents level of significance level (**** - P < 0.0001, *** - P = 0.0001-0.001, ** - P = 0.001-0.01, * - P = 0.01-0.05, ns is not significant).

DISCUSSION

Radioactive exposures cause oxidative stress in cells that might contribute to acute injury as well as chronic systemic issues that include carcinogenesis and exacerbation of inflammatory responses that lead to tissue dysfunction [7]. Therefore, anti-oxidative compounds that protect from these exposures have to

satisfy criteria such as practical route of administration, frequent intake, and low toxicity unlike conventional cysteamine compounds to protect from the acute and chronic effects of exposures [17]. These criteria align well with natural endogenous compound supplements which thus might serve as effective radioprotectors. In this study, treatments of phenylacetate, ursodeoxycholate, and tauroursodeoxycholate were applied to the clonogenic HCT116 p53 wt cell line to screen compounds to assess cell viability as an indicator of radioprotective, radiomitigative, and inhibitory bystander effects. The phenylacetate and tauroursodeoxycholate confer pre-irradiation effectiveness up to 2 Gy of external beam dose, with no post-irradiation radioprotection as well as absence of inhibitory bystander effects from the exposure. The linear-quadratic α/β ratio of these compounds in radioprotective experiments demonstrates notable reductions which suggests that it confers protection from single particle interactions at the low dose portion ($\leq$ 2 Gy) of the non-linear regression curve which is in line with the analysis. Therefore, the chemical basis of this protection suggests the compounds might serve as effective radioprotectors because of an anti-oxidative ability that serves to scavenge free radicals in the medium and not interferences in cell transduction signals that mitigate cellular responses to exposures via the inhibition of apoptotic signal transduction in cells. This study addresses only the basic screening of radioprotective compounds. The chemical basis of the radioprotection of the candidates thus warrants an additional extensive elucidation

of the molecular mechanisms in which some information from the literature on these compounds might serve useful.

The tauroursodeoxycholate and ursodeoxycholate are both bile acids that are important in cholestasis, that is, the regulation of cholesterol in vivo [32]. Though their role in pharmacology is to regulate the levels of cholesterol, studies have shown there are other properties of bile acids that might be important such as anti-oxidative, anti-carcinogenic, and anti-inflammatory qualities [33, 34, 35]. For instance, bile acids inhibit inducible nitric oxide synthase expression, production of nitric oxide, and integrate into membranes which might play some role in radioprotection [36, 37]. Given the compounds are ambiphilic this suggests that the compound might be able to protect from ROS as well as lipid peroxidation therefore have various modes of radioprotection. The conventional free radical scavenging radioprotector compounds operate in a dose dependant manner thus high amounts of free radical scavengers confer high radioprotection [15]. This mechanism is in line with the finding that the tauroursodeoxycholate, which has the highest NIC value of the compounds, might operate via free radical scavenging capabilities. Though the compound might operate in this manner, it has to possess sufficient kinetic reactivity to neutralize the hydroxyl radical which is the principal product of the gamma irradiation that reacts with biological targets via indirect action [11]. The sulfur containing tauroursodeoxycholate might

possess sufficient reactivity similar to the sulphydryl group of cysteamine, therefore exhibit properties that allow it to neutralize the effects of chronic ROS exposures that might cause oxidative stress to the cells [38]. The tauroursodeoxycholate compound is a taurine conjugate of ursodeoxycholate, therefore the incorporation of the sulfur containing taurine functional group suggests that it aids the radioprotective capability of the compound [39]. The ursodeoxycholate expresses no level of radioprotective effects, therefore, the additional tauro functional group might play noteworthy role in the elucidation of the mechanism of the compound and chemical basis of its role in cells. Though the ursodeoxycholate does not exhibit radioprotection as per the criteria of the experiment, the results do not suggest necessarily that the compound might be of no use in radioprotection as studies demonstrate that the compound is anti-carcinogenic and anti-inflammatory from in vivo experiments [40].

Phenylacetate is a metabolite of phenylalanine, therefore it is an endogenous metabolite of an important dietary substituent [41, 42]. The phenylacetate is an intermediate product to the production of phenylacetyglutamine that results from the conjugation of phenylacetate to l-glutamine. Therefore, it is responsible in the clearance of excess nitrogen in the cell, via removal of l-glutamine, which is the partial basis of its radioprotective effects by reduction of reactive nitrogen species in the cellular environment [43]. The underlying basis of the anti-carcinogenic

properties of phenylacetate in chronic dose rate, low dose radioactive exposure studies is that highly proliferative cells, specifically cancerous cells, utilize l-glutamine as an additional energy source. Thus, the phenylacetate binds to l-glutamine to generate phenylacetylglutamine a non-useable waste product that inhibits the level of l-glutamine in cells [44, 45]. Though this might suggest that this an impairment to the cell, the level of compound at the NIC value is not sufficient to cause toxic responses in the cell, therefore the anti-oxidative capability of the phenylacetate might be advantageous to the cell response. Despite the low NIC value, phenylacetate might possess potent anti-oxidative reactivity to effectively neutralize the ROS effects. The criteria of this study uses clonogenic assays to measure dose dependent growth arrest that might not necessarily be indicative of radiomitigative effects of an anti inflammatory and anti-carcinogenic compound. Therefore, the non post-irradiation effects of phenylacetate as well as the ursodeoxycholate and tauroursodeoxycholate do not undermine studies that show these effects.

The compounds show no modulatory response with respect to the radiation induced bystander effect - this suggests no intervention of the compounds in the process of bystander cell signalling in the radiation response. Though the bystander effect might contribute to the cellular response, the beneficial as well as negative outcome of the effect is context dependent [46]. Therefore, it is difficult

to ascertain whether an important aspect of radioprotection is the inhibition of bystander responses. This is because cellular communication via radiation induced bystander signalling might be beneficial in recovery responses to low doses and it might also be negative in that overt localized high doses of radiation elicit inflammatory responses as well as apoptotic responses that might necessitate interventional compounds [47]. In the case of eliminating the negative effects of bystander signalling, radioprotective compounds might suffice to quell the production of bystander signals in directly irradiated cells via antioxidant activity therefore not require direct inhibition of molecular bystander targets. Otherwise, the use of compounds to modulate cellular signalling requires the inhibition of cellular targets responsible in bystander signal production in directly irradiated cells, inhibition of the molecular signal, as well as inhibition of receipt of the signal in recipient cells. Given the bystander signal encompasses multiple molecules that are responsible in mediating various cellular responses, these bystander signals might have multitude of cellular targets as well as roles. Therefore, cellular responses are susceptible to exacerbation of issues from use of interventional compounds. Thus, in terms of the natural product radioprotector it might be necessary that it has no effect on bystander mechanisms in order to be effective.

Therefore, phenylacetate and tauroursodeoxycholate are of particular appeal as these candidates satisfy radioprotective criteria as well as lack of interference in bystander signal processes. Despite the lack of radiomitigative effects, the compounds might have utility through frequent dietary intake thus confer persistent radioprotection regardless of exposure. These particular candidates also demonstrate other suitable factors such as low toxicity, anti-inflammatory, and anti-carcinogenic effects that appeal to the field of radioprotection. Chemical radioprotection of the compounds with respect to clonogenic survival of cells warrants studies on their molecular mechanisms. These studies might allow us to screen specific compounds that possess similar attributes as well as perhaps produce modifications to these to aid their effect.

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Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the research, authorship and/or publication of this article.

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Chapter 6: General Discussion including Concluding Remarks/ Implications

Objective of this thesis

The objective of this thesis was to examine select natural dietary compounds for their radioprotective properties in vitro. These compounds were selected on the basis of their use in the treatment of CFIDS (Chronic Fatigue Immune Dysfunction Syndrome) given CFIDS encompasses symptoms similar to those from ionizing radiation (IR) exposures. Therefore, compounds that address CFIDS symptoms might also have utility in dietary chemical radioprotection. Specifically, this thesis examines the radioprotective effects of plant metabolites – curcumin, andrographolide, d-limonene – and endogenous metabolites – phenylacetate (PA), ursodeoxycholate (UDC), tauroursodeoxycholate (TUDC) – in clonogenic in vitro experiments. These compounds are natural consumer products that are commercially available with non toxic antioxidant, anti-inflammatory, and anti-carcinogenic qualities and therefore are a valid platform to screen for radioprotective compounds. Ultimately, this thesis examines whether these CFIDS treatments provide an attractive basis on which to screen radioprotective compounds in vivo.

Major Findings

Section I

Section I (Chapter I,II) of this PhD thesis is concerned with the chemoprotective properties of compounds on the basis of preferential toxicity towards p53 deficient and mutant cell lines. Specifically, this section examines clonogenic inhibitory effects of exogenous metabolites (curcumin, andrographolide, d-limonene) and endogenous metabolites (PA, UDC, TUDC) on HCT116 p53 null, HT29, and HCT116 p53 wt human colon carcinoma cell lines. The data clearly demonstrate in vitro chemoprotective effects with particular emphasis on the exogenous compounds that have high Non-Minimal Inhibitory Concentration (NIC) over Minimal Inhibitory Concentration (MIC) ratio values. The results indicate that compound toxicity is dependent on p53 status in isogenic HCT116 cell lines.

These chemoprotective effects are in agreement with literature studies that show anticarcinogenic effects in each of curcumin, andrographolide and d-limonene (Chen et al 1999; Chao et al 2010; Karlson et al 1996). Despite their effectiveness, the narrow margins between the NIC/MIC ratio values as well as high cytotoxicity of curcumin and andrographolide constrain their use in vivo. Therefore, the dose discrepancy values between p53 proficient and p53 deficient cell lines from these compounds are too low to confer high therapeutic gain in vivo. The clonogenic NIC values of d-limonene might be compatible with

effective, chronic chemoprotection in vivo given the appreciable NIC gap between cell lines. Unfortunately, the d-limonene does not demonstrate radioprotective effects which diminish its use in this regard as shown in Section II (Chapter I).

The notable chemoprotective results of d-limonene suggest that there are xenohormetic anticarcinogenic mechanisms which might be impacting the cell line. Xenohormesis is the phenomenon in which plants produce compounds that impact cellular mitogenic activity to target herbivorous organisms that consume these plants. Therefore, these plant metabolites act as pesticides (Surh 2011). In the literature, d-limonene has demonstrated inhibition of k-RAS (Kirsten rat sarcoma viral oncogene homolog) gene products of the oncogenic KRAS mutation in the HCT116 cell line (Karlson et al 1996). Section I experiments demonstrate there is notable toxicity among exogenous compounds that might also contribute partly to this k-RAS inhibitory effect. These compounds warrant the use of another cell line to examine this effect in the context of radioprotection. On the contrary, the endogenous compounds do not demonstrate this magnitude of toxicity in experiments. Of these compounds, only the TUDC demonstrates novel results of chemoprotection alongside radioprotection [Section I (Chapter II), Section II (Chapter II)]. The PA does not demonstrate chemoprotection in these experiments, although there is evidence in the literature of inhibition of cellular

transformation in vitro (Shack et al. 1996). Therefore, PA and TUDC, are compounds worthy of further examination in the field of dietary radioprotection.

Section II

Section II of this PhD thesis examines the radioprotective, radiomitigative, as well as inhibitory effects of compounds on bystander soluble factor intercellular communication. Notably, PA confers radioprotection up to 0.5 and 2 Gy of acute external beam IR. Minor radioprotective effects are seen using TUDC at 0.5 Gy exposures (Chapter II). Experiments demonstrate no radiomitigative effects among the compounds. Section II of this PhD thesis also examines the inhibitory effects of compounds on bystander soluble factor intercellular communication. None of the six compounds showed either inhibition of bystander soluble factor signal production or inhibition of the receipt of bystander signals in HCT116 p53 wt experiments. No other experiments have looked at the effects of these compounds on soluble factor bystander effects. The lack of bystander effects is important in terms of mechanisms. The literature shows that exosomes mediate the communication of bystander signals between cells (Xu et al. 2015). Therefore, the experiments suggest compounds do not interfere with exosome intercellular communication. In the literature, there are reports of bystander mechanisms affecting inflammatory processes resulting from IR exposures (Prise 2009). To examine immunomodulatory effects separate from BE, experiments have to utilize in vitro cell lines derived from components of the immune system in vivo.

These particular compounds have roles in the treatment of CFIDS which warrants an experiment on this effect.

Implications of the results for radiobiology

This thesis addresses several important issues in radiobiology

1. The variation in effectiveness of radioprotective/radiosensitising compounds with radiation dose: The results for the six compounds were analysed with respect to dose of radiation to see if there was any evidence of variation in modulation of radiation response depending on the applied radiation dose. This is important because there is evidence that low dose effects and high dose effects cause activation of different cellular responses, meaning a simple linear extrapolation from high dose to low dose effects is not possible. The data are presented in Figure 1 which shows the % change in cloning efficiency in the presence versus absence of each compound for each radiation dose. The data show significant increases in response for compounds PA and TUDC pre-irradiation administration peaking after a 1 or 2Gy dose and declining at higher doses. There is no effect of pre irradiation administration of d-limonene at any dose but there is a significant decrease in survival in cells receiving d-limonene with post irradiation. The decrease in survival is greatest at doses of 1 and 2 Gy. No changes in response were seen for curcumin, andrographolide, and UDC. The results suggest that there is a change in mechanism around the 2Gy dose point after which the protective or toxic effects are negated. The reason for this is unknown but may be because a

transition point occurs in the survival curve around this point as the dose response relationship becomes exponential.

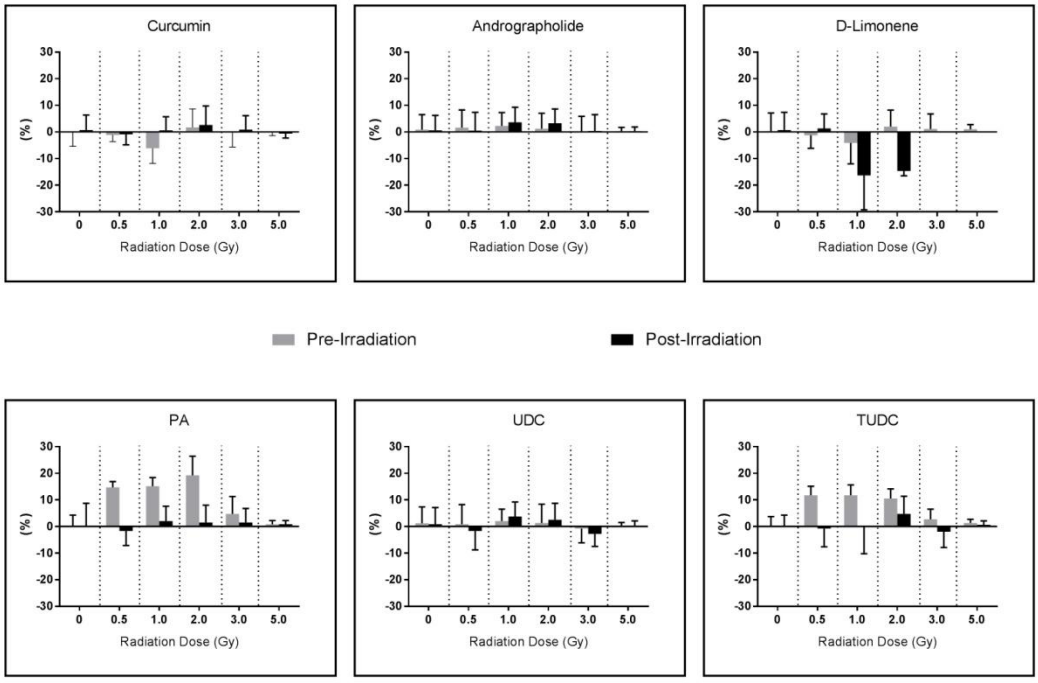

FIG. 1: Summary results of compound radioprotective effects in terms of percent change at each radiation dose, when the compound is present (± Standard Deviation).

2. The extent to which bystander effects mirror (or not) direct effects of radiation: the thesis looked at the ability of the compounds to modulate direct radiation response as well as the bystander effect. Most bystander studies in the literature are mechanistic in design and seek to inhibit certain processes in cells to help define bystander mechanisms. Few look at the ability of known radioprotective compounds to independently modulate direct and bystander responses. The results

in this thesis suggest that while there are direct effects of both endogenous and exogenous compounds on direct radiation response, the bystander effect is not changed. The results presented in Figure 2 show this clearly. There are no significant effects either using the bystander medium from donors receiving the chemical before irradiation or in recipients which got the chemical added to the transferred medium from irradiated cells. This important finding shows that direct and bystander effects do not mirror each other and is further evidence that independent mechanisms underlie bystander effects. This is to my knowledge, the first rigorous demonstration of this with proven radiomodulatory compounds.

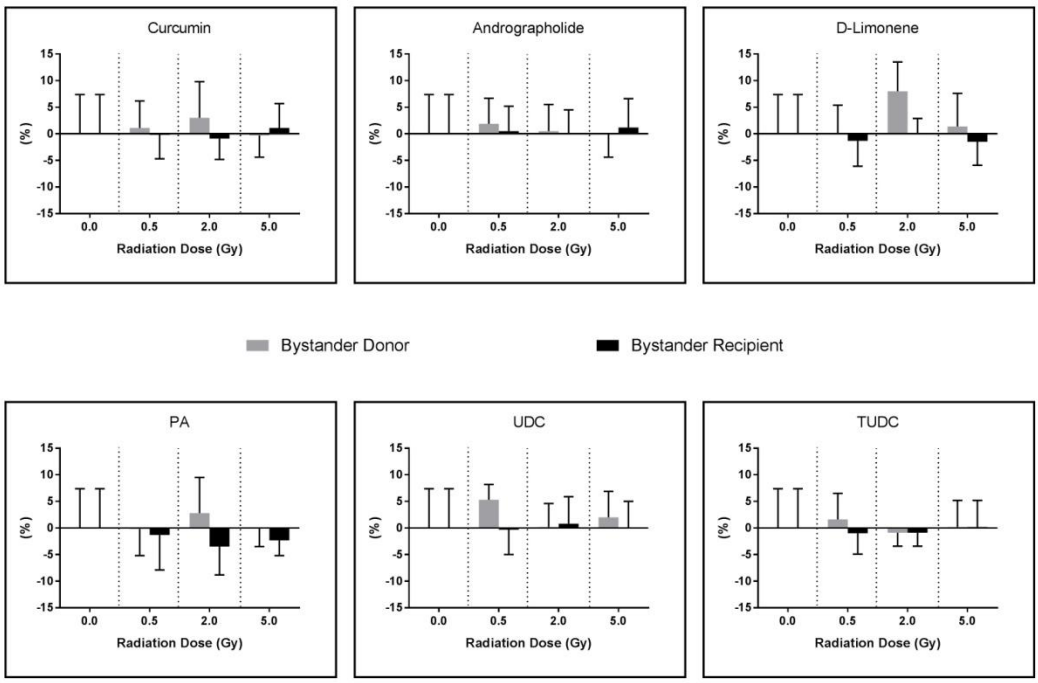

FIG. 2: Summary results of compound radioprotective effects in terms of percent change at each radiation dose, when the compound is present (± Standard Deviation).

3. The importance of p53 in modulating survival of cells exposed to the natural compounds: P53 has been shown previously to be essential for expression of a bystander response following irradiation (Takahashi et al. 2008, Mothersill et al, 2011, Le et al, 2017). It is also important in determining the survival of cells exposed to stressors including chemicals (Lotem et al. 1996; Brantley-Finley et al. 2003; Gasparian et al. 2011). Usually the assumption is that toxicity is higher when p53 is present since it halts cell division (G1 block) or directs cells into an apoptotic pathway. However little is known about the effects of p53 in dose ranges where the natural compound produces protective effects. In this thesis experiments were done with chemicals known to have radioprotective properties over a wide range of doses from non-toxic to toxic [Section I (Chapter I, II)].

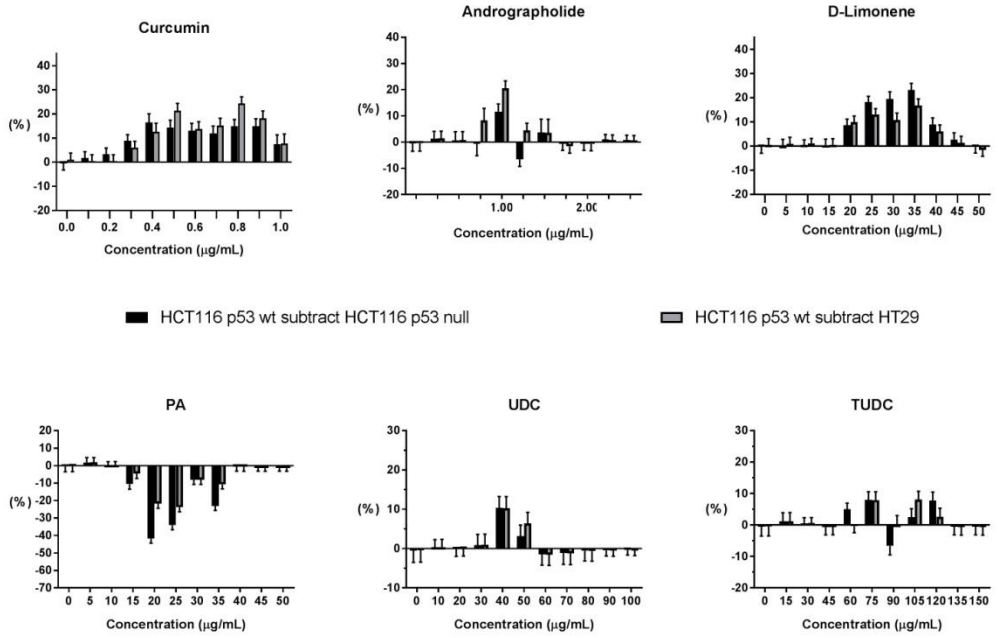

FIG. 3: Summary results of compound chemoprotective effects in terms of percent difference (± Standard Deviation) when p53 is present in the isogenic cell line.

The results show the chemoprotective effects of compounds calculated as the difference between HCT isogenic cell lines at each dose of the compound (% survival of p53+/+ line minus % survival of p53-/- cell line. Therefore, positive bars favour 'chemoprotective' effects due to the presence of p53 whereas negative bars show less protective effect when p53 is present. These results suggest that curcumin displays consistent chemoprotection along the dose range which demonstrates that the interaction of curcumin with p53 is very prominent in the mechanism of chemoprotection regardless of dose of compound. This suggests that p53 interactions with the compound dominate the outcome. Similar effects with d-limonene suggest that the compound is chemoprotective over the compound doses with p53 as the main mode of regulation in cytotoxicity effects. The remainder of the compounds, except PA, display chemoprotective effects with variable responses which are dependent on the dose. PA is the only compound where presence of p53 reduces the survival relative to when p53 is not expressed. The HT29 cell line with mutation in p53 demonstrates an intermediate toxicity between the HCT isogenic cell lines that suggests that this has an important role in dose dependant effects. The interesting question of how the p53 status might modify the radioprotective effects of the drugs could not be

addressed in this thesis due to lack of time however, the work using the p53 positive cell line suggests that radioprotective effects do occur for PA and TUDC but none of the compounds altered the bystander signal strength using the p53 positive line.

Further Experiments

To demonstrate further effectiveness in the field of radioprotection, experiments involving the use of PA and TUDCA require in vitro cell lines with carcinogenic mutations aside from loss of p53 function. Experiments in which compound treated 'regular' tissue derived cells are exposed to IR to evaluate frequency of cellular transformation are also of importance. Despite the chemoprotective effects of PA and TUDCA in vitro, factors that influence / impair / constrain in vivo application include compound modification, digestion, low solubility, absorption as well as microenvironment of the target sites (ex. pH). These factors warrant studies of effective delivery systems that address these issues.

Possible future experiments would be to examine PA in Nrf network effects. In brief, under conditions of regular redox, the cellular cytosolic transcription factor NF-E2- related factor 2 (Nrf2) is in association with the suppressor protein Kelchlike ECH-associated protein 1 (Keap1) that is maintained at low expression levels via proteasomal degradation. The radioprotective molecules might interact with protein thiols in the Keap1-Nrf2-ARE signaling networks to activate ROS responses. The phosphorylation of the Nrf2 by protein kinases imports the Nrf2 into the cellular nucleus. Nrf2 stimulates stress inducible gene expression via interaction with the antioxidant response element (ARE). This interaction also induces phase II enzymes that include glutathione S-transferases (GST), UDPglucuronosyl transferase (UGT), antioxidant enzymes such as glutathione

peroxidases (GPx), superoxide dismutase (SOD) as well as peroxiredoxin. In addition, Nrf2 activation involves genes that stimulate cellular redox regulation including glutathione synthetase, thioredoxin, thioredoxin reductase and NAD(P)H: quinone oxidoreductase 1 (NQO1) (Wang 2019). The compounds might stimulate some portion of this response that contributes to their radioprotective effects.

With respect to radioprotection, the examination of energy production in radioprotective PA treatments requires an experiment to evaluate the hormetic response of mitochondria in cells. Subcellular responses regarding mitochondrial has shown the diversity and importance of mitochondria in dictating overall health and dictating individual outcome to chronic stressors (Herst et al. 2017). In studies of mitochondria, it has been found that low to moderate exposures to oxidative stress increase mitochondrial MMP and trigger the mitochondria to compensate and undergo adaptive responses, via biogenesis (Yamamori et al. 2017). Upon exposures to acute sources of ROS, mitochondria depolarize favouring an apoptotic mechanism that might confer protection from lethal mutations thereon. This criterion is an important distinction between chronic, low dose irradiation and acute, high dose irradiation. Interestingly, cytosolic p53 has been found to translocate and to stabilize and repair mitochondrial DNA suggesting a role for it in radiation induced effects (Yamamori et al. 2015). Although such might confer protection on the cellular level, it must be noted that

chronic as opposed to intermittent exposures might facilitate and promote an unwanted phenotype contributing similar to a lesion, therefore this warrants further research.

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