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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headlines

- A higher retention of beneficial nutrients can be achieved by microwaving or steaming watercress instead of boiling it.

- To date, experimental results suggest a dose-dependent response of cancer cells to watercress and phenethyl isothiocyanate (PEITC). PEITC has been studied for its potential for chemoprevention.

Background

This PhD project is based on a collaboration between the Department of Food and Nutritional Sciences, University of Reading, the Institute of Molecular Medicine at the University of Lisbon, the Radiotherapy Department of the University Hospital of Santa Maria, and Vitacress Ltd. All work carried out at the University of Reading is funded by AHDB Horticulture.

The study plans are to look at the health outcomes of 200 early stage breast cancer patients commencing a six-week radiotherapy course and 200 healthy controls; with this phase of work being conducted by the University of Lisbon and the University Hospital of Santa Maria. Blood and urine samples from trial patients are analysed at the Department of Food and Nutritional Sciences at Reading University.

The study aims to find out if the consumption of watercress has beneficial health impacts on breast cancer patients referred for radiotherapy *vs* a second group of breast cancer patients that will maintain their diets whilst receiving the radiotherapy treatment. The potential role of watercress consumption in protecting normal cells from collateral damage, whilst potentially enhancing the susceptibility of cancer cells to radiotherapy treatment will be evaluated. Watercress will be tested in its natural form as a food item that will supplement the usual diet.

Work at Reading includes:

1. Volunteers in the human trial will consume watercress prepared using different methods. Watercress preparation may impact on the abundance of its

phytochemical composition like glucosinolates, flavonols, polyphenols and carotenoids. Watercress samples are and will continue to be prepared in the Food Processing Hall of the Food and Nutritional Sciences Department and analysed for the major phytochemical components.

2. An *in vitro* study looking at the effects of crude watercress extracts and PEITC on Michigan cancer foundation-7 (MCF-7) breast cancer cells will be performed. Cells cultured with watercress extract will be exposed to a source of radiation (mimicking the conditions of radiotherapy). Endpoints used to draw conclusions on the possible synergistic effect of watercress and radiation in cancer will include measuring the degree of DNA damage in the cells via the Comet assay, cell cycle assays and 1H NMR spectroscopy of cell extracts and cell media.

3. Metabonomic approaches primarily based on 1H nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS) will be used to characterise the plasma and urinary metabolic phenotypes of the patients before and after radiotherapy. This will allow the biochemical modifications associated with radiotherapy to be explored and the ability of watercress to modulate such responses. This has potential to provide mechanistic insights into how watercress may improve the restorative effects of radiotherapy in breast cancer patients. Blood and urine samples will be collected at the beginning and at the end of radiotherapy treatment (RT), 3 months, 1 year and 3 years after the end of RT. The results will also provide data on the levels of potentially beneficial phytochemicals derived from watercress consumption including folate, carotenoids, flavonoids and glucosinolates. Metabonomics simultaneously measures thousands of low molecular weight metabolites providing holistic information on the biochemical status of the body. High-resolution 1H NMR spectroscopy, mass spectrometry and mathematical modelling approaches will be coupled to determine the metabolic profile in the biological samples from the patients and hence, potentially elucidate the biomolecular mechanisms underlying the watercress-associated improvement in therapeutic outcome.

4. DNA damage in lymphocytes collected from the patients before and three months after the intervention with watercress will be assessed via the Comet assay. DNA damage will be measured using the alkaline version (single-cell gel electrophoresis) of the Comet assay that quantitates basal levels of DNA damage by measuring strand breaks in DNA at alkali labile sites.

These results will provide metabolic and genetic information on the mechanism of action of watercress exposure in breast cancer cells and preliminary indications on whether it can potentially be used as a therapeutic adjuvant in early breast cancer treatment. Moreover, this study will contribute to the development of a multidisciplinary nutritional intervention protocol, feasible and adequate to patients and researchers. It will also promote awareness of other health professionals to the relevance of nutrition as a supplementary therapy of cancer patients.

Summary

In this PhD project, breast cancer patients referred for radiotherapy are consuming watercress following their treatment as a part of their normal diet. To date the number of patients recruited in the study is 42, which is far lower than the 200 hoped for at the beginning of the study. This is due to several issues in the hospital where the study is taking place which is not allowing a higher rate of volunteer recruitment. Therefore as far as this PhD is concerned, any samples that have or will be collected in Portugal until the end of January 2016 will be sent to Reading for all the analyses. Metabonomic analysis, in addition to clinical measurements, will be used to evaluate the effect of watercress consumption on disease outcomes and therapeutic efficiency. Metabonomics is a powerful approach because metabolites and their concentrations directly reflect the underlying biochemical activity and state of cells / tissues. Metabonomics allows holistic information on the biochemical status of breast cancer patients to be obtained and the ability of watercress to improve the toxic effects of radiotherapy to be explored. DNA damage parameters will also be assessed.

The volunteers in the human intervention trial are using a variety of preparation methods for watercress, which could impact on the abundance of the beneficial phytochemicals. Tests to determine the effects of a range of preparation methods were carried out in Year 1. Watercress was processed using different domestic

cooking methods and the major phytochemical components were quantified using a range of analytical techniques. The results to date suggest that boiling of watercress has a detrimental effect on the levels of beneficial phytochemical whereas microwaving and steaming have little effect suggesting that they should be used as the preferred method of watercress preparation. A manuscript with the results from this study has been submitted for publication at the Journal of Food Chemistry.

At the molecular level, the *in vitro* study being performed is looking at the effects of watercress extracts on a breast cancer cell line exposed, or not, to a radiation source to mimic the radiotherapy conditions. To date the experiments looking at the metabolic effects of different doses of watercress and PEITC have been performed and analysed and the results suggest a dose dependent response of cancer cells to watercress and PEITC. Watercress and PEITC cause the release of different metabolites, which are related to the detoxification mechanisms, and regulation of the oxidative status of the cancer cells. Modification of these pathways by watercress and PEITC is of great importance as they are considered to be hallmarks of cancer progression.

Cell cycle experiments that help us understand at which point of the cancer cell's life cycle watercress and PEITC can inhibit growth and proliferation have also been performed and the results are being analysed. The earliest the watercress or PEITC stop the cell cycle the better the chances of cancer cell death are. Preliminary data suggest that PEITC is a strong inhibitor of cell cycle early on.

DNA damage is a very important step in cancer initiation and progression. The higher the level of DNA damage in a cancer cell the better the chances of the cell undergoing apoptosis, the programmed cell death. We are measuring the levels of DNA damage using the Comet assay.

All of the above experiments are also being performed in a healthy breast cell line to examine whether any observed effects of watercress and PEITC are specific to cancer cells or not.

Collectively, these studies will further our understanding of the biomolecular mechanisms of radiotherapy in breast cancer and the potential for its effectiveness to be improved by watercress consumption.

Financial Benefits

None to date but growers may benefit if peer reviewed science which substantiates the health properties of watercress is generated. Such information can be used in PR campaigns aimed at raising consumer awareness of the nutritional benefits of watercress and its potential cancer fighting properties. Past campaigns based upon research demonstrating anti-cancer properties have seen sales increases of up to 40% with a sustained uplift for months afterwards. This is significant to the watercress industry whose sales exceed £40M at Retail Sale Value (RSV). In addition the shelf space of multiple retailers is under intense competitive pressure; watercress needs to maintain its exposure and national publicity to keep it on retail shelves.

Action Points

None at this stage.

SCIENCE SECTION

Introduction

The increasing interest in healthy diets and the shift towards natural foods has put watercress under the 'microscope' of the scientific community as well as the health-conscious public. Watercress is an exceptionally rich source of phytochemicals such as glucosinolates, carotenoids, flavonols, vitamins and minerals. Recent studies have provided evidence for a strong anti-oxidative potential of watercress [1, 2], and showed that several watercress components have anti-proliferative effects and are associated with the inhibition of the three stages of carcinogenesis: initiation, proliferation and metastasis [3-5].

The inverse relationship between cancer development risk and healthy nutrition has been well established. Cancer builds upon damage to cellular DNA resulting from carcinogenic environmental factors, in which nutrition plays a major role. Many diets and lifestyle factors can influence the development of cancer, a disease expected to affect 1 in 3 people over the next years. Epidemiological studies associate a higher intake of cruciferous vegetables with a reduced risk of various types of cancers including breast cancer [6].

Watercress is a rich source of glucosinolates, which are further hydrolysed to isothiocyanates. It is the biological activity of the subsequent isothiocyanate hydrolysis products in humans that are of most interest in terms of conferring health benefits to humans. It is these metabolites rather than the glucosinolate precursors that reach the target sites. Phenethyl isothiocyanate (PEITC) is the primary isothiocyanate derived from watercress with it being the main dietary source of PEITC and it is this compound that the majority of watercress-related health benefits are attributed to. Research has highlighted a favourable role of watercress or watercress specific components such as PEITC and flavonoids, in antigenotoxic and anticancer processes both *in vivo* and *in vitro*.

Boyd *et al.* [3] showed Inhibition of three stages of carcinogenesis: initiation, proliferation and metastasis was shown in HT29 colon cancer cells after incubation with watercress extract. In the same study, watercress extract was protective against oxidative damage induced by known genotoxic compounds like 4-Hydroxy Nonenal

(4-HNE), faecal water and hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS) produced during mitochondrial metabolism.

In the present study we employ metabonomics technologies for phenotyping the metabolic responses of MCF-7 breast cancer cells to watercress extract and PEITC treatment. A well-recognised tool for metabonomics is NMR spectroscopy, which is limited in sensitivity but shows high metabolite specificity and remarkable versatility of acquisition conditions, allowing us the potential of metabolic biomarker or pathway discovery.

Materials and methods

Cell culture

Cell line

Human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC; Manasses, USA). The cells were maintained in complete Dulbecco;s modified Eagle's Medium (DMEM; Lonza Group Ltd, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies, UK), 2mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin (Sigma Aldrich, Dorset, UK) and non-essential amino acids. Cells were grown in a 37°C incubator supplied with 5% CO_2.

Cell culture conditions

Cells were maintained in 75 cm^2 tissue culture flasks and routinely passaged at 60-70% confluence. Media was aspirated from flasks and Trypsin-Versene® (EDTA) Mixture (3 ml; 0.05% (w/v) trypsin and 0.02% (w/v) versene (EDTA); Lonza Group Ltd.) was used to rinse away any remaining cell debris before being discarded. Trypsin-Versene® (EDTA) Mixture (5 ml) was added to the flask and incubated at 37ºC/5% CO2 for a minimum of 3 min to detach cells. Following this complete DMEM (5 ml) was added to the flask to inactivate the trypsin and collect the cells. The required volume of cell suspension to produce the desired dilution was removed and either discarded or transferred to a 15 ml centrifuge tube where the cells were then counted and used for an experiment. Complete DMEM (20 ml) was added to the remaining cells in the flask and incubated at 37ºC/10% (v/v) CO2.

Compounds and extracts

Analytical grade compounds

Phenethyl isothiocyanate (PEITC) was purchased from Sigma. 10 mM PEITCstock solution was made in DMSO fresh on the day of use.

Watercress extracts

Green and red watercress samples were obtained directly from Vitacress Salads Ltd. (Andover, UK). Samples were snap frozen in liquid nitrogen and stored at -80ºC. 2 g of leaf and 2 g of stem were weighed and placed in a 20 ml syringe (BD Biosciences, Oxford, UK) that had had the plunger removed and a circular 25 mm glass microfiber filter (Whatman, Dassel, Germany) placed at the bottom. The syringe was then placed inside a 50 ml centrifuge tube without the lid and centrifuged at 2600 rpm for 30 to collect the extract. This crude watercress extract was then filtered through a 0.22 µm filter (Whatman) and used immediately.

^{1}H NMR spectroscopy-based metabonomic analysis

The metabolic profiles of MCF-7 cells were analysed using ^{1}H NMR spectroscopy. Cells were treated at 1x10^{6} cells per well into 6 well plates at 80% confluency. The cells were then exposed to the watercress extract at 6.25, 12.5, 25 and 50 µl/ml of extract and PEITC at 5, 10, 20, 30 µM for 24 hours. Media was transferred into eppendorf tubes and cells on the surface of the plate were washed twice using 1ml cold (4ºC) PBS and were then quenched using 1ml of ice-cold methanol. The cells were allowed to lyse for 2 minutes and were detached from the plate using a Starstedt cell scraper and transferred into an Eppendorf tube. Methanol quenching was repeated to maximise metabolite recovery. A vacuum concentrator (SpeedVac) was used to dry down the cell suspensions before reconstitution in 80 µl of phosphate buffer (pH 7.4) in 100% deuterium oxide containing 1 mM of the internal standard, 3-(trimethylsilyl)-[2,2,3,3,-^{2}H$_{4}$]-propionic acid (TSP). For every sample, a standard one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (recycle delay (RD)-90°-t1–90°-tm-90°-acquire free induction decay (FID)). For each spectrum 512 scans and 8 dummy scans were obtained, collected in 64K data points with a spectral width of 12.001 ppm. ^{1}H NMR spectra were manually corrected for phase and baseline distortions and referenced to the TSP singlet at δ 0.0. Spectra were digitized using an in-house MatLab (vesion

R2012a, The Mathworks, Inc.; Natwick. MA) script. Multivariate modeling, including principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) [7], was performed on the samples in using scripts provided by Korrigan Sciences Ltd., United Kingdom.

Results

The metabolic profiles of MCF-7 cells treated with increasing concentrations of watercress and PEITC were characterised by ^{1}H NMR spectroscopy. Unsupervised hierarchical clustering of the metabolites identified and quantified suggests a clear separation of all treatments and doses (Figure 1). MCF-7 breast cancer cells respond to watercress and PEITC treatments in an anti-parallel manner. Amino acids such as threonine, glutamine, valine, isoleucine, leucine, phenylalanine, tyrosine and methionine appear to be increased with the highest doses of PEITC with concurrent decreases in glutathione, N-acetylglutamate, glutamate, phosphocholine, glycine, aspartate, the NADP+ cofactor and acetate. The metabolic perturbations induced by watercress treatments are the opposite suggesting a different way of action in the cells by exposing them to the whole watercress extract.

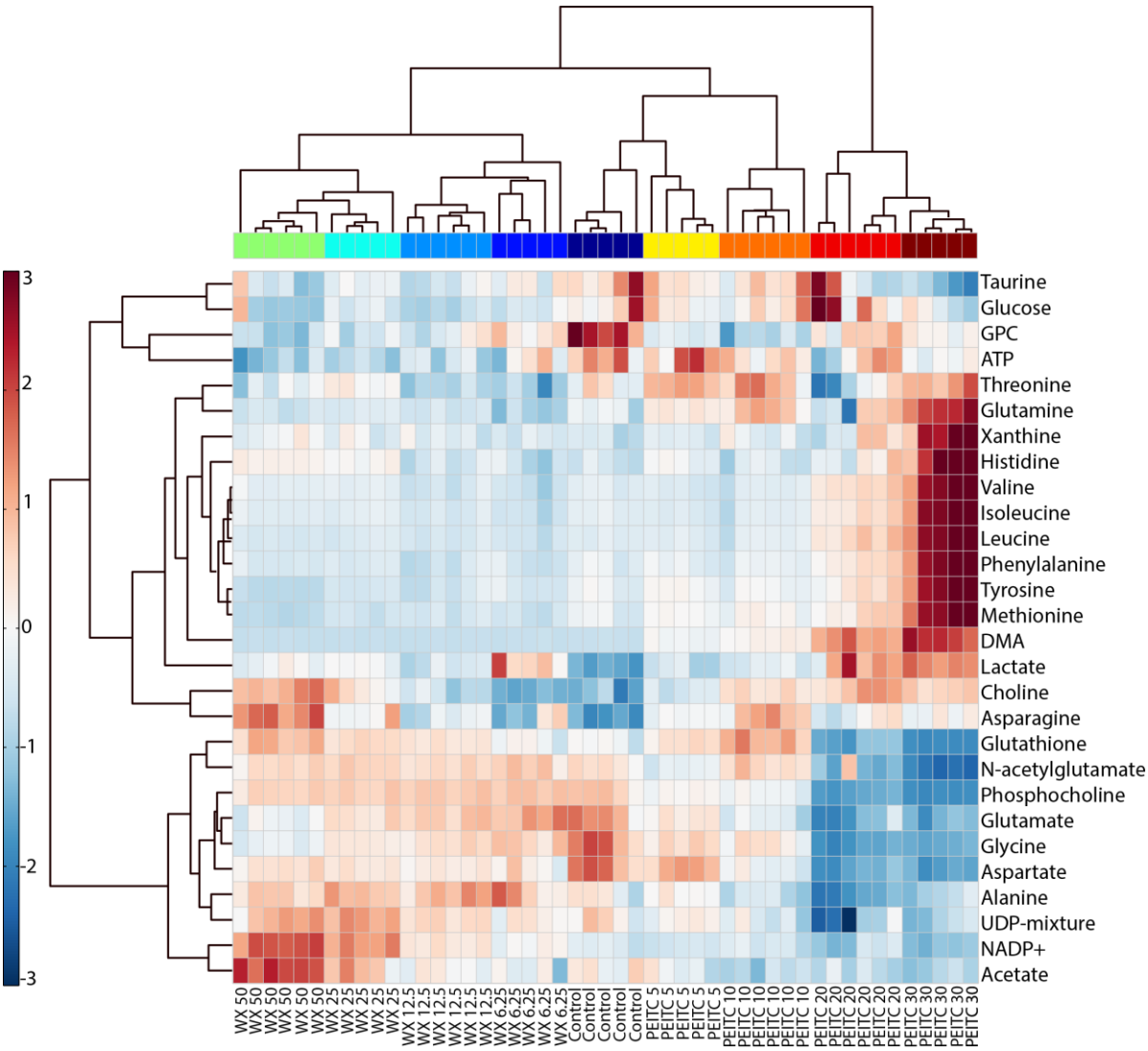


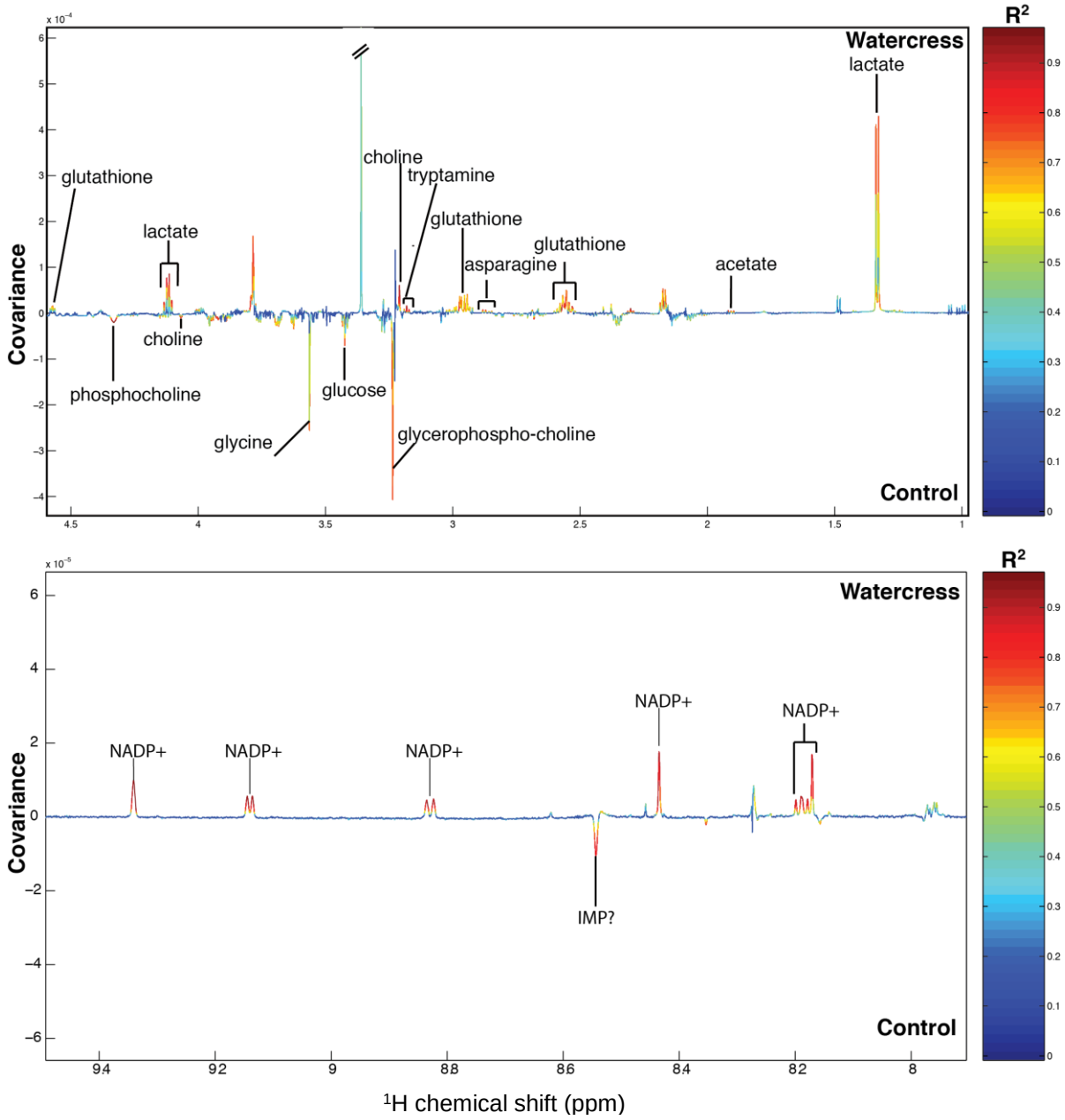
Figure 1 Unsupervised hierarchical clustering heat-map of metabolites from MCF-7 cells treated with watercress (WX) extract or PEITC at different concentrations. Each row represents a metabolite and each column represents a sample. The row Z-score (scaled expression value) of each metabolite is plotted in red-blue colour. The red colour indicates metabolites that are high in abundance and blue indicates metabolites low in abundance.

Supervised profiling of the metabolic responses of MCF-7 cells to watercress& PEITC

The supervised analysis revealed significant differences in the response of MCF-7 breast cancer cells after incubation with the watercress extract (Figure 2A). Data presented in this section are from the highest doses for both watercress and PEITC. Untreated cells had a higher content of choline-derived metabolites such as phosphocholine and glycerophosphocholine, glucose and glycine. Treatment of the

cells with watercress led to increased quantification of lactate, asparagine, choline, glutathione and NADP+.

A



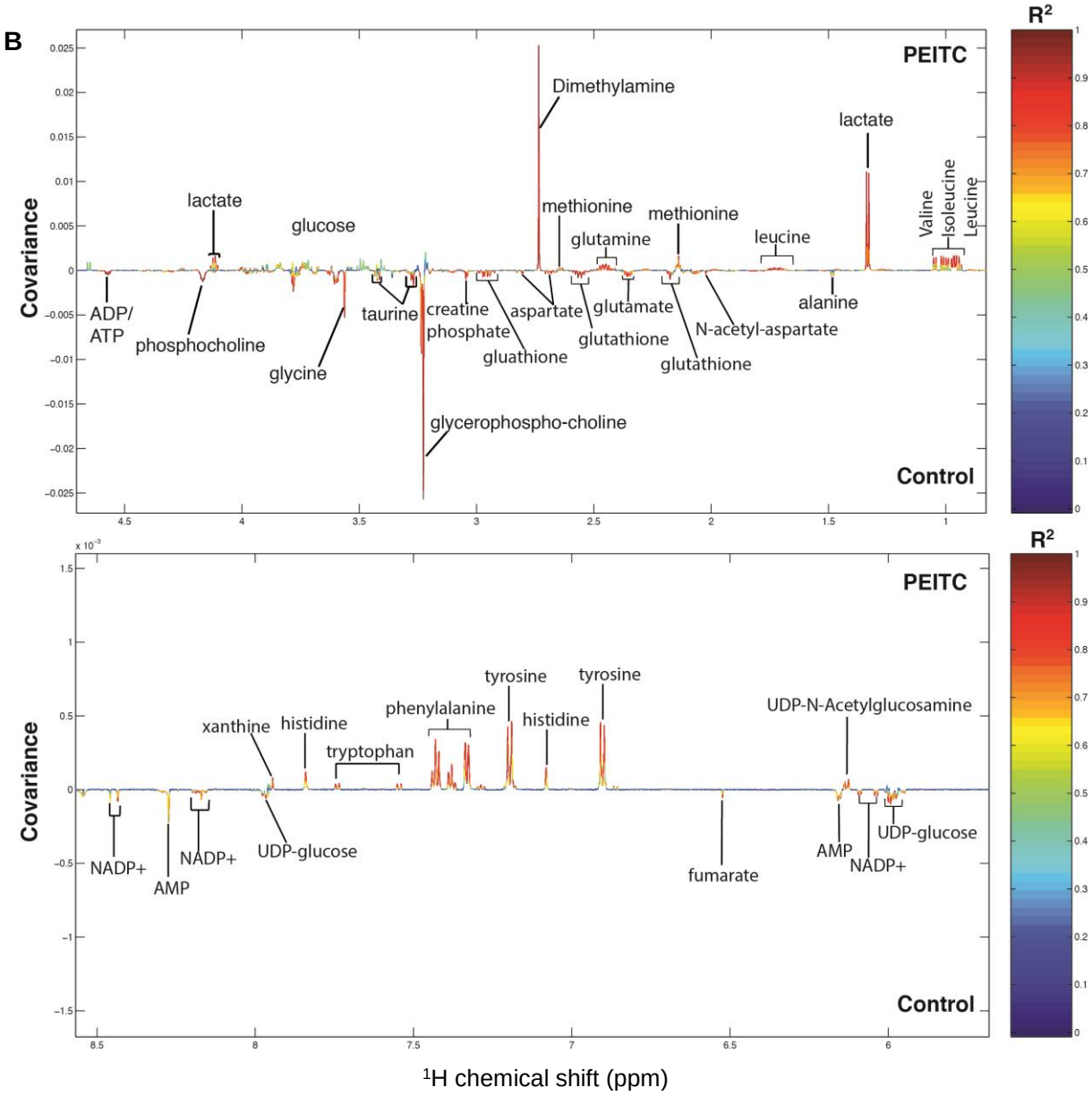


Figure 2 Plot of OPLS-DA coefficients comparing the metabolic profiles of A) control and watercress treated (50µl/ml) MCF-7 cells and B) control and PEITC treated (30µM) MCF-7 cells.

Metabolic profiling of the impact of PEITC treatment in breast cancer cells suggests that it induces strong perturbations in the biochemical signature of these cells. These changes are characterised by increased amino acids such as valine, leucine, isoleucine, methionine, histidine, tryptophan and phenylalanine. Comparing the untreated cells to the PEITC treated cells; it appears that untreated cells are higher in glutathione, glutamate, alanine, aspartate, choline-derived metabolites, taurine, creatine, glycine, NADP+ and fumarate.

Table 1 Comparisons between treatments with the corresponding Q2Ŷ (goodness of model prediction) and the P-values for each model.

Model	$Q^2\hat{Y}$	P-value
Control x WX 6.25 µl/ml	0.5868	0.001
Control x WX 12.5 µl/ml	0.7979	0.001
Control x WX 25 µl/ml	0.6491	0.01
Control x WX 50 µl/ml	0.7545	0.01
Control x PEITC 5 µM	0.5596	0.004
Control x PEITC 10 µM	0.7146	0.003
Control x PEITC 20 µM	0.9317	0.001
Control x PEITC 30 µM	0.9285	0.001

Discussion

On the biochemical level our results suggest that watercress and PEITC treatments can have multiple effects on the metabolic phenotype of MCF-7 breast cancer cells at different dose levels. The main objective was to enhance our understanding as to which are the main differences of a sole isothiocyanate such as PEITC, which is a very important component of PEITC and the whole watercress extract. It is worth noting that the watercress extract used in this study potentially lacks PEITC as the vegetable material was snap-frozen in liquid nitrogen before extraction. Due to its high volatility PEITC is difficult to be maintained in a watercress extract. This will essentially allow us to isolate the effects of PEITC alone and also to examine the way watercress extract, which is rich in other compounds such as phenols, carotenoids and vitamins act on breast cancer cells.

Isothiocyanates have originally been characterised as natural antioxidants that can reduce the levels of ROS and act as potential chemopreventive components of a diet. They act as antioxidants at low doses in normal cells. At higher doses isothiocyanates can increase the levels of ROS by depleting the cell of its antioxidant level. This observation is consistent with the pro-oxidant phenotype seen with the PEITC treated cells. Isothiocyanates are very electrophilic and can therefore quickly conjugate with thiols. Glutathione (GSH) is the most abundant thiol in the cells therefore after diffusion in the cells isothiocyanates form GSH-dithicarbamate conjugates[8]. GSH is an important antioxidant and its exhaustion by the higher doses of PEITC as observed in our results lowers the redox buffering potential of the cell and essentially induces elevations in intracellular ROS formation in the mitochondrial

space. Cancer cell lines containing low GSH levels have been demonstrated to be much more sensitive than control cells to the effect of irradiation. Generation of excess ROS is considered to play a role in the isothiocyanates-mediated apoptosis in cancer cells. Watercress on the other hand seems to be working in an anti-oxidant manner by increasing the intracellular levels of glutathione. This is potentially suggestive of lower levels of ROS in the cell. This is not necessarily a negative result since functions such as tumour growth, development and metastasis can be initiated by higher levels of ROS. A number of studies suggest that increases in ROS scavenging enzymes such as glutathione inhibit the growth of cancer cells [9, 10].

In our results we also observe clear shifts in amino acids like glutamine, glutamate, alanine, glycine, aspartate, asparagine and others. These amino acids are formed from TCA cycle intermediate or are involved in the formation of some of the TCA cycle intermediates [11]. The dramatic increase of a number of these amino acids in the cells treated with the highest doses of PEITC suggests they are not being used by the TCA cycle which is suggestive of a disrupted mitochondrial function since the TCA occurs in the mitochondrial matrix. Furthermore, shifts in these amino acids have been identified in DNA damage and repair pathways. Therefore it is safe to conclude that treated cells have altered mitochondrial function in relation to the control cells. Altered mitochondria activity can lead to modified production of free radicals and ROS from normal respiratory activity, and thus increased sensitivity to radiation and DNA damaging chemotherapeutic drugs.

The increases in lactate concentration in both watercress and PEITC treatment can be explained by the inhibition of oxidative phosphorylation during which glucose is converted to pyruvate and ATP in the presence of oxygen. Cancer cells produce lactate from glycose following anaerobic glycolysis at a high rate to fulfil their high demands in ATP. This phenomenon is called the ‘Warburg effect’ and it is a commonly observed phenotype in cancer cells. This effect can appear as a consequence of damage to the mitochondria in cancerous cells or as an adaptation to the prevalent hypoxic microenvironment of tumours. Interestingly watercress treated cells and cells treated with the highest doses of PEITC are devoid of ATP suggesting an energetically starved phenotype that could lead to cell death. Our results are in consistence with a study where prostate cancer cells treated with PEITC exhibited suppressed glycolysis, they were depleted of ATP, had increases in lactate and eventually cell death [12]. This is only primary *in vitro* of PEITC and watercress efficacy in terms of glycolysis inhibition, therefore it is important to confirm these observations in animal models as well as in clinical trials involving cancer patients.

Conclusions

^{1}H NMR spectroscopy-based metabonomic analysis was successfully applied to evaluate the response of MCF-7 breast cancer cells to watercress extract and PEITC. ^{1}H-NMR spectroscopy assisted metabonomics identified prominent targets of watercress and PEITC effect in breast cancer cells, including glutathione and glucose metabolism both which are considered to be hallmarks in cancer progression. Multivariate data analysis revealed metabolic transitions in these two hallmarks that are related to the dose of both watercress and PEITC. It is critical that more studies investigate the metabolic behaviour of potential therapeutic agents in cancer models. There is a clear need to understand the metabolic behaviour of cancer cells in order begin to understand how to effectively manage cancers therapeutically.

Knowledge and Technology Transfer

Knowledge transfer activities involve presenting the work carried out in conferences and academic publications. Scientific posters of this work have been presented in the Glucosinolates conference in Wageningen October 2014, and the Metabolomics society meeting in London in September 2014. The completed work is expected to be orally presented at the Metabolomics 2016 conference in Dublin. A manuscript entitled ‘’Effects of domestic processing methods on the phytochemical content of watercress (*Nasturtium officinale)”* has been submitted in the Journal of Food Chemistry.

Glossary

Metabolites: small biological compounds involved in biochemical processes and pathways. Examples include cholesterol, glucose and amino acids

Metabolome: A collection of all metabolites observed in a biological system under a given set of conditions.

Metabolomics: the analysis, quantification and interpretation of metabolite levels in biological samples with the aim of characterising the metabolome.

Metabonomics: The study of changes in metabolite levels in response to drugs, diseases or toxicity, usually used when determining such changes using NMR.

Metabolic profiling: Analysis of a group of metabolites that are a part of a particular group or class (e.g. sugars, lipids) or linked to a specific pathway.

NMR spectroscopy: Some atomic nuclei possess a non-zero magnetic moment. This property is quantised and leads to discrete energy states in a magnetic field. Nuclei such as 1H, 13C, 15N, 19F and 31P can undergo transitions between these states when radiofrequency pulses of appropriate energy are applied. The exact frequency of a transition depends on the type of nucleus and on its electronic environment in a molecule. For example, 1H nuclei in a molecule give NMR peaks at frequencies (chemical shifts) characteristic of their chemical environment. In metabonomics, it is the patterns that occur when many different biochemical entities are detected simultaneously in a mixture using 1H NMR that are interpreted

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