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Chemopreventive Potential of Dietary Polyphenols

by

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Submitted for the Degree of Doctor of Philosophy

School of Biomedical and Molecular Sciences
University of Surrey

March 2005

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ABSTRACT.

The chemopreventive potential of a wide variety of structurally diverse polyphenols were initially evaluated for their apoptotic-inducing activity, using HCT-8 ileocecal adenocarcinoma cells. Apoptosis was measured by fluorescence microscopy and morphological criteria. (-)-Epigallocatechin-3-gallate (EGCG), theaflavins, curcumin and the grape seed polymer were found to be the most potent inducers of apoptosis, at concentrations physiologically relevant to humans. In contrast to the observation in HCT-8 cancerous cells, polyphenol-induced apoptosis was substantially lower in ICE-6 non-cancerous epithelial cells.

The aforementioned “active” apoptotic polyphenols were subsequently assessed for their ability to activate direct and indirect caspases. Activation of downstream caspase-3 and -7, and upstream caspase-9, in HCT-8 cells, implies that an intrinsic (intracellular) caspase cascade is involved in polyphenol-induced apoptosis.

Potential pro-oxidant and/or antioxidant mechanisms and interactions, through which polyphenols may initiate apoptosis in HCT-8 cells, were investigated by assessing the impact of various agents, including antioxidant enzymes and hydrogen peroxide (H2O2), upon apoptotic induction. Catalase and superoxide dismutase substantially reduced EGCG-induced apoptosis. In contrast, a substantial rise in EGCG-induced apoptosis was observed in the presence of both H2O2 and Fe3+. Moreover, H2O2 production was apparent in the presence of EGCG. These data thus suggest that polyphenols induce apoptosis via a pro-oxidant mechanism, which appears to involve the generation of H2O2 and transition metals in a Fenton type reaction.

A range of naturally-occurring and synthetic polyphenolic compounds was assessed for their ability to bind to the Ah receptor, as agonists and/or antagonists, in H1L1.1c2 cells, using the chemically activated luciferase gene expression (CALUX) assay. Although the flavonoid 3',4'-dimethoxyflavone appears to competitively antagonise 2,3,7,8-tetrachlorodibenzo-p-dioxin binding at the Ah receptor, the other flavonoids tested failed to indicate reliable or reproducible interactions with the Ah receptor.
ACKNOWLEDGEMENTS.

First and foremost, I would like to thank my supervisors Costas Ioannides and Miloslav Dobrota, for their continuing support and guidance throughout this project, particularly through ill health and bereavement.

My thanks extend to POLYBIND participants, Celestino Santos Buelga, Denis Barron, Veronique Cheynier, Sheryl Lazarus, Conrad Astill, Mike Saltmarsh and, in particular Mike Clifford, either for providing novel polyphenolic compounds, or for their invaluable knowledge of polyphenol chemistry. I also want to thank the EU for their generous funding, without which I could not have undertaken this project.

I would like to thank Kamal Ivory, Flore Depeint, Chris Shotton, Dave Lamb and especially Helen Coley, for their help in understanding the intricate workings of a flow cytometer, as well as Helen for her invaluable cell culture instruction. Many thanks go to George Kass for his apoptosis guidance, particularly pertaining to DNA fragmentation gels, Fas expression and fluorescence microscopy. Also thanks go to Dave Lewis for his time, logP knowledge and use of his PrologP Pallas computer software, Nick Plant for invaluable advice on cell re-isolation, Mike Denison, via email in California, for his essential assistance with the CALUX assay, and Aldo Roda for donating H1L1.1c2 cells. I would also like to thank, Julie Howarth and Richard Hinton for their time and advice on immunocytochemistry techniques and image capture.

My greatest appreciation goes to my cousins; Simon Goodacre, who has taken time to frequent the library, on my behalf, in the endless quest for interlibrary loans, for which I am truly grateful. Also, to Alison Daniel, who, collated the references into a comprehensible form, and by proof reading a subject she knows little about, has vastly improved my English grammar, and thus the presentation of this manuscript. A million thank yous, Ali.

Finally, my better half Mark Belchamber, who, as well as being a dab hand with a scanner, has kept me fed and watered, and been a perpetual source of love and support.
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ABBREVIATIONS.

α-MEM  Minimum essential medium alpha
AFC    7-amino-4-trifluoromethyl coumarin
Ah     Aryl hydrocarbon
AhRC   Ah receptor complex
AIF    Apoptosis inducing factor
AOM    Azoxymethane
AP-1   Activator protein-1
Apaf-1 Apoptotic protease-activating factor-1
ARNT   Ah receptor nuclear translocator
ATP    Adenosine triphosphate
B1     Epicatechin-4,8-catechin
B2     Epicatechin-4,8-epicatechin
B7     Epicatechin-4,6-catechin
B'2G   Epicatechin gallate-4,8-catechin
B[α]P  Benzo[α]pyrene
Bcl-2  B-cell lymphoma/leukaemia-2
BSA    Bovine serum albumin
C1     Epicatechin-4,8-epicatechin-4,8-epicatechin
CA     Cholic acid
CALUX  Chemically activated luciferase gene expression
CAT    Chloramphenicol acetyltransferase
CBG    Cytosolic β-glucosidase
CDCA   Chenodeoxycholic acid
cdk    Cyclin dependant kinase
CHX    Cycloheximide
Cl 1   Chrysin
Cl 2   8-C-(3,3-dimethylallyl)-chrysin
Cl 3   8-C-(1,1-dimethylallyl)-chrysin
Cl 6   5-7-dihydroxy-3,2',4',5'-tetramethoxyflavone
Cl 7   5-7-dihydroxy-3,2',3',4'-tetramethoxyflavone
Cl 8   5-7-dihydroxy-3,3',4',5'-tetramethoxyflavone
cki    Cyclin dependant kinase inhibitor
COMT   Catechol-O-methyl transferase
COX1   Cyclooxygenase-1
COX2   Cyclooxygenase-2
Cu     Copper
CYP450 Cytochromes P450
DAB    3,3'-Diaminobenzidine
DCA    Deoxycholic acid
DED    Death effector domain
DHC    Dihydrochalcones
DMAB   3,2'-Dimethyl-4-aminobiphenyl
DMBA   7,12-Dimethylbenz[a]anthracene
3',4'-DMF 3',4'-Dimethoxyflavone
DMSO   Dimethyl sulphoxide
DNA    Deoxyribonucleic acid
DPX    Distrene, tricresyl phosphate and xylene mounting medium
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>DR3</td>
<td>Death receptor 3</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECACC</td>
<td>European collection of cell cultures</td>
</tr>
<tr>
<td>ECG</td>
<td>(-)-Epicatechin-3-gallate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGCG</td>
<td>(-)-Epigallocatechin</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>Fe</td>
<td>Iron</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Ferrous ion</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>Ferric ion</td>
</tr>
<tr>
<td>FIGE</td>
<td>Field inversion gel electrophoresis</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FOL</td>
<td>Flavonoids</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin selective antibiotic</td>
</tr>
<tr>
<td>GCG</td>
<td>(-)-Gallocatechin-3-gallate</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H&lt;sub&gt;A&lt;/sub&gt;</td>
<td>Heterocyclic amines</td>
</tr>
<tr>
<td>HAH</td>
<td>Halogenated aromatic hydrocarbons</td>
</tr>
<tr>
<td>HCA</td>
<td>Hydroxycinnamic acids</td>
</tr>
<tr>
<td>(H&lt;sub&gt;C&lt;/sub&gt;H&lt;sub&gt;CHO&lt;/sub&gt;)&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Para-formaldehyde</td>
</tr>
<tr>
<td>HCT-8</td>
<td>Human ileocecal adenocarcinoma cell line</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'&lt;sub&gt;2&lt;/sub&gt;-2-ethanesulphonic acid</td>
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<tr>
<td>HIL1.1c2</td>
<td>Recombinant mouse hepatoma cell line</td>
</tr>
<tr>
<td>HSB</td>
<td>Hydroxystilbenes</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock proteins (x2 90 kDa)</td>
</tr>
<tr>
<td>HO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Hydroperoxyl radical</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitory κB</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>ICE-6</td>
<td>Non-cancerous rat epithelial cell line</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin-1β-converting enzyme</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IFV</td>
<td>Isoflavones</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>IND</td>
<td>Indoles</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IQ</td>
<td>2-Amino-3-methylimidazo[4,5-f]quinoline</td>
</tr>
<tr>
<td>JUK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>logP</td>
<td>Partition coefficient (octanol/water)</td>
</tr>
<tr>
<td>LPH</td>
<td>Lactase phloridzin hydrolase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mdm2</td>
<td>Mouse double minute 2</td>
</tr>
<tr>
<td>MMTV LTR</td>
<td>Mouse mammary tumour virus–long terminal repeat</td>
</tr>
<tr>
<td>3'M-4'NF</td>
<td>3'-methoxy-4'-nitroflavone</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>MPT pore</td>
</tr>
<tr>
<td>Mr</td>
<td>Relative molecular mass</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NQO1</td>
<td>NADP(H) oxidoreductase</td>
</tr>
<tr>
<td>'O2</td>
<td>Singlet Oxygen</td>
</tr>
<tr>
<td>'OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-Hydroxy-2'-deoxyguanosine</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>O2·⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl group</td>
</tr>
<tr>
<td>OMM</td>
<td>Outer mitochondrial membrane</td>
</tr>
<tr>
<td>PAD</td>
<td>Phenolic acids and derivatives</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PAI-2</td>
<td>Plasminogen activator inhibitor-2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PhIP</td>
<td>2-Amino-1-methylimidazo[4,5-b]pyridine</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RO-H2O</td>
<td>Reverse-osmosis water</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SF</td>
<td>Synthetic flavonoids</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Sodium-dependant glucose transporter</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SULT</td>
<td>Phenol sulphontransferases</td>
</tr>
<tr>
<td>tBid</td>
<td>Truncated Bid</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TCDF</td>
<td>2,3,7,8-Tetrachlorodibenzo-p-furan</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis inducing ligand</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]-aminomethane</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UGT</td>
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</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
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<td>X-associated protein 2</td>
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CHAPTER 1.

General Introduction.
1.1 DIET AND CANCER.

Cancer can be broadly defined as a disease of the somatic cells, which undergo uncontrolled proliferation, and in the process are altered into abnormal cells. These transformed cells have the ability to metastasise, enabling the spread of cancer to other areas of the body. The four main characteristics of cancer cells are, uncontrolled proliferation, loss of function, invasiveness and metastasis (Rang et al., 1996), which distinguishes them from normal cells. Such abnormalities tend to occur due to problems in the regulatory processes of the cell cycle. Normally, the mitotic entry and S phase (deoxyribonucleic acid (DNA) synthesis) checkpoints within the cell cycle regulate the replication of healthy cells and, if DNA damage is found at a checkpoint, the cell either undergoes DNA repair before the cycle is continued, or, if damage is extensive and repair is not possible, the cell undergoes apoptosis. However, in cancerous cells proliferation proceeds irrespective of DNA damage, due to altered feedback controls, abnormal production of growth factors, the inactivation of tumour suppressor genes, and/or the activation of proto-oncogenes (Rang et al., 1996).

Diet is reported to play an important role in both cancer development and prevention. Whilst some dietary factors may protect against or reduce the risk of cancer, others may increase the risk, however, it is notable that diet is only one of several factors involved in cancer development. Due to their mutagenic properties, compounds such as heterocyclic amines (HA) and polycyclic aromatic hydrocarbons (PAH), found predominately in fried meat, cooked at household temperatures, barbecued foods and smoked fish, are likely to act as initiators in the carcinogenic process, causing mutations in critical tumour suppressor genes and/or proto-oncogenes, which can lead to uncontrolled proliferation of the now mutated cells (Kleman & Overvik, 1995; Skog &
Chapter 1. General Introduction.

Jagerstad, 1998). For example, analysis of HA-induced squamous cell carcinomas in the Zymbal glands has shown mutations in the Ras and p53 oncogenes, both of which are thought to undergo multiple alterations during human colon carcinogenesis (Kleman & Overvik, 1995). Furthermore, food-derived HA are premutagens, and thus have to undergo metabolic activation by hepatic cytochrome P450 (CYP450) enzymes, before they are able to form DNA adducts, leading to DNA mutation(s) and, consequently, carcinogenesis (Kleman & Overvik, 1995). Fortunately, there are elements of a healthy diet that can aid the prevention of cancer development instigated by some of the aforementioned compounds. A wide range of dietary constituents have demonstrated cancer chemopreventive potential, including the micronutrients vitamin C and E, and phytochemicals such as isothiocyanates and polyphenols (Pezzuto, 1997; Ahmad et al., 1998; Singh & Lippman, 1998a; 1998b; Manson, 2003).

1.2 POLYPHENOLS AS CANCER PREVENTION AGENTS.

Due to the high incidence of cancer worldwide, a great deal of research is being undertaken into cancer chemoprevention, with emphasis being placed on the use of natural dietary chemicals. A variety of foods and beverages, to include fruits, vegetables, wine and tea, all with potential chemopreventive properties, are currently being investigated (Katiyar & Mukhtar, 1997; Mukhtar & Ahmad, 2000). Diets rich in fruits and vegetables have been strongly associated with a reduced risk of a number of common cancers. The majority of phytochemicals present in food, and thus the most widely considered for their chemopreventive qualities, are the plant polyphenols.

Plant polyphenols are products of plant secondary metabolism originating as a result of biochemical evolution, allowing individual plants to synthesise complex molecules as
Chapter 1. General Introduction.

defence agents capable of bestowing plants with anti-fungal, anti-bacterial and anti-viral properties, as well as having the ability to act as feeding deterrents to herbivores (Haslam, 1998; Bennick, 2002). However, extensive research has revealed that polyphenolics are also capable of affording similar protection to humans (Wargovich, 1997; Haslam, 1998; Le Marchand, 2002; Lambert & Yang, 2003).

1.2.1 Polyphenols: common features and properties.

Polyphenols are defined most simply and concisely as, “water soluble phenolic compounds having molecular weights between 500 and 3,000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins” (Bate-Smith & Swain, 1962). There are several common features that distinguish this class of widely distributed higher plant secondary metabolites from other phytochemicals. In their natural state, some minimal solubility is obtainable in aqueous media, although, certain pure polyphenols may well be difficult to solubilise. The natural molecular weight of plant polyphenols can range from ~200 to 3000-4000; nevertheless certain oligomeric compounds may exceed this, and, in general, they contain 12-16 phenolic groups and 5-7 aromatic groups, per 1000 relative molecular mass (Haslam, 1998).

In addition to phenolic reactions, polyphenols are also capable of precipitating certain alkaloids, gelatin and other proteins, from solution. Complexation with peptides and proteins is predominantly driven by polyphenol hydrophobicity, thus facilitating a decrease in the surface area of hydrophobic groups exposed to aqueous solution (Haslam, 1974; Hagerman & Butler, 1981; Luck et al., 1994; Haslam, 1998). Even though polyphenols may encompass a wide range of chemical structures,
fundamentally, their basic structures are based on either the condensed proanthocyanidins (catechin), or galloyl and hexahydroxydiphenoyl esters and their derivatives (ellagic acid), or the more recently recognised minor class, based on the phlorotannin isolates from several genera of red-brown algae, with a phloroglucinol subunits linked by C–C and C–O bonds (Haslam, 1998). Examples of biologically active polyphenols, and their chemical class, commonly available in the human diet, are listed in Table 1.1.

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Chemical Class</th>
<th>Dietary Source</th>
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<tr>
<td>EGCG</td>
<td>Flavan-3-ols</td>
<td>Green Tea and Cocoa</td>
</tr>
<tr>
<td>Theaflavin</td>
<td>Flavan-3-ols</td>
<td>Black Tea</td>
</tr>
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<td>Quercetin</td>
<td>Flavonols</td>
<td>Tomatoes and Onions</td>
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<td>Kaempferol</td>
<td>Flavonols</td>
<td>Horseradish and Kale</td>
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<tr>
<td>Naringenin</td>
<td>Flavanones</td>
<td>Grapefruit</td>
</tr>
<tr>
<td>Genistein</td>
<td>Isoflavones</td>
<td>Soya Beans</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Phenolic Acids and Derivatives</td>
<td>Turmeric</td>
</tr>
<tr>
<td>Phloridzin</td>
<td>Dihydrochalcones</td>
<td>Apples</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Hydroxystilbenes</td>
<td>Grapes</td>
</tr>
</tbody>
</table>

Table 1.1: Examples of biologically active polyphenols and their dietary sources.

1.2.2 Chemopreventive activity.

In the search for new drugs and medicines, natural plant products have been at the forefront of development in the pharmaceutical industry. In recent years, considerable attention has been focused on the potential ability of naturally occurring plant polyphenols to act as chemopreventive agents (Ahmad et al., 1998; Haslam, 1998). Continuing research is being carried out on a wide range of polyphenols, including resveratrol ($trans$–3,5,4$'$–trihydroxystilbene), a natural phenolic substance found in grapes and wine, which has been shown to have anti-tumour activity via an apoptotic pathway (Kwang-Seok, et al., 1999). A variety of isothiocyanates, found in cruciferous vegetables such as cauliflower, cabbage and broccoli, are reported to modify carcinogen
metabolism, which results in an increase in carcinogen excretion or detoxification and a reduction in the formation of carcinogen DNA-adducts (Singh & Lippman, 1998b). Moreover, cocoa and chocolate, derived from the cocoa bean, are rich in (+)-catechin, (-)-epicatechin and proanthocyanidins, which, in epidemiological studies, are reported to increase plasma antioxidant levels, a desirable defence against reactive oxygen species (ROS) (Weisburger, 2001). Curcumin, the yellow pigment found in turmeric, is reported to effectively inhibit carcinogenic activity, in vitro, by a number of mechanisms, to include cell cycle arrest and induction of apoptosis (Manson, 2003). There is also substantial interest in the chemopreventive actions of green, oolong and black tea polyphenols (Mukhtar & Ahmad, 1999; Pan et al., 2000a; Wang & Bachrach, 2002; Lambert & Yang, 2003).

Natural plant polyphenols are of great importance in cancer research, primarily due to their non-toxic nature; equally they are inexpensive and widely accepted by the public. In addition, such work is encouraged by the incentive that polyphenols are much less likely to cause severe side effects, similar to those experienced by conventional cancer therapies, where patients undergoing radiation or chemotherapy encounter many unpleasant side effects such as nausea, vomiting, hair loss, impaired wound healing and possible sterility (Rang et al., 1996; Ahmad et al., 1998).

1.2.3 Tea: production and consumption.

It is generally accepted that, next to water, tea is the most extensively consumed beverage on the planet, with a per capita consumption of approximately 120 ml per day. Moreover, to sustain this degree of consumption, a staggering 2.5 million metric tonnes of dried tea leaves are produced annually across the globe, of which 20 % is green tea,
which is produced in a few countries, and generally consumed in China, Japan, India and certain areas of North Africa and the Middle East. Less than 2 % becomes oolong tea, which is produced and consumed in both South-Eastern China and Taiwan. The remaining 78 % is converted into black tea, which is primarily consumed in the West and some areas of Asia (Ahmad et al., 1998; Mukhtar & Ahmad, 2000).

Production of green or unfermented tea involves the rapid steaming or pan frying of freshly gathered *Camellia sinensis* leaves, which inactivates a number of oxidation enzymes, thus inhibiting fermentation, before drying. Oolong or semi-fermented tea is prepared by steaming the leaves shortly after the rolling process, inactivating enzymes in the leaf and terminating oxidation early, thus obtaining only partial fermentation. Consequently, oolong tea retains characteristics of both black and green tea. Black or fermented tea undergoes a long process in which the leaves are left to wither until only 55 % of the original leaf weight remains, before they are rolled and crushed, initiating polyphenol oxidation, ahead of firing which brings fermentation to a conclusion. This process transforms the colourless, water-soluble monomeric catechins found in green tea, via polyphenol oxidase-dependent oxidative polymerisation, into thearubigins and theaflavins, which give black tea its distinctive colour, taste and aroma (Ahmad et al., 1998; Haslam, 1998; Mukhtar & Ahmad, 2000).

Green tea comprises a similar chemical composition to fresh tea leaves, with polyphenolic compounds accounting for up to 60 % of dry weight. Major flavan-3-ol compounds, (-)-epicatechin, (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and EGCG, constitute some 20-30 %, imparting bitterness and astringency into a green tea infusion. Following oxidation, 10-20 % dry weight of black tea comprises
thearubigins, and 0.3-2 % is theaflavins, consisting of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate (Figure 1.1) (Ahmad et al., 1998; Haslam, 1998; Yang et al., 2002).

![Chemical structures of major polyphenols in green, black, and oolong tea](image)

Black and green tea also contains approximately 3 % flavonols (\(^{\text{w/w}}\)), predominately present as glycosides rather than in their aglycone form. These include the major tea flavonols of quercetin, kaempferol and myricetin, which may be associated with a
number of different sugar moieties, including glucose, rhamnose, galactose, arabinose and fructose, as mono-, di- or tri-glycerides (Wang et al., 2000; Yang et al., 2002). Oolong tea contains a mixture of green tea monomeric catechins and black tea theaflavins and thearubigins, in addition to the characteristic compounds of dimeric catechins and proanthocyanidins, epigallocatechin esters and theasinensins (Ahmad et al., 1998; Yang et al., 2002). In addition, black, green and oolong tea also contain small amounts of methyl xanthines. Caffeine makes up approximately 5-10 % (w/w), although these levels may vary between tea infusions, depending on leaf size, brewing time, and water temperature (Marks, 1992; Yang et al., 2001a; 2002).

1.2.4 Anti-oxidant properties.

Defined as a disturbance in the pro-oxidant and antioxidant balance within a cell, organ or organism, in favour of the former, resulting in potential cellular damage (Sies, 1985), oxidative stress has been implicated in an array of pathologies, including carcinogenesis. Reactive products of oxygen, to include superoxide anion (O$_2^{−}$), singlet oxygen (O$_2^{·}$), hydroxyl radical (·OH), hydroperoxyl radical (HO$_2^{·}$) and H$_2$O$_2$, are produced within all aerobic cells during normal metabolic processes, such as detoxification reactions in the liver by CYP450 enzymes. Furthermore, a major source of intracellular O$_2^{−}$ occurs via “leakage” of electrons onto oxygen (O$_2$) from various components of cellular electron transport chains, in both mitochondria and endoplasmic reticulum (Figure 1.2) (Halliwell & Gutteridge, 1990; Chandra et al., 2000). Exogenous sources of ROS are generated following exposure to a toxic insult, such as environmental pollutants, alcohol, cigarette smoke, asbestos, ionising radiation, and subsequent to a bacterial, fungal or viral infection. All have the potential to induce cellular damage, by means of protein damage, DNA strand breakage and lipid
peroxidation, as demonstrated by the H$_2$O$_2$-induced apoptosis in a variety of cancer cell lines (Burdon, 1995; Dumont et al., 1999; Halliwell & Gutteridge, 1999; Barbouti et al., 2002; Ogawa et al., 2003).

Figure 1.2: Generation of ROS and the main defence mechanisms against ROS-induced damage (adapted from Mates & Sanchez-Jimenez, 2000). CAT, catalase; CuZn-SOD, copper-zinc superoxide dismutase; ER, endoplasmic reticulum; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidised glutathione; Mn-SOD, manganese superoxide dismutase.

Fortunately, intracellular antioxidants provide a defence against oxidative attack (Figure 1.2). Hydrophilic scavengers, to include glutathione (GSH) and ascorbic acid, commonly found in cytosolic, mitochondrial and nuclear aqueous cellular compartments, are highly important free radical scavengers, with the ability to continuously scavenge ROS such as HO$_2^-$ and $^1$O$_2$ (Chaudiere & Ferrari-Iliou, 1999; Chandra et al., 2000). In addition, protective antioxidant enzymes scavenge ROS by specific enzymic degradation. For example, dismutation of O$_2^{2-}$ to H$_2$O$_2$ is ensured by superoxide dismutase (SOD), whilst H$_2$O$_2$ can either undergo dismutation to O$_2$ and water by catalase, or using GSH, reduction by glutathione peroxidase (GPx), to oxidised.
glutathione (GSSG) and water (Brunori & Rotilio, 1984; Chandra et al., 2000; Mates, 2000; Mates & Sanchez-Jimenez, 2000).

Additionally, polyphenolic compounds may provide an antioxidant defence against oxidative stress, primarily due to their antioxidant properties via the mechanisms of free radical scavenging and/or metal chelation. Since transition metals such as iron (Fe) and copper (Cu) can participate in ROS generation, chelation of these metals will decrease the $O_2$ toxicity to cells. Moreover, preferential binding to ferrous ion (Fe$^{2+}$) changes the redox potential, by converting a Fe$^{2+}$ ion to its ferric state (Fe$^{3+}$), and thus inhibiting oxidative damage (Deng et al., 1997; Haslam, 1998; Khokhar & Apenten, 2003). Therefore, much attention has focused on the use of natural antioxidants, such as plant polyphenols, with the potential ability to protect from free radical damage by the aforementioned antioxidant mechanisms.

The structural groups, reported as important determinants for antioxidant potential via free radical scavenging and/or Fe-binding, include the presence of a 3',4'-dihydroxy (catechol) structure on the B-ring, the presence of 3-hydroxyl substituent, a 2,3 double bond in conjugation with a C4 keto group, as in quercetin (Figure 1.3), and a large number of hydroxyl (OH) groups, as in tannic acid. Conversely, the presence of a 3',4',5'-trihydroxy (gallo) group on the B-ring is reportedly associated with reduced Fe-binding (Khokhar & Apenten, 2003; Deng et al., 1997). In general, phenolic compounds assessed for antioxidant activity, demonstrated augmented activity, which was associated with an increased number of OH groups and a reduction in glycosylation (Fukumoto & Mazza, 2000). Moreover, the same workers reported that, at concentrations below 500 $\mu$M, most of the phenolic compounds investigated exhibited
pro-oxidant activity, whereas α-tocopherol and synthetic antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, did not (Fukumoto & Mazza, 2000).

![Quercetin Diagram](image)

**Figure 1.3: Quercetin, an example of general features of flavonoid structure, nomenclature and numeration** (Kroon et al., 2004). Commonly a flavonoid backbone is based upon a C₆-C₃-C₆ skeleton, with a chroman ring (C ring) and two aromatic rings (A and B rings), containing positions to which functional groups, such as hydroxyls, may attach.

Paradoxically, there is also considerable interest in the potential of polyphenolics to exhibit pro-oxidant properties (Long et al., 2000; Li & Xie, 2000; Loo, 2003). Polyphenols, including EGCG and quercetin are reported to generate H₂O₂, after addition to a variety of commonly used cell culture media (Long et al., 2000; Sakagami et al., 2001). Moreover, black tea theaflavins may also produce H₂O₂, subsequently leading to apoptosis in several cell lines, most likely due to their pro-oxidant activity (Feng et al., 2002). Collectively, these studies allude to the possibility that apoptosis induced by polyphenols may be as a consequence of increased extracellular levels of H₂O₂ (Long et al., 2000; Sakagami et al., 2001; Feng et al., 2002). For the most part, the potential pro-oxidant or antioxidant activity of polyphenols is dependent on a number of factors. In addition to metal reducing and chelating behaviour, their pH, solubility characteristics, stability within tissues and cells, and bioavailability should also be considered (Feng et al., 2002).
1.2.5 Polyphenol bioavailability.

Dietary polyphenols may well possess particular properties, which are reportedly beneficial to human health. During this study, experiments were performed in vitro, particularly with relation to the accessibility of a compound to the individual cell. Nevertheless, to understand the potential chemopreventive activity of polyphenols in vivo, their bioavailability, including absorption in the gastrointestinal (GI) tract, distribution to the tissues, metabolism and cellular uptake, must also be considered (Walgren et al., 2000; Rechner et al., 2002; Catterall et al., 2003; Day et al., 2003).

Some polyphenols present in foods may be considered non-absorbable, others are deemed to permeate, and passively diffuse across the cell membrane, hence, the cell permeability of a polyphenol is dependent upon its chemical structure (Scalbert & Williamson, 2000). To ascertain whether a compound will passively diffuse across a biological membrane, the octanol/water partition coefficient (logP), in which an increasing partition coefficient denotes increasing hydrophobicity, can be measured in the absence of other parameters, providing clues to a compound's bioavailability. For example, glycosylation can influence polyphenol chemical, physical and biological properties, and thus to passively traverse a biological membrane, the predicted first step in polyphenol absorption (Figure 1.4) is enzymic removal of the attached sugar moiety by β-glucosidases (Hollman & Katan, 1999; Scalbert & Williamson, 2000; Aherne & O'Brien, 2002). Moreover, galloyl substitutions can also potentially influence polyphenol bioavailability, although to not such a dramatic extent as glycosylation, since they only result in minor alterations to the partition coefficient (Scalbert & Williamson, 2000; Yang et al., 2001a; 2002).
Although flavonoid glycosides are generally hydrophilic, and therefore unable to passively traverse biological membranes, uptake studies indicate that, to a small extent, the sodium-dependent glucose transporter, SGLT1, partakes in the active transport of quercetin glucosides (Gee et al., 1998; Day et al., 2003). In addition, highly hydrophilic quercetin glucuronides may actively enter cells via membrane transporters.
similar to OATP2, an organic anionic transporter, found predominantly in hepatocytes (O’Leary et al., 2003; Walle, 2004). However, deglycosylation in the small intestine by lactase phlorizin hydrolase (LPH), and subsequent diffusion of the aglycone, is reported to be the major route of absorption for quercetin glucosides in vivo (Day et al., 2003). Furthermore, efficient absorption of certain glycosides is reportedly suppressed by efflux through the anionic transporter, multidrug resistance protein (MRP) 2, which is surprising, considering that flavonoid glycosides are not anionic in nature. The MRP isoforms MRP1 and MRP2 have also been shown to efflux green tea polyphenols, including ECG and EGCG, with the suggestion that EGCG metabolites may also be exported by MRP energy-dependent efflux (Hong et al., 2002; Walle, 2004).

In general, polyphenol metabolism follows a similar pathway to many drugs. Deconjugation and absorption are dependent upon polyphenol structure, for example, quercetin glucoside, is absorbed from the small intestine either as a result of active transport, or luminal deglycosylation by LPH, and subsequent passive diffusion of the aglycone. Due to the presence of β-glycosides, polyphenolic compounds such as rutin, can only be absorbed from the colon after deglycosylation by the colonic gut microflora, however, the liberated aglycone will also be degraded at the same time. Any flavonoid glycosides that are absorbed intact, by active transport, are usually deglycosylated by endogenous broad-specificity cytosolic β-glucosidase (CBG) in the liver or enterocytes (Hollman et al., 1997; Day et al., 2000; Kroon et al., 2004). Regardless of the mechanism of absorption, following deconjugation, the aglycone, in this case, quercetin, is subjected to three main types of conjugation reaction, namely glucuronidation, sulphation and methylation. Although conjugation rates vary depending upon, species, sex, and food intake, conjugation reactions with glucuronic
acid and/or sulphate seem to be the most common metabolic pathway for flavonoids in humans (Day et al., 2000; Aherne & O’Brien, 2002; Manach et al., 2004). The UDP-glucuronyltransferases (UGT), a large family of membrane-bound enzymes, generally situated in the endoplasmic reticulum, catalyse polyphenol glucuronidation reactions, which occur predominately by the UGT1A subfamily in the liver, kidney and intestine. Phenol sulphontransferases (SULT), a small group of widely distributed cytosolic enzymes, are then involved in polyphenol sulphation. Methylation occurs in a wide range of tissues, by catechol-O-methyl transferase (COMT). Polyphenolics that are not absorbed in the stomach or small intestine are generally carried to the colon, where they are degraded by colonic gut microflora into more simple compounds, such as phenolic acid, before being excreted via urine or bile (Scalbert & Williamson, 2000; Yang et al., 2001a; 2001b; O’Leary et al., 2003; Manach et al., 2004; Walle, 2004).

One study compared the metabolism of the proanthocyanidin dimer B3, trimer C2 and polymer, isolated from willow tree catkins, to that of (+)-catechin, in rats. Rats fed the (+)-catechin monomer excreted large amounts of the parent compound and its 3’-O-methyl derivative in the urine, however, in rats fed proanthocyanidins neither was detected. Moreover, several microbial metabolites were detected in the urine, the yield of which significantly decreased as the degree of polymerisation increased (Gonthier et al., 2003). The metabolic fate of (+)-catechin, (-)-epicatechin, EGC, ECG and EGCG, were investigated in a number of different studies. After the oral administration, to rats, of (+)-catechin or (-)-epicatechin, more efficient absorption was observed by (+)-catechin, as demonstrated by a higher excretion in the urine and a greater absorption rate constant, in comparison with (-)-epicatechin (Catterall et al., 2003). Moreover, Kohri et al. (2001) reported that, following oral administration to rats, EGCG appears
not to be absorbed intact, but first undergoes microbial degradation by the colonic gut microflora before being absorbed, which is also believed to occur with (+)-catechin and (-)-epicatechin (Catterall et al., 2003). Additionally, in rats, derivatives were conjugated, principally with glucuronic acid and to a lesser extent with sulphates. In contrast, sulphation is reported to predominate in humans (Baba et al., 2001; Kohri et al., 2001; van Amelsvoort et al., 2001; Vaidyanathan & Walle, 2002; Catterall et al., 2003). Therefore, it is increasingly apparent that to fully appreciate the potential biological activity of polyphenols, the activity of their metabolites must also be considered.

1.3 CHEMOPREVENTIVE POTENTIAL OF POLYPHENOLS.

The numerous animal studies undertaken with the aim of investigating the chemopreventive effects of polyphenols on solid tumours have been promising, many demonstrating a reduction in tumour size. Whilst some studies have focussed on the mechanisms of action of polyphenols in vivo, others have looked at the biological response of polyphenols upon ultraviolet (UV)-B or chemically induced solid tumours (Table 1.2).

1.3.1 UVB-induced tumours: impact of polyphenolics.

Several studies have been undertaken to investigate the potential inhibitory effects of polyphenols upon the growth of UVB-induced tumours (Lu et al., 1997; 2002; Conney et al., 1999; Wei et al., 2002). Chronic exposure to solar radiation, including UVB, is reportedly the primary cause of skin cancer. However, prevention is possible by oral or topical application of antioxidants, including those found in tea, which may function, primarily by regulating cell cycle progression and induction of apoptosis (Ichihashi et
al., 2003). One study, investigating the inhibitory effect of black tea on the growth of established skin tumours in mice, revealed that, following oral administration of black tea, as the sole source of drinking fluid for 11-15 weeks, UVB-induced tumour growth was inhibited by 70%. Further histological examination of the tumours revealed that tea-treated mice had a 58 and 54% reduction in the number of non-malignant tumours and squamous cell carcinomas per animal, respectively (Lu et al., 1997). Moreover, black tea administration reduced the size of non-malignant tumours and carcinomas by 60 and 84%, respectively, as well as increasing the apoptotic index by approximately 40%, implying that black and green tea may potentially inhibit cell proliferation, and enhance apoptosis in mice bearing either non-malignant or malignant tumours (Lu et al., 1997). In a similar study Conney et al. (1999) reported that, following oral administration of black or green tea over a 23-week period as their sole source of drinking fluid, mice, which were administered tea, not only had fewer UVB-induced tumours, but these were considerably smaller compared to those in the control mice (Conney et al., 1999). The same workers also reported, after histologic examinations, that administration of black tea could inhibit DNA synthesis and enhance apoptosis, in both non-malignant and malignant tumour bearing mice (Conney et al., 1999). These findings were later corroborated by Lu et al. (2002), wherein UVB irradiated mice, topically treated with EGCG for 18 weeks, demonstrated a decrease in the number of non-malignant and malignant tumours per mouse, by 55 and 66%, respectively. Subsequent immunohistochemical analysis of the epidermis showed EGCG to increase apoptosis, as measured by caspase-3 positive cells, in both non-malignant tumours and squamous cells carcinomas by 72 and 56%, respectively, whilst no apoptotic response was observed in non-tumour areas of the epidermis, indicating that EGCG may only induce apoptosis in cancerous cells (Lu et al., 2002).
Table 1.2: Summary of evidence for the chemopreventive effects of polyphenolic compounds and extracts, in animal studies. AOM, azoxymethane; DMAB, 3,2'-dimethyl-4-aminobiphenyl; DMBA, 7,12-dimethylbenz[a]anthracene; PhIP, 2-amino-1-methylimidazo[4,5-b]pyridine; UVB, ultraviolet-B.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carcinogenic Insult</th>
<th>Chemopreventive Effect</th>
<th>Tissue</th>
<th>Animal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black &amp; green tea</td>
<td>UVB</td>
<td>↓ Tumour size</td>
<td>Skin</td>
<td>Mouse</td>
<td>Conney et al. (1999)</td>
</tr>
<tr>
<td>Black tea</td>
<td>UVB</td>
<td>↑ Tumour size</td>
<td>Skin</td>
<td>Mouse</td>
<td>Lu et al. (1997)</td>
</tr>
<tr>
<td>EGCG</td>
<td>UVB</td>
<td>↓ Tumour numbers</td>
<td>Skin</td>
<td>Mouse</td>
<td>Lu et al. (2002)</td>
</tr>
<tr>
<td>Genistein</td>
<td>UVB</td>
<td>↑ Inhibition of oxidative DNA damage</td>
<td>Skin</td>
<td>Mouse</td>
<td>Wei et al. (2002)</td>
</tr>
<tr>
<td>Hesperidin-rich fruit juice</td>
<td>AOM</td>
<td>↑ Apoptosis in tumours Suppressed tumours</td>
<td>Colon</td>
<td>Rat</td>
<td>Tanaka et al. (2000)</td>
</tr>
<tr>
<td>Black &amp; green tea extracts</td>
<td>AOM</td>
<td>↑ Apoptosis in tumours Tumour numbers</td>
<td>Colon</td>
<td>Rat</td>
<td>Caderni et al. (2000)</td>
</tr>
<tr>
<td>Black &amp; green tea extracts</td>
<td>AOM</td>
<td>↓ Tumour numbers</td>
<td>Colon</td>
<td>Rat</td>
<td>Yamane et al. (1991)</td>
</tr>
<tr>
<td>Green tea &amp; curcumin</td>
<td>DMBA</td>
<td>↓ Tumour incidence</td>
<td>Mouth</td>
<td>Hamster</td>
<td>Li et al. (2002)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>DMBA, PhIP</td>
<td>No significant effect against tumours</td>
<td>Prostate</td>
<td>Rat</td>
<td>Imaida et al. (2001)</td>
</tr>
<tr>
<td>Turmeric</td>
<td>DMBA</td>
<td>Suppressed tumours</td>
<td>Skin</td>
<td>Mouse</td>
<td>Azuine &amp; Bhide (1992)</td>
</tr>
<tr>
<td>Red wine &amp; black tea polyphenols</td>
<td>AOM</td>
<td>↑ COX2, GST &amp; iNOS expression</td>
<td>Colon</td>
<td>Rat</td>
<td>Luceri et al. (2002)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>AOM</td>
<td>↑ Apoptosis in tumours</td>
<td>Colon</td>
<td>Rat</td>
<td>Kawamori et al. (1999)</td>
</tr>
</tbody>
</table>

The soybean isoflavone, genistein, has also been shown to inhibit UVB-induced carcinogenesis in hairless mice (Wei et al., 2002). In these studies, UVB exposure significantly augmented H$_2$O$_2$ production in the skin of irradiated mice, however, topical pre-treatment with 10 µM genistein, 1 hour before UVB exposure, inhibited UVB-induced H$_2$O$_2$ production through either direct scavenging of ROS or indirect inhibition of neutrophil recruitment. In addition, the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a hallmark of oxidative DNA damage, was inhibited by genistein. Taken together, these observations suggest that the anti-photocarcinogenic action of genistein is most likely due to its antioxidative properties (Wei et al., 2002).
1.3.2 *Chemopreventive effects of polyphenols in chemically induced tumours.*

Numerous studies have been conducted to evaluate the influence of polyphenols upon chemically induced tumours (Kawamori *et al*., 1999; Caderni *et al*., 2000; Luceri *et al*., 2002). Studies investigating the chemopreventive efficacy of a variety of green tea polyphenols, given as a drinking solution, upon azoxymethane (AOM)-induced colon carcinomas, demonstrated that, irrespective of tea polyphenol used, no significant difference was found in the size or occurrence of tumours in the colon or small intestine, compared to control rats (Weisburger *et al*., 1998). The same workers also observed that, although certain CYP450 isoforms and glutathione-S-transferase (GST) were suppressed by tea, conversely, CYP2E1, the principal isoenzyme required for AOM activation to methylazoxymethanol, was not affected. Thus, tea polyphenols appear unable to modify AOM-induced colon carcinogenesis, since they are unlikely to inhibit the biochemical mechanism associated with AOM metabolism (Weisburger *et al*., 1998). On the contrary, investigations by Caderni *et al.* (2000) demonstrated the clear inhibition of AOM-induced intestinal carcinogenesis, following oral administration of black and green tea extracts, by a mechanism thought to involve increased apoptosis in tumours. This was corroborated by Tanaka *et al.* (2000), who observed an increase in the apoptotic index of AOM-induced colonic adenocarcinomas in rats, given hesperidin-rich mandarin juices, by oral administration.

In a separate study, investigating the effect of both tea and curcumin upon 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamsters, following topical application of DMBA, hamsters received either oral administration of green tea, topical administration of curcumin or a combination of the two (Li *et al*., 2002). The combination treatment gave rise to a significant decrease in both oral
tumour and squamous cell carcinoma incidence, as well as a reduction in total tumour volume. Neither agent alone was more effective than both in combination (Li et al., 2002). The same researchers reported that, in the presence of tea alone, or in combination with curcumin, a significant increase in the apoptotic index in squamous cell carcinomas was evident, indicating that green tea and curcumin may provoke an inhibitory response against oral carcinogenesis in the promotion stage, which might be related to both suppression of cell proliferation and induction of apoptosis (Li et al., 2002).

Initial studies, investigating the chemopreventive effects of turmeric in chemically induced tumours given in the diet, reported a significant inhibition of DMBA-induced skin and benzo[a]pyrene (B[a]P)-induced forestomach tumours, in female Swiss mice, as observed by a significant decrease in hepatic CYP450 enzyme activity and a related concomitant increase in GSH content and GST activity. This implies that turmeric is an effective anticancer agent at both initiation and progression stages of carcinogenesis (Azuine & Bhide, 1992). In addition, curcumin, the major polyphenol found in turmeric, is reported to have a chemopreventive effect in chemically induced tumours, in mice (Huang et al., 1997). In this study, following administration of curcumin, B[a]P-induced forestomach and N-ethyl-N'-nitro-N-nitroguanidine (ENNG)-induced duodenal carcinogenesis were inhibited, as observed by a decrease in tumour volume and tumour numbers (Huang et al., 1997). In contrast, the potential chemopreventive efficacy of curcumin was investigated using the 3,2'-dimethyl-4-aminobiphenyl (DMAB)- and 2-amino-1-methylimidazo[4,5-b]pyridine (PhIP)-induced prostate carcinogenesis models, in which neither summational nor synergistic chemoprevention was evident (Imaida et al., 2001). These encouraging results, for the chemopreventive
action of polyphenolic compounds, supported by other experimental studies (Dreosti et al., 1997; Lou et al., 1999; Mahmoud et al., 2000; Luceri et al., 2002), demonstrate that polyphenolics are likely to afford protection in a variety of solid tumour systems, however, further studies are required to fully elucidate their mechanisms of action, including those leading to inhibition of cell proliferation and DNA synthesis, and the induction of apoptosis.

1.3.3 Gerlans protocol.

The chemical-induced carcinogenesis Solt and Faber procedure (Solt et al., 1977) was modified and improved by Lans et al. (1983), with dramatic results, demonstrating that, after the original administration of diethylnitrosamine and 2-acetylaminofluorene, exposure of rats to phenobarbital encouraged hepatocellular carcinoma promotion (Lans et al., 1983). Now known as the Gerlans protocol, this procedure yields phenotypically altered hepatocytes, which can occupy approximately 40% of liver volume after only seven days of phenobarbital treatment. It has been used as a model to confirm that genetic alterations occur at a chromosomal level (micronuclei expression), during the different steps of chemically-induced rat hepatocarcinogenesis (van Goethem et al., 1993). Moreover, the inhibitory effects of black tea extract, oolong tea extract and the green tea polyphenols, epicatechin, EGC, ECG and EGCG, in F344 rats exhibiting aberrant hepatic foci, were investigated using the Gerlans protocol. A decrease in the number and the area of preneoplastic foci was noted, in both initiation and promotion phases of carcinogenesis. This indicates that any potential chemopreventive activity afforded by the abovementioned polyphenols against chemically-induced hepatocarcinogenesis, in this model at least, appears somewhat weak (Matsumoto et al., 1996).
1.3.4 Inhibition of carcinogenesis by polyphenols: proposed mechanisms.

The vast array of dietary polyphenols currently under investigation for their anticarcinogenic potential are reported to mediate their response by multiple mechanisms, which are generally antioxidant in nature (Yang et al., 2002; Manson, 2003). Although the antioxidant potential of many polyphenols appear strong, the in vivo antioxidative effect is relatively weak, mainly as a result of low bioavailability (Yang et al., 2002). However, UVB-induced skin carcinogenesis elicited by a series of oxidative events, including H₂O₂ production, was attenuated by direct free radical scavenging of H₂O₂, by the soy isoflavone genistein (Wei et al., 2002).

Studies investigating the chemopreventive effect of tea polyphenols on chemically-induced tumours, induced by chronic administration of carcinogens such as PhIP and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), in rodents, reported an inhibition of CYP450 Phase I enzymes, after polyphenol treatment (Xu & Dashwood, 1999; Le Marchand, 2002). In addition, similar studies have shown that polyphenols increase the activity of Phase II conjugation enzymes, including GST, glucuronidases and SULT, thus increasing the elimination of genotoxic intermediates (Xu & Dashwood, 1999; Yang et al., 2001a; 2001b; 2002; Le Marchand, 2002; Ioannides & Yoxall, 2003).

A variety of different polyphenolic compounds have been shown to modulate proto-oncogenes, tumour suppressor genes and signal transduction pathways, leading to the inhibition of cell proliferation, transformation and angiogenesis, and the induction of apoptosis (Luceri et al., 2002). Certain compounds, including curcumin, quercetin, resveratrol and apigenin, have demonstrated anti-inflammatory activity by inhibiting arachidonic acid metabolism and, as a consequence, the associated enzymes of
lipooxygenase, phospholipase A\textsubscript{2} and cyclooxygenase-2 (COX2). Chronic inflammation is reported to play a role in carcinogenesis and thus polyphenols may be considered as potential COX2 inhibitors. Furthermore, isoflavones such as genistein, daidzein and lignans (enterodiol), more commonly regarded as phytoestrogens, may potentially inhibit mammary and prostate carcinogenesis, probably due to modulation of oestrogenic activity (Jang \textit{et al.}, 1997; Yang \textit{et al.}, 2001b; Le Marchand, 2002; Luceri \textit{et al.}, 2002). Moreover, resveratrol, found in various plants including grapes, is believed to inhibit events associated with tumour initiation, promotion and progression, by induction of phase II metabolising enzymes (anti-initiation), inhibition of cyclooxygenase-1 (COX1) (anti-promotion) and inhibition of preneoplastic legions in DMBA-treated mice (anti-progression) (Jang \textit{et al.}, 1997). In addition, polyphenolic compounds extracted from black tea and red wine, inhibited tumour promotion in rat AOM-induced colon carcinogenesis. This was associated with a significant increase in COX2, inducible nitric oxide synthase (iNOS) and GST gene expression, in comparison to corresponding healthy mucosa, suggesting a possible therapeutic activity for these polyphenolic compounds (Luceri \textit{et al.}, 2002).

Another potential mechanism is thought to involve tumour necrosis factor (TNF-\textalpha), an essential cytokine, considered to have activity as an endogenous tumour promoter. EGCG and other green tea polyphenols were reported to inhibit tumour promotion and chemical carcinogenesis via inhibition of TNF-\textalpha gene expression, in a dose-dependent manner (Fujiki \textit{et al.}, 1999; Fujiki & Suganuma, 2002). Similar findings were observed by Suganuma \textit{et al.} (2000), who reported that green tea extract, administered in the drinking water of transgenic mice, inhibits TNF-\textalpha expression and reduces TNF-\textalpha protein levels.
In addition, suppression of nuclear factor κB (NF-κB) inducing kinase (NIK), and/or mitogen-activated protein kinase (MAPK) signal transduction pathways, including extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JUK) and p38, by polyphenols, has been reported to prevent nuclear translocation of transcription factors, such as activator protein-1 (AP-1) and NF-κB. Given that AP-1 and NF-κB are considered anti-apoptotic and promote survival in cancer cells, their inhibition by polyphenols may potentially enhance apoptotic activity (Cross et al., 2000; Lin, 2002; Yang et al., 2002; Lin et al., 2003; Manson, 2003). Similar observations were made in studies investigating the anticancer effects of EGCG in UVA-damaged and transformed cells (Kim et al., 2001a; Wang & Bachrach, 2002). After reporting that EGCG reduced UVA-induced skin damage in hairless mice, further studies, by the same workers, in fibroblast cells, demonstrated that UVA-induced activation of AP-1 and NF-κB was blocked following EGCG treatment (Kim et al., 2001a). In addition, following exposure to EGCG, transformed fibroblasts were reported to inhibit MAPK activity, resulting in DNA fragmentation (Wang & Bachrach, 2002). Furthermore, polyphenolics are reported to aid chemoprevention, via modulation of cell cycle control via cyclin-dependent kinases (cdk) and their inhibitors (cki), leading to cell cycle arrest and ultimately apoptosis (Yang et al., 2002; Manson, 2003). Hence, to fully appreciate and understand polyphenolic chemopreventive mechanisms of action, including induction of apoptosis and modulation of cell cycle regulation, both in vivo and in vitro studies would benefit from integration.

1.4 MECHANISMS OF APOPTOSIS.

The main component of chemoprevention investigated during this study was the involvement of apoptosis in the modulation of cancer. Hence, subsequent sections will
focus on the mechanisms of apoptosis and the potential chemopreventive action of polyphenols as a result of apoptotic induction.

Cell death can occur in one of two ways. Necrosis generally appears as a result of acute cellular dysfunction, following severe stress insult or toxic exposure, and is a passive process associated with the rapid depletion of cellular adenosine triphosphate (ATP). Cells undergoing necrosis are morphologically characterised by an increase in cell volume, along with their intracellular organelles, they swell until the plasma membrane ruptures, releasing intracellular proteins into the extracellular space, thus affecting neighbouring cells or attracting pro-inflammatory cells, which may lead to further tissue injury (Halestrap, 2000; Chandra et al., 2000). The second and non-inflammatory form of cell death is known as apoptosis, occurring during numerous pathologies in multicellular organisms, and constituting a common mechanism of cell replacement and tissue remodelling, as well as being important in removing cells that are moderately damaged due to mild insults, such as hypoxia (Halestrap, 2000; Chandra et al., 2000).

1.4.1 Apoptotic cell morphology.

Originating from the Greek “ptosis” meaning a “fall” or “drop”, apoptosis is the term coined by Kerr et al. in 1972 to describe the distinctive morphology of a particular kind of dying cell. Apoptotic cell death occurs in two phases, an initial commitment or initiation phase followed by the execution phase, which is characterised by a series of stereotypic morphological changes, to include cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation and formation of “apoptotic bodies” (Figure 1.5). Moreover, apoptotic morphology appears comparable in both vertebrate and invertebrate cells, regardless of the initial apoptotic signal, suggesting the existence of a
common biochemical pathway. Early on in apoptosis, the nucleus shrinks, leading to chromatin condensation and fragmentation. Subsequently, contact with neighbouring viable cells is lost at the cell membrane which, already contracted due to loss of microvilli, begins to ruffle and bleb. The endoplasmic reticulum becomes dilated, while the cytoplasm condenses, leading to compaction of cytoplasmic organelles within the cell. The plasma membrane and, consequently, the entire cell, are then further broken down into membranous packages, known as apoptotic bodies. After which cell surface markers, such as adhesion molecules and externalised phosphatidyl serine, are recognised by the immune system, resulting in phagocytosis of the apoptotic cell, thus preventing the inflammatory responses associated with necrosis (Gill & Dive, 2000; Johnson, 2001; Curtin & Cotter, 2003).

![Diagrammatic representation of apoptotic cell morphology](image)

Figure 1.5: Diagrammatic representation of apoptotic cell morphology (adapted from Curtin & Cotter, 2003). Key morphological changes that occur during apoptosis are outlined above, sequentially.

### 1.4.2 Caspases: the enzymes of death.

Studies in *Caenorhabditis elegans* revealed that a death gene identified as *ced-3* encoded a protein essential for apoptosis during hermaphrodite development. Moreover, in 1993, when cloned and sequenced, *ced-3* was found to share sequence homology with the mammalian protease ICE (interleukin-1β-converting enzyme or
caspase-1). Although caspase-1 has no obvious role in apoptosis, it has been shown to play an important role in inflammation. Since caspase-1 was first identified, 14 mammalian proteases, 11 of human origin, now commonly termed caspases, (cysteinyl aspartate-specific proteases), have been identified, many of which play a pivotal role in apoptosis (Bratton & Cohen, 2001; Zimmermann et al., 2001; Curtin & Cotter, 2003; Degterev et al., 2003).

![Figure 1.6: The structural organisation of caspases](adapted from Zhivotovsky et al., 2003). Pro-caspases can be classified as either initiators or effectors, depending on the size of pro-domain they contain. Active caspases are tetramers, composed of two large and two small subunits. kDa, Kilodalton.

Caspases are constitutively synthesised as zymogens (inactive pro-enzymes), which are activated by a variety of apoptotic stimuli, following proteolytic cleavage at a specific aspartate cleavage site between the large (17-21 kilodalton (kDa)) and small (10-13 kDa) subunits, and generally in association with the proteolytic removal of the caspase N-terminal pro-domain. Depending upon the size of these pro-domains, caspases can be sub-divided into two groups. Caspases, such as, -3 and -7, which contain a short N terminal pro-domain of less than 30 amino acids, are considered as effector caspases. Those containing longer pro-domains of more than 100 amino acids, including caspase-8 and -9, are regarded as initiator caspases (Figure 1.6) (Zimmermann et al., 2001; Zhivotovsky, 2003). Initiator caspases are capable of either directly or indirectly activating effector caspases, in a sequential manner, thus forming a signalling cascade.
that transmits and amplifies the death signal (Figure 1.7) (Cohen, 2000). In the course of the cascade, caspases are also responsible for inactivation, by cleavage, of a number of critical cellular substrates, including poly(ADP-ribose) polymerase, lamins and DNA-dependent protein kinase, all contributing to the aforementioned morphological changes observed in apoptotic cells (Degterev et al., 2003; Zhivotovsky, 2003).

1.4.3 Caspase-activated pathways.

Subsequent to the initiation phase, wherein an apoptotic signal is received, the execution phase, involves either an extrinsic death-receptor-induced or an intrinsic chemical/stress-induced pathway (Figure 1.7). The former occurs via a number of cell surface death receptors belonging to the TNF gene superfamily. The best characterised of the death receptors are Fas (also known as Apol and CD95), TNF, TNF-related apoptosis inducing ligand (TRAIL) and death receptor 3 (DR3). Triggering of these receptors by their corresponding ligands results in receptor trimerisation, which in turn leads to the intracellular clustering of their death domains. In the case of the Fas, an adapter protein known as Fas-associated death domain (FADD) protein, containing its own death effector domain (DED) is recruited by the receptor. Consequently, recruitment of pro-caspase-8 and immediate proteolytic cleavage yields activated caspase-8, and subsequent activation of downstream effector caspases (Bratton et al., 2000; Cain, 2000; Hersey & Zhang, 2001; Zimmermann et al., 2001).

The intrinsic chemical/stress apoptosis pathway, induced by a variety of stimuli downstream of the mitochondria, including DNA-damaging agents, cytotoxic stress and the loss of extracellular growth factors, is mediated via mitochondrial damage and outer mitochondrial membrane (OMM) permeabilisation, leading to the release of pro-
apoptotic molecules into the cytosol. At present, there are two recognised mechanisms of OMM permeabilisation. One mechanism involves the induction of mitochondrial permeability transition (MPT), in which a non-specific pore on the inner mitochondrial membrane, called the MPT pore (MPTP), is opened in response to high mitochondrial calcium concentrations. The subsequent hyperpolarisation across the inner mitochondrial membrane (IMM), due to a loss in normal proton gradient, leads to an influx of fluid into the mitochondrial matrix, which generates a large increase in osmotic pressure, and causes the organelle to swell. The IMM, due to its extensively folded cristae can compensate, but unable to do so, the OMM eventually ruptures, leading to the release of cytochrome c and apoptosis inducing factor (AIF) from the mitochondria (Kroemer et al., 1997; Halestrap, 2000; Bratton & Cohen, 2001; Zimmermann et al., 2001; Orrenius, 2004). The second mechanism involves modulation of OMM permeabilisation by the B-cell lymphoma/leukaemia-2 (Bcl-2) protein family. Wherein anti-apoptotic members, including Bcl-2 itself and Bcl-XL, and pro-apoptotic members, including Bax and Bad, by inhibition or promotion, respectively, tightly regulate the release of proteins, such as cytochrome c, from the mitochondria (Bratton et al., 2000; Zimmermann et al., 2001; Orrenius, 2004). In both mechanisms, once cytochrome c is released into the cytosol, it forms a complex with apoptotic protease-activating factor-1 (Apaf-1) and pro-caspase-9, known as the apoptosome. Only then is caspase-9 activated, leading to the subsequent activation of caspase-3 and thus the executionery machinery of apoptotic cell death (Gill & Dive, 2000; Halestrap, 2000; Hersey & Zhang, 2001; Parone et al., 2003; Orrenius, 2004).

In a twist that demonstrates the complexity and inter-relatedness of the cascade, caspase-8, which is primarily associated with receptor mediated apoptosis, can, in some
cell types, by caspase-8-mediated Bid cleavage, produce truncated Bid (tBid); through subsequent oligomerisation and activation of Bax or Bak. Bid potentially triggers the release of cytochrome c from the mitochondria, consequently activating caspase-9 and engaging the intrinsic apoptosis pathway. Hence, a caspase amplification loop is established, whereby both caspase-8 and -9 can contribute to the activation of downstream effector caspases, ensuring cell destruction by apoptosis (Bratton & Cohen, 2001; Zimmermann et al., 2001; Degterev et al., 2003; Orrenius, 2004).

Extrinsic pathway

Intrinsic pathway

Death ligand

Death-receptor-independent stimuli

FADD

Pro-caspase-8

Bcl-2

Caspase-8

tBid

Bax

Mitochondrion

Cytochrome c

Apaf-1

Mitochondrial apoptosome

Caspase-9

Caspases 3/6/7

Death substrates

Apoptosis

Figure 1.7: Intrinsic and extrinsic apoptotic pathways (adapted from Daniel et al., 2003). The extrinsic pathway (left) is initiated via a death receptor, such as Fas, which leads to the activation of caspase-8. The intrinsic pathway (right) is triggered by a variety of stimuli, including DNA-damaging agents and cytotoxic drugs, leading to cytochrome c release, formation of the apoptosome and caspase-9 activation. Both pathways ultimately cause the activation of effector caspases. The intrinsic pathway can be amplified by cleavage of Bid to truncated Bid (tBid). ATP, adenosine triphosphate; CARD, caspase-recruitment domain; DED, death-effector domain; DD, death domain; FADD, Fas-associated death domain.
1.4.4  *p53: the tumour suppressor gene.*

Due to the fact that cancer occurs as a consequence of several independent genetic events, and not one single genetic alteration, the activation of a single oncogene, in the absence of a compensating mutagen, is likely to result in apoptotic cell death, thus cell proliferation is prevented by a mechanism of tumour suppression. The p53 tumour suppressor gene encodes a transcription factor, generally present intracellularly, in normal cells, at low basal concentrations, owing to negative regulation by the ubiquitin-ligase mouse double minute 2 (Mdm2). However, when cells are exposed to DNA-damaging agents, p53 becomes functionally activated and protein concentrations rise, due to an increase in p53 protein translation and half-life by post-translational stabilisation (Hickman *et al*., 2002; Gostissa *et al*., 2003; Brooks & Gu, 2003; Labazi & Phillips, 2003). Once activated, p53 regulates the expression of several proteins mediating signal pathways associated with either cell cycle regulation or apoptosis. p53 is reported to regulate Bcl-2 family members, Bax being the first pro-apoptotic factor to be identified as a target for p53 transactivation. In contrast, p53 appears to repress the expression of the anti-apoptotic factor, Bcl-2, thus p53 contributes to apoptosis by suppressing Bcl-2 survival signals. In addition, p53 was reported to induce Fas-activated apoptosis, implying that p53 may be involved in the induction of the caspase cascade. Moreover, Apaf-1 has also been identified as a direct target of p53, further implicating p53 as a potential inducer of the cascade. However, at present, the precise role of p53 in apoptosis is not entirely clear, and questions remain as to whether the individual genes activated by p53 are able to function as tumour suppressors themselves (Gill & Dive, 2000; Bratton & Cohen, 2001; Johnson, 2001; Hickman *et al*., 2002; Gostissa *et al*., 2003; Labazi & Phillips, 2003).
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1.5 POLYPHENOL-INDUCED APOPTOSIS.

Chemoprevention has emerged as potentially a practical approach to reducing cancer incidence. Chemopreventive agents, including polyphenols that can modulate apoptosis, thereby affecting steady-state cell populations, may be potentially useful in the management and therapy of cancer. To this end, research has been undertaken, using a variety of different cell types, to investigate the influence of a wide range of polyphenols, including resveratrol, flavone, prenylated compounds and, most notably, EGCG, upon apoptosis (Table 1.3) (Singh & Lippman, 1998b; Le Marchand, 2002; Manson, 2003).

<table>
<thead>
<tr>
<th>Polyphenol</th>
<th>Concentration</th>
<th>Cell Line</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>100 μM</td>
<td>KATO III stomach cancer</td>
<td>DNA frag</td>
<td>Hibasami et al. (1998a)</td>
</tr>
<tr>
<td>EGCG</td>
<td>100μM</td>
<td>DU 145 prostate cancer</td>
<td>↑ ROS &amp; mitochondrial depolarisation</td>
<td>Chung et al. (2001)</td>
</tr>
<tr>
<td>Theaflavin</td>
<td>50-100 μM</td>
<td>W138 virally transformed lung</td>
<td>DNA frag</td>
<td>Lu et al. (2000)</td>
</tr>
<tr>
<td>Theasinensin A</td>
<td>25 μM</td>
<td>Hep G2</td>
<td>↑ Bax expression</td>
<td>Pan et al. (2000a)</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>20 μg/ml</td>
<td>U937 histolytic lymphoma</td>
<td>Cyt c release &amp; casp-9 activation</td>
<td>Kuo et al. (2002)</td>
</tr>
<tr>
<td>Piceatannol</td>
<td>100 μM</td>
<td>Caco-2 &amp; HCT-116 colon cancer</td>
<td>Inhibits cell proliferation</td>
<td>Wolter et al. (2002)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>10 μM</td>
<td>KATO III &amp; HCT-116</td>
<td>↓ Bcl- X&lt;sub&gt;i&lt;/sub&gt; expression, casp-8 &amp; 3 activation</td>
<td>Moragoda et al. (2001)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>50 μM</td>
<td>Caki renal</td>
<td>DNA frag, cyt c release, ↓ Bcl-2 &amp; Bcl-X&lt;sub&gt;i&lt;/sub&gt; expression</td>
<td>Woo et al. (2003)</td>
</tr>
<tr>
<td>Curcumin</td>
<td>25 μM</td>
<td>HL-60 myeloid leukaemia</td>
<td>Cyt c release &amp; casp-8 mediated Bid cleavage</td>
<td>Anto et al. (2002)</td>
</tr>
<tr>
<td>Genistein</td>
<td>10-70 μg/ml</td>
<td>DU145 &amp; LNCaP prostate</td>
<td>Casp-3 activation &amp; inhibition</td>
<td>Kumi Diaka et al. (2000)</td>
</tr>
<tr>
<td>Quercetin, Apigenin &amp; Myricetin</td>
<td>60 μM</td>
<td>HL-60</td>
<td>Cyt c release, casp-9 &amp; 3 activation</td>
<td>Wang et al. (1999)</td>
</tr>
</tbody>
</table>

Table 1.3: Summary of evidence of apoptotic induction by polyphenolic compounds in cell culture studies. The use of different polyphenol concentrations and a variety of cell lines are highlighted. Casp, caspase; cyt c, cytochrome c; DNA frag, DNA fragmentation.
1.5.1 *Apoptotic activity of green, oolong and black tea polyphenols.*

A number of studies have been conducted using the major polyphenols found in black, green and oolong tea, or extracts of the aforementioned teas, to determine their effect upon apoptosis. Studies undertaken with black tea and its constituents illustrate a growth inhibitory effect by theaflavin, thearubigin, theaflavin-3-monogallate, theaflavin-3'-monogallate and theaflavin-3,3'-digallate, in a variety of human cancer cell lines, but not in their normal counterparts (Hibasami *et al.*, 1998b; Lu *et al.*, 2000; Das *et al.*, 2002). In addition, apoptosis was observed, in the form of apoptotic bodies, in cells treated with theaflavin and its digallate (Hibasami *et al.*, 1998b). Moreover, theaflavin-3'-monogallate-induced apoptosis was demonstrated in cancerous cells (Hibasami *et al.*, 1998b). In contrast, theaflavin-3-monogallate and theaflavin-3,3'-digallate had no effect upon apoptosis (Lu *et al.*, 2000).

An investigation into the growth inhibitory effects of a number of tea compounds, in human cancer cell lines, was undertaken. Theasinensin A, a condensation product of two EGCG molecules, found in oolong tea, theaflavin, and a mixture of the mono- and digallates all displayed strong inhibitory responses against U937 cell growth, whereas theaflavin-3,3'-digallate and EGCG exhibited lower activities, but were less effective against JURKAT cells (Pan *et al.*, 2000a). Furthermore, theasinensin A reportedly induced apoptosis in U937 cells via a loss in mitochondrial membrane potential, cytochrome c release and caspase-9 activation (Pan *et al.*, 2000a). In addition, an oolong tea polyphenol extract, containing oolong tea polyphenol trimer as the major component, impaired cell growth and induced apoptosis in KATO III cells, as exemplified by the production of apoptotic bodies and DNA fragmentation, in a time- and concentration-dependent manner (Hibasami *et al.*, 2000). In human U937 histolytic
lymphoma cells, the tea polyphenols EGCG, theaflavin, theaflavin-3,3'-digallate and theasinensin D induced apoptosis, as revealed by chromatin condensation, DNA fragmentation and caspase inhibition (Saeki et al., 1999a). The same researchers also reported that theasinensin D, exhibiting a 8.5-fold increase in chromatin condensation, was the most potent apoptotic inducer of the compounds studied, at the same concentration. Taken together with the fact that theaflavin-3,3'-digallate has the same number of hydroxyl groups as theasinensin D, but exhibits much less activity, this indicates that the number of and three-dimensional localisation of phenolic groups in a compound may be important in determining apoptotic activity (Saeki et al., 1999a).

Caffeine, a key component in a number of popular beverages, most notably coffee and tea, is reported to contribute to the chemopreventive effect of green and black tea (Lou et al., 1999; Lu et al., 2002; Fernandez et al., 2003; He et al., 2003). In one study, where green and black teas were given orally to rats, in the drinking fluid, both inhibition of tumour formation, and reduction in tumour size were observed (Lou et al., 1999). The same workers reported that decaffeinated teas were inactive or less active than regular tea at inhibiting tumour formation. However, when caffeine was added back into the decaffeinated tea, its original chemoprotective activity was restored, implying that caffeine may potentially be more active than EGCG (Lou et al., 1999). In a similar study, investigating the chemoprotective effect of caffeine or EGCG, both compounds displayed increased apoptosis in UVB-induced squamous cell carcinomas, following direct administration to the skin of hairless mice (Lu et al., 2002). Taken together, these and other studies demonstrate a chemopreventive effect by tea for skin carcinogenesis, although, it is unclear which compounds within tea are responsible for the chemoprotection. When applied topically, both caffeine and EGCG inhibited
tumour formation, but after oral administration, caffeine appeared far more effective. Hence, the bioavailability of tea polyphenols has to be taken into account when considering the comparative contribution of caffeine as a potential chemopreventive agent (Yang et al., 2002). Furthermore, caffeine may be responsible for apoptosis induction. In studies investigating the apoptotic effect of caffeine, DNA laddering and other characteristics of apoptosis were reported to occur via the activation of p53, Bax and caspase-3 (He et al., 2003; Ito et al., 2003). However, in vitro studies demonstrating caffeine-induced apoptosis utilised either high µM or mM concentrations of caffeine (Fernandez et al., 2003; Ito et al., 2003), which have no relation to the amount of caffeine ingested with tea or found as a final concentration in plasma, after tea consumption (Marks, 1992).

1.5.2 EGCG-induced apoptosis.

A study investigating the influence of green tea catechin extract and EGCG, the major polyphenol in green tea, in KATO III human stomach cancer cells, reported the presence of DNA oligonucleosomal-sized fragments, characteristic of apoptosis, following exposure to EGCG (Hibasami et al., 1998a). However, in this study EGCG concentrations of 100µM were utilised, which are unlikely to be achieved in the stomach, following dietary intake (Scalbert & Williamson, 2000). Meanwhile, EGCG-treated LNCaP, PC-3 and DU145 prostate cancer cells, and peripheral blood T lymphocytes from adult T-cell leukemia patients, all exhibited apoptotic cell death, by illustrating changes in nuclear morphology and DNA fragmentation (Paschka et al., 1998; Li et al., 2000). In addition, EGCG-induced apoptosis, via cell cycle dysregulation, has been reported in a variety of cancer cell lines, instigated by the upregulation of cki, p21 and p27, and the down modulation of cyclin D1, cyclin E and
cdk2, cdk4, and cdk6 expression, but not of cyclin D2. As a result, the formation of cyclin-cdk complexes in phase G1 of the cell cycle is impaired, causing G1 phase arrest, which may ultimately lead to apoptosis (Lin et al., 1999; Ahmad et al., 2000; Ahn et al., 2003; Gupta et al., 2003). Chung et al. (2001) alluded to the fact that catechins, such as EGCG, induce apoptosis in DU145 human prostate cancer cells, largely due to an increase in ROS formation and mitochondrial depolarisation, indicating that the presence of a gallate moiety and OH group may generate an effective apoptotic catechin isomer. Moreover, in K-562, chronic myelogenic human leukaemia cells EGCG-induced apoptosis is reportedly mediated by activation of caspase-8 and -3 (Roy et al., 2003).

Kuo and Lin (2003), investigating the antiproliferative activity of EGCG in HepG2 cells, reported a key role for p53, which leads to G1 phase cell cycle arrest and subsequently apoptosis. The same workers also reported that EGCG-induced apoptosis was associated with Fas binding, indicating the activation of the extrinsic apoptotic pathway (Kuo & Lin, 2003). Moreover, induction of apoptosis by the green tea catechins EGCG and EGC in LoVo human colon carcinoma cells was triggered by a rapid, transient increase in cell ceramide content. This was attributed to hydrolysis of the phospholipid sphingomyelin, in which ceramide serves as a second messenger, linking cell-surface receptors and environmental stresses through to the nucleus, resulting in DNA degradation and subsequently apoptosis (Tan et al., 2002).

Active in immortalised cells, but not in normal cells, the cancer-associated enzyme, telomerase, is involved in telomere maintenance, via de novo telomeric DNA synthesis, yielding telomere stabilisation and elongation. Studies were undertaken to investigate
telomerase inhibition as a potential apoptotic mechanism of polyphenols, such as EGCG, in telomerase-dependent and -independent cancer cells. Data indicated that polyphenols degraded by structural rearrangement, rather than intact molecules, were very effective at inhibiting telomerase, resulting in telomere shortening and subsequent cellular damage (Naasani et al., 2003).

1.5.3 Polyphenols from grapes and wines.

Resveratrol, a hydroxystilbene found in a number of plants including grapes, is reported to induce apoptosis, as demonstrated by enhanced Bax expression, in Hep-G2 p53-positive, but not p53-negative cells, indicating the presence of a potential p53-dependent apoptotic-signalling pathway (Kuo et al., 2002). In addition, resveratrol caused an increase in the expression of the cki, p21, leading to phase G1 cell cycle arrest in treated cells. However, over-expression of Bcl-2, in cells such as U937, may antagonise the apoptotic effect of resveratrol, by interfering with cytochrome c release and caspase-3 activation, both indicative of apoptosis (Park et al., 2001). Moreover, studies undertaken on whole wine and wine polyphenols, have shown that piceatannol, a natural analogue of resveratrol, not only inhibits cell proliferation in a time- and concentration-dependent manner in Caco-2 and HCT-116 colon cancer cells, but also initiates cell cycle arrest in S phase, as revealed by flow cytometry (Wolter et al., 2002). A similar study, using the prostate cancer cell lines PC3, LNCaP and DU145, revealed a direct inhibition of cell proliferation, by a variety of wine polyphenols, including resveratrol, quercetin and (-)-epicatechin, at low concentrations, mediated by the production of nitric oxide (NO). However, this inhibitory response occurred with varying effect in the three different prostate cancer cell lines tested, indicating a cell line-specific response (Kampa et al., 2000).
1.5.4 *Curcumin-induced apoptosis.*

Induction of apoptosis by curcumin, a major biologically active component found in turmeric, was investigated in a variety of cancer cell lines, which demonstrated caspase-3 activation, however, reports on the upstream mechanism of action vary, depending on the cell line employed (Bielak-Zmijewska *et al.*, 2000; Bush *et al.*, 2001; Moragoda *et al.*, 2001; Anto *et al.*, 2002; Duvoix *et al.*, 2003; Woo *et al.*, 2003). Bush *et al.* (2001) reported that curcumin triggers apoptosis in eight different human melanoma cell lines via activation of caspase-8. In addition, Fas expression was induced, at least 2-fold, in melanoma cells after exposure to curcumin (Bush *et al.*, 2001). Moreover, curcumin-induced apoptosis of KATO III gastric and HCT-116 colon cancer cells reportedly involves both caspase-3 and -8 activity, implying activation of an extrinsic apoptotic pathway (Moragoda *et al.*, 2001). Woo *et al.* (2003) reported that curcumin-induced apoptosis in human renal Caki cells was mediated via the release of cytochrome c, and thus likely to occur via an intrinsic pathway. However, in human myeloid leukaemic cell lines, curcumin is reported to induce apoptosis via caspase-8-mediated Bid cleavage, initiating cytochrome c release and subsequent caspase-3 activation, culminating in apoptosis (Anto *et al.*, 2002). In contrast, similar studies undertaken in K562 leukaemia cells demonstrated both caspase-8 and -9 activation, thereby implying that curcumin might induce apoptosis by involving both extrinsic and intrinsic apoptotic pathways (Duvoix *et al.*, 2003).

1.5.5 *Other polyphenolic compounds.*

Other polyphenols whose apoptotic activity was investigated include the soy isoflavones, genistein and daidzein, which are reported to inhibit cell proliferation, in a dose-dependent manner, with subsequent arrest in phase G2/M of the cell cycle, in
murine and human melanoma cell lines (Wang et al., 2002). However, when studies were conducted in the prostate cancer cell lines DU145 and LNCaP, genistein demonstrated characteristics of apoptosis, via caspase-3 activation and, inhibition by Z-VAD-FMK (Kumi-Diaka et al., 2000). Similarly, Kobayashi et al. (2002), examining the effect of a number of flavonoids on cell cycle progression in LNCaP cells, observed a genistein-induced G2/M phase arrest, associated with suppression of cyclin B and an increase in p21 expression.

Apoptotic cell death has also been observed in human oral tumour cells incubated with a variety of flavones, flavonols and isoprenoid-substituted flavonoids, such as apigenin, artonin E and gancaonin O, as observed by DNA fragmentation and caspase-3 activation, both characteristic of apoptosis (Sakagami et al., 2000). Meanwhile, apigenin and other structurally related flavonoids, including quercetin, myricetin, kaempferol, genistein and EGCG, found in a broad range of fruit and vegetables, for example tomatoes, grapes, kale and strawberries, were assessed for apoptosis in HL-60 cells. The flavonoids reportedly stimulated loss of mitochondrial membrane potential, cytochrome c release, and activation of caspase-9 and -3, thus inducing apoptosis via an intrinsic pathway (Wang et al., 1999). The same workers also reported that the effectiveness of individual compounds to induce apoptosis appeared dependent on the numbers of OH groups in the 2-prenyl group, as well as the absence of a 3-hydroxyl substituent in the chemical structure (Wang et al., 1999). In addition, studies investigating the potential influence of the core structure of the flavone subgroup upon apoptosis in HT-29 colon cancer cells, using 2-prenyl-4H-1-benzopyran-4-one (flavone), reported that apoptotic subG1 peaks were observed (Wenzel et al., 2000). On the whole, the concentrations of polyphenols demonstrated to induce apoptosis, in vitro,
exceeded those normally found in plasma and tissues (Kim et al., 2000; Scalbert & Williamson, 2000). In addition, recent human studies have shown that plasma and tissues are exposed to polyphenols as neither aglycones nor their original dietary form, but predominately as polyphenol conjugates (Kroon et al., 2004). Therefore, future research should focus less on “consumed” parent compounds, but more on the biological activities of physiologically relevant polyphenolic compounds, including conjugated analogues and metabolites, at appropriate concentrations.

1.6 THE ARYL HYDROCARBON RECEPTOR: RELEVANCE TO COLON CARCINOGENESIS.

The cytosolic aryl hydrocarbon (Ah) receptor, a ligand-dependent transcription factor, ubiquitously expressed in mammalian tissues, plays an essential role in the metabolism of a variety of xenobiotics. High affinity ligands, such as PAH, indolocarbazoles and halogenated aromatic hydrocarbons (HAH), as well as polychlorinated dibenzofurans, biphenyls, and dibenzo-p-dioxins, in particular the chemical 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), all compete for the Ah receptor binding site. Furthermore, the Ah receptor is an orphan receptor, with no identified endogenous ligand. Currently, the highly toxic dibenzo-p-dioxin TCDD is the highest known affinity ligand for the receptor (Hankinson, 1995; Denison et al., 2002; Denison & Nagy, 2003).

1.6.1 Ah receptor pathway.

The Ah receptor and its associated proteins are members of a small family known as the basic helix-loop-helix transcription factors. Generally found in the cytosol, close to the plasma membrane, in the absence of ligand, the Ah receptor exists as a multiprotein complex associated with two 90 kDa heat shock proteins (HSP90), that inhibit constitutive dimerisation with the Ah receptor nuclear translocator protein (ARNT), the
X-associated protein 2 (XAP2), which is essential for both HSP90 and Ah receptor binding, and a recently identified 23 kDa co-chaperone protein, p23 (Denison et al., 2002; Petrulis & Perdew, 2002; Denison & Nagy, 2003). Upon ligand binding, HSP90 and associated proteins may become dissociated from the Ah receptor, triggering translocation of the Ah receptor across the nuclear membrane into the nucleus. Alternatively, following ligandation, the Ah receptor may undergo a conformational change, exposing nuclear localisation sequence(s), inducing nuclear translocation of the multiprotein complex, which becomes dissociated from the HSP90, XAP2 and p23 protein complex upon interaction with ARNT (Hankinson, 1995; Denison et al., 2002; Petrulis & Perdew, 2002; Denison & Nagy, 2003). However, following extensive research, controversy still remains on whether dissociation of the Ah receptor from the protein complex occurs in the cytosol or nucleus (Hankinson, 1995; Sogawa & Fujii-Kuriyama, 1997; Petrulis & Perdew, 2002). In spite of this, the general view is that, once in the nucleus and dissociated from the protein complex, the liganded Ah receptor binds to ARNT, forming the heterodimeric transformed Ah receptor complex (AhRC). In this high affinity DNA-binding form, the AhRC binds to the xenobiotic response elements (XRE) of specific target genes, where it initiates transcription of the CYP1A1 and other target genes (Figure 1.6). Liganded receptors failing to dimerise with ARNT, and AhRC incapable of binding to DNA, are expelled to the cytosol via a nuclear export pump for degradation (Hankinson, 1995; Gonzalez & Fernandez-Salguero, 1998; Denison et al., 2002; Denison & Nagy, 2003).
Figure 1.8: Activation of gene expression by the Ah receptor (adapted from Denison & Nagy, 2003). AhR, Ah receptor; ARNT, Ah receptor nuclear translocator protein; CYP1A1, cytochrome P450 isoenzyme 1A1; hsp90, 90 kDa heat shock proteins; mRNA, messenger ribonucleic acid; XAP2, X-associated protein 2; XRE, xenobiotic response elements.

Moreover, the liganded Ah receptor complex modulates the expression of a variety of different genes, displaying pleiotropic biological effects, following Ah receptor TCDD ligandation, to produce the hepatotoxic consequences of porphyria, immunotoxicity, developmental and reproductive toxicity, in addition to a number of genes and gene products involved in cell proliferation and differentiation (Table 1.4) (Hankinson, 1995; Sogawa & Fujii-Kuriyama, 1997; Safe, 2001). Hence, at the cellular level, the Ah receptor appears to act as an environmental sensor, committing cells exposed to toxic chemicals to cell cycle arrest prior to DNA replication. For instance, the Ah receptor can reportedly function as an efficient inhibitor of cell cycle progression by forming protein complexes with the retinoblastoma protein, able to prevent phase G1 cell cycle progression, in some cases leading to cellular apoptosis (Elferink et al., 2001; Puga et al., 2002).
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Table 1.4: Genes regulated by the Ah receptor. IL-1β, interleukin-1β; PAI-2, plasminogen activator inhibitor-2; TGF, transforming growth factor.

1.6.2 Xenobiotic metabolism.

Many xenobiotics are hydrophobic in nature and, thus, have the potential to accumulate to toxic concentrations in the body, unless converted to more hydrophilic chemicals, which can be readily excreted in the urine or faeces (Figure 1.9). Biotransformation is initially achieved by CYP450 enzymatic phase I metabolic transformations, via reduction, oxidation or hydrolysis reactions, therefore altering the biological activity of the parent compounds. However, many xenobiotics are believed to be pro-carcinogens, and, thus, instead of CYP450-induced detoxification, biotransformation results in the production of toxic reactive intermediates (Hankinson, 1995; Denison & Whitlock, 1995; Vermeulen, 1996; Delescluse et al., 2000). In most cases, the chemically reactive (toxic) intermediates will be detoxified at their site of formation, but under circumstances of inefficient detoxification, oxidation or reduction reactions may occur, leading to cell damage, cell death and ultimately chemical carcinogenesis. Thereby implicating the CYP1 phase I drug-metabolising enzyme subfamily in the metabolic activation of pro-carcinogens, into chemically reactive ultimate carcinogens, for example benzo[a]pyrene to (+)-anti-benzo[a]pyrene-7R,8S-diol-9S,10R-epoxide, respectively (Vermeulen, 1996). The aforementioned xenobiotic agent now has the
potential to mutate DNA, thereby causing the activation of protooncogenes and/or the inactivation of tumour suppressor genes. Fortunately, the harmful electrophilic derivatives formed via CYP1A, CYP2B, CYP3A and CYP2E metabolism are usually transformed via conjugation reactions with phase II metabolising enzymes, such as UGT and GST, which inactivate them. After conjugation, previously harmful compounds are now sufficiently polar to be excreted safely from the body, via biliary and renal routes (Nebert & Jensen, 1979; Hankinson, 1995; Denison & Whitlock, 1995; Vermeulen, 1996; Delescluse et al., 2000).

Metabolism by phase I and II enzymes occurs predominately in the liver, where a large number of CYP450 isoenzymes have been recognised, whilst in extra hepatic tissues, particularly the colon, an appreciably lower rate of transformation has been observed (Ahmad et al., 1998). The majority of tumours in the digestive tract appear to develop in the colon, where UGT activity is low and direct exposure to potentially toxic procarcinogens is high. However, for the most part, dietary intake includes a wide variety of polyphenolic compounds capable of preventing colon carcinogenesis in animal
studies, either by potentially inhibiting expression of phase I CYP450 enzymes or enhancing the activity of phase II detoxification enzymes (Ahmad et al., 1998; van der Logt et al., 2003).

1.6.3 Potential of polyphenols as Ah receptor ligands.

Induction of CYP450 activity by polyphenols proceeds via various mechanisms, including direct stimulation of gene expression via a specific receptor, such as the Ah receptor. Structure-function studies undertaken with a large number of HAH and PAH Ah receptor ligands indicate that the receptor binding pocket favours hydrophobic compounds with maximal van der Waals dimensions of $14 \times 12 \times 5$ Å, chemical characteristics also common in flavonoids (Ashida et al., 2000; Hodek et al., 2002; Denison & Nagy, 2003). However, flavonoid glycosides, the principal form of flavonoids in the diet, which contain a large sugar moiety, appear to have lower binding affinities for the Ah receptor than their corresponding aglycone (Ashida et al., 2000; Denison & Nagy, 2003). Numerous polyphenolic compounds have been studied thus far for their Ah receptor binding affinity, antagonism of TCDD binding to the receptor, and their ability to induce AhRC and thus CYP1A1 expression (Gasiewicz et al., 1996; Henry et al., 1999; Zhou & Gasiewicz, 2003; Palermo et al., 2003). In one study, ellipticine and flavone derivatives were investigated for their ability to stimulate or inhibit XRE binding, and thus their potential as Ah receptor agonists or antagonists, wherein compounds exhibiting extremely potent antagonist activity, also demonstrated excellent Ah receptor binding pocket characteristics (Gasiewicz et al., 1996). Similar observations have been made in studies investigating flavone derivatives, such as 3'-methoxy-4'-nitroflavone, 3'-methoxy-4'-aminoflavone and 3'-methoxy-4'-azidoflavone, which exhibited potent antagonist, yet little agonist activity (Lu et al., 1996; Henry et
The same workers also reported that effective Ah receptor antagonists appear to require the presence of a 3'-methoxy group, as well as a 4' substituent, with one or more terminal atoms that have a high electron density (Lu et al., 1996; Henry et al., 1999). These studies were later corroborated by Lee & Safe (2000), reporting the pure antagonistic properties of the polyphenolic 3',4'-dimethoxyflavone (3',4'-DMF) against TCDD. In addition, other polyphenols, including EGCG and whole tea, have been demonstrated to antagonise the effects of TCDD at the Ah receptor (Ashida, 2000; Amakura et al., 2002).

Furthermore, following Ah receptor binding, possibly involving a hydrogen bond, polyphenolic compounds appear to inhibit HSP90 dissociation, thus preventing subsequent nuclear translocation and dimerisation with ARNT. However, reports remain contradictory as to whether this occurs solely with agonistic or antagonistic ligands (Henry et al., 1999; Ashida, 2000; Ashida et al., 2000; Henry & Gasiewicz, 2003).

1.7 PROJECT AIMS.

Several studies demonstrate a chemopreventive effect by naturally-occurring dietary polyphenols against carcinogenesis. However, there are large differences in the quantity of polyphenols and actual compounds consumed in Northern and Southern Europe, with lower risks of carcinogenesis attributed to a Mediterranean diet. POLYBIND is one of the largest collaborative and multidisciplinary EU projects to be undertaken on phytochemicals and human health. It aims to investigate the process of polyphenol metabolism and examine whether polyphenols can alter the rate of carcinogen metabolism. As well as investigate the potential mechanisms by which
polyphenols affect cell signalling, during cell proliferation and apoptosis, and to assess whether polyphenols are able to modulate early events in colon carcinogenesis.

Strong experimental evidence demonstrates, both in vivo and in vitro, that a number of different polyphenols exhibit apoptotic-inducing activity. This has been observed in a variety of cancer cell lines, wherein curcumin, quercetin and green tea galloylated catechins appear the most active. The main purpose of this study, as part of the POLYBIND project, is to investigate the apoptotic-inducing activity of a range of commercially available and novel polyphenolic compounds, some of which will be supplied by other POLYBIND partners, with a view to identifying the most potent apoptotic inducers. This will be achieved by using the human ileocecal adenocarcinoma cell line, HCT-8, chosen to complement other studies on the POLYBIND project, which will employ commonly used carcinoma cell lines of intestinal origin, including human colon HT-29 and Caco-2 cells. In addition, the relationship between polyphenol structure and the modulation of apoptosis will be explored. Polyphenols shown to have a significant effect upon apoptosis in adherent cells will be subjected to further investigation, particularly with respect to the mechanisms of action involved in apoptotic induction.

Also, a range of polyphenolic compounds will be investigated for their influence upon the Ah receptor, which is now implicated in carcinogenesis. This will be achieved by determining the ability of polyphenols to bind the Ah receptor agonistically. The ultimate aim is to determine whether polyphenols can impede, or reduce the binding of TCDD to the Ah receptor, and thus elicit some chemopreventive activity.
CHAPTER 2.

Apoptotic Activity of Naturally-Occurring and Synthetic Polyphenols.
2.1 INTRODUCTION.

Chemoprevention involves the utilisation of chemical agents, natural or synthetic, to suppress, reverse or prevent the development of cancer. Intervention with chemopreventive compounds can potentially directly modulate one of the stages of carcinogenesis, by mechanisms such as the prevention of DNA damage, suppression of cell hyperproliferation, and modulation of cell differentiation (Singh & Lippman, 1998a; Kellen, 1999). “So-called” chemopreventive blocking agents may well alter carcinogen metabolism, by preventing metabolic activation via phase I enzymes, enhancing detoxification by induction of phase II enzymes, as well as inhibiting further DNA damage, in subsequent stages of carcinogenesis. Since all the initiating events in carcinogenesis are unlikely to be prevented, suppressing agents, with the potential to suppress or inhibit growth of the transformed cells, and inhibit angiogenesis, may also be employed in chemoprevention (Singh & Lippman, 1998a). Many types of cancer are reported to either over express or constitutively upregulate MAPK, protein kinase B (PKB), AP-1 and NF-κB, key molecules involved in cell proliferation and survival, indicating that their inhibition, or downregulation, by chemopreventive suppression agents is likely to stimulate transformed cells into cell cycle arrest or apoptosis. In addition, suppression agents have the potential to directly modulate important molecules involved in cell cycle control and apoptosis, including cdk, cki and the Bcl-2 family (Kellen, 1999; Lambert & Yang, 2003; Manson, 2003).

Apoptosis (as described in 1.4) occurs in two phases, an initiation and execution phase. The execution phase, which occurs either by an extrinsic death-receptor-induced or an intrinsic chemical/stress-induced pathway, follows the initiation phase, where the cell receives an apoptotic signal (Hersey & Zhang, 2001; Zimmermann et al., 2001;
Orrenius, 2004). The execution phase is characterised by a series of stereotypic morphological changes, including chromatin condensation, internucleosomal DNA fragmentation and cell shrinkage, leading to the formation of apoptotic bodies, as well as a number of biochemical events, such as increases in intracellular calcium, chromatin cleavage by endogenous endonucleases, and exposure of surface glycoproteins, leading to cell phagocytosis (Gill & Dive, 2000; Johnson, 2001; Curtin & Cotter, 2003).

The majority of techniques currently available to detect apoptosis are associated with morphological or biochemical changes that occur during the execution phase. Methods for the detection of apoptotic cells generally include the use of DNA dyes, such as propidium iodide (PI) and Hoescht 33342, and fluorescence microscopy, to study nuclear morphology, analysis of DNA degradation by agarose gel electrophoresis or the comet assay, and DNA end-labelling techniques such as TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling). In addition, flow cytometry, in which light scatter is used to detect morphological changes in size and density in apoptotic cells, is employed, after cells are stained with DNA intercalating dyes, including PI, ethidium bromide and acridine orange (Sgonc & Gruber, 1998; Foster, 2000).

There is strong experimental evidence, both in vivo and in vitro (as described in 1.3 and 1.5), that a number of polyphenols exhibit apoptosis-inducing activity. Caderni et al. (2000), investigating the effect of black tea extracts, green tea extracts and wine extracts on AOM-induced intestinal carcinogenesis in rats, concluded that both the black tea and wine extracts, but not the green tea extracts, were able to protect against AOM-induced carcinogenesis. It was also observed, following histopathological evaluation, that
tumours from black tea-treated rats, and to a lesser extent those from rats treated with wine extracts, had a significantly greater apoptotic index than tumours from control animals. In a separate study, Mahmoud et al. (2000) investigated the response of the polyphenolic compounds curcumin, quercetin and rutin using Min/+ mice, which develop multiple intestinal adenomas as a result of a germline mutation in one adenomatous coli (Apc) allele. Significant tumour inhibition was observed by curcumin, associated with an increase in apoptosis, however, quercetin and rutin failed to alter tumour incidence, possibly due to their poor absorption by the GI tract.

In addition to numerous animal studies (as described in 1.3), flavonoids have also been reported to suppress carcinogenesis in cell lines derived from a variety of different origins (as described in 1.5). A number of polyphenols, including EGCG and resveratrol, are reported to modulate cell growth and apoptosis in a variety of tumour cell lines, with little or no effect in their normal counterparts (Huynh & Teel, 2000; Lu et al., 2000; 2001; Hsu et al., 2002). Apoptotic induction, by 10 μM of resveratrol, has been observed in SV40 virally transformed WI38 cells, although no apoptosis was seen in healthy WI38 cells, even at higher concentrations (Lu et al., 2001). Moreover, Ahmad et al. (2000), investigating the effects of EGCG in cancer and non-cancer cell lines, reported that treatment of human epidermoid carcinoma cells with EGCG resulted in a concentration-dependent inhibition of proliferation, cell cycle arrest at phase G1 and apoptosis, none of which was evident in normal human epidermal keratinocytes.

A selection of polyphenolic compounds (section 2.2.2) were investigated to determine which are effective inducers of apoptosis in the GI tract, in vitro, using the human ileocecal adenocarcinoma cell line, HCT-8. Polyphenols were chosen as a result of their
chemical class, application within the POLYBIND project, published apoptotic data and availability. During this study, the distinctive apoptotic morphology of HCT-8 cells was analysed using the DNA binding dye, Hoescht 33342, and fluorescence microscopy. In addition, cellular apoptosis was assessed by agarose gel electrophoresis, the TUNEL assay and flow cytometric analysis (Sgonc & Gruber, 1998).

2.2 MATERIALS AND METHODS.

2.2.1 Cell culture of HCT-8 and ICE-6 cells.

Materials.

- Phosphate buffered saline (PBS): Oxoid Ltd, Basingstoke, UK.
- RPMI 1640 media with L-glutamine, DMEM media with L-glutamine & D-glucose, trypsin-EDTA (ethylenediamine tetraacetic acid) 1X and foetal bovine serum (FBS): Invitrogen Ltd, Paisley, UK.
- 50 units penicillin- 50 µg/ml streptomycin- 100 µg/ml neomycin antibiotic solution and sodium pyruvate: Sigma-Aldrich Company Ltd, Poole, UK.

Method.

Human ileocecal adenocarcinoma, HCT-8 cells, obtained from the European Collection of Cell Cultures (ECACC), were cultured in RPMI 1640 media, supplemented with 10 % FBS, 1 mM sodium pyruvate and penicillin-streptomycin-neomycin antibiotic solution. The non-cancerous rat epithelial, ICE-6 cells, also obtained from ECACC, were grown in DMEM media, supplemented with 10 % FBS and penicillin-streptomycin-neomycin antibiotic solution; both cell cultures were maintained under standard conditions of 37 °C and 5 % CO₂ in a humid environment.
2.2.2 Polyphenol Treatment of HCT-8 and ICE-6 cells.

Materials.

- Staurosporine, caffeine, (+)-catechin, (-)-epicatechin, rutin, naringin, ellagic acid, curcumin, resveratrol, ferulic acid, caffeic acid, naringenin, quercetin, myricetin, genistein, indole-3-carbinol, chlorogenic acid, α-naphthoflavone, β-naphthoflavone, phloridzin, gallic acid and eriodictyol: Sigma-Aldrich Company Ltd, Poole, UK.
- Theaflavins, thearubigins and theafulvins: Donated by Emma Copeland, University of Surrey, UK.
- EGCG, EGC, ECG, (-)-gallocatechin-3-gallate (GCG), whole green tea, whole black tea, polyphenol-free green tea and polyphenol-free black tea: Donated by Dr. Conrad Astill, Unilever UK, Colworth, UK.
- Kaempferol: Indofine Chemical Company Inc, Somerville, NJ, USA.
- Epicatechin-4,8-catechin (B1), epicatechin-4,8-epicatechin (B2), epicatechin-4,6-catechin (B7) and epicatechin gallate-4,8-catechin (B'2G): Donated by Prof. Celestino Santos Buelga, University of Salamanca, Spain.
- Epicatechin-4,8-epicatechin-4,8-epicatechin (C1), grape seed proanthocyanidin oligomer and polymer, quercetin-3-glucuronide and whole red wine extract: Donated by Dr. Veronique Cheynier, INRA, Montpellier, France.
- Quercetin-3'-sulphate and quercetin-7-sulphate: Donated by Prof. Denis Barron, University of Lyon, France.
- Cocoa octamer: Donated by Sheryl Lazarus, Mars Confectionery, Slough, UK.
Figure 2.1: Chemical structures of flavonoids assessed for apoptotic activity. Dr. Conrad Astill, Prof. Denis Barron, Prof. Celestino Santos Buelga, Dr. Veronique Cheynier and Emma Copeland donated compounds that were not commercially available.

Figure 2.2: Chemical structures of commercially available polyphenols assessed for apoptotic activity.

Method.

HCT-8 and ICE-6 cells were cultured in 6-well plates or 25 cm² culture flasks until 80% confluent, then washed in PBS before addition of fresh media containing the polyphenols (compounds with known structural formulae are shown in Figures 1.1, 2.1 and 2.2). Initially, cells were incubated for 24 hours in the presence of polyphenols, at concentrations of 200-500 µM, in media containing 10% FBS. However, because of the high affinity of polyphenols for protein, on subsequent studies, cells were incubated with 0.5-50 µM of polyphenols in FBS-free media for 24 hours, under standard
conditions of 37 °C and 5 % CO₂ in a humid environment. Negative control cells were incubated with media and the vehicle only, whilst positive control cells were treated with 0.5-1 μM of staurosporine, an established inducer of apoptosis.

2.2.3 Measurement of HCT-8 and ICE-6 cell viability.

Materials.

- Trypan Blue: Sigma-Aldrich Company Ltd, Poole, UK.

Method.

Cell viability of HCT-8 and ICE-6 cells was determined by the trypan blue exclusion method. After incubation with polyphenols (as detailed in 2.2.2), both detached and attached cell fractions were harvested as cell suspensions, with the attached fraction being harvested by incubation with trypsin-EDTA for 5-10 minutes at 37 °C, following gentle washing with PBS. A 200 μl aliquot from each fraction was kept to determine cell viability count via the trypan blue exclusion method. Cell numbers and viability were counted in 10 μl of cell suspension, mixed with trypan blue (4 %) at a ratio of 1:1 (v/v) (diluted in reverse-osmosis water (RO-H₂O)), using a Modified Fuch’s Rosenthal counting chamber, under phase contrast light microscopy. Non-coloured cells were scored as live/early stage apoptosis and blue cells were scored as necrotic/late stage apoptosis.

2.2.4 Assessment of Apoptosis by Fluorescence Microscopy.

Materials.

- Para-formaldehyde (H.CHO)ₙ: VWR International Ltd, Poole, UK.
- Hoechst fluorescent DNA-binding dye 33342 (bisBenzimide): Sigma-Aldrich Company Ltd, Poole, UK.

Method.

When the removal of aliquots for cell viability was completed, cell suspensions were centrifuged, fixed overnight using 4 % (H.FCHO)₆ (at a ratio of 1:1 %) (diluted in RO-H₂O) at room temperature, and subsequently resuspended in 100 μl PBS, before nuclear integrity was assessed by staining with Hoechst 33342 dye. An aliquot of cell suspension (20 μl) was mixed with 3 μl of Hoechst 33342 dye, and 10 μl of the mixture was applied to a clean glass slide with coverslip, and visualised using a Zeiss Axiovert fluorescence microscope (Jones et al., 1998). Percentage apoptosis was determined by counting a total of 200 nuclei, per sample, from randomly selected fields of view (typically 5-10 fields of view counted for each attached fraction). Non-apoptotic cells showed intact homogeneous nuclei of uniform size and were regularly observed attached to other cells, whilst apoptotic cells were identified by distinctly fragmented and condensed nuclei, displaying either cell shrinkage, membrane blebbing, condensation of chromatin on the nuclear envelope (halo effect) or apoptotic bodies.

2.2.5 TUNEL Assay.

Materials.

- TdT-FragEL DNA Fragmentation Kit; containing Proteinase K (in 10 mM Tris, (tris[hydroxymethyl]-aminomethane)), 5X TdT (terminal deoxynucleotidyl transferase) equilibration buffer (1 M sodium cacodylate, 0.15 M Tris, 1.5 mg/ml BSA (bovine serum albumin), 3.75 mM cobalt chloride), TdT labelling reaction mix, TdT enzyme, stop buffer (0.5 M EDTA), blocking buffer (4% BSA in PBS), 50X conjugate (peroxidase streptavidin), DAB (3,3'-diaminobenzidine), H₂O₂/urea tablets, methyl green counterstain (0.3 %) and
positive and negative control slides (HL-60 cells): Oncogene Research Products, Merck Biosciences Ltd, Nottingham, UK.

- Xylene and methanol: Fisher Scientific UK Ltd, Morecambe, UK.
- Ethanol: Hayman Ltd, Witham, Essex, UK.
- 30 % H$_2$O$_2$, poly-L-lysine and Tris: Sigma-Aldrich Company Ltd, Poole, UK.
- NaCl, DPX (distrene, tricresyl phosphate and xylene mounting medium) and (H.CHO)$_n$: VWR International Ltd, Poole, UK.

Method.

To enhance cell adherence, HCT-8 cells were grown on poly-L-lysine coated glass coverslips in 6-well plates until 80 % confluent. They were then incubated for 24 hours with polyphenols in FBS-free media, after which cells were washed in PBS and fixed in (H.CHO)$_n$ for 20 minutes. Cells were rehydrated in 1X Tris-buffered saline (TBS: 20 mM Tris pH 7.6, 140mM NaCl) for 15 minutes, followed by 5 minutes only in 20 μg/ml Proteinase K, to increase cell membrane permeability, before being washed in 1X TBS. Endogenous peroxidases, which may interfere with the streptavidin-horseradish peroxidase conjugate, were inactivated by incubating cells in 3 % H$_2$O$_2$ for 5 minutes, followed by washing in 1X TBS. Cells then underwent equilibration and labelling reactions by incubation for 30 minutes in 1X equilibration buffer. The excess buffer was removed by aspiration, and immediately 60 μl of working TdT labelling reaction mix (the TdT labelling reaction mix was mixed with the TdT enzyme at a ratio of 19:1), which binds to exposed 3'-OH ends of DNA fragments, was added to each sample, covered with a Parafilm square, to assure an even distribution of the reaction mixture and prevent evaporation, and incubated in a humidity chamber at 37 °C for 1.5 hours. After removal of Parafilm,
samples were washed in 1X TBS and the reaction was terminated by incubating for 5 minutes in stop solution, followed by a second 1X TBS wash. Detection of biotinylated nucleotides was carried out by an initial 10-minute incubation with blocking buffer, which was aspirated off, and immediately followed by the addition of 100 μl of 1X streptavidin-horseradish peroxidase conjugate (50X conjugate diluted 1:50 in blocking buffer), and further incubation in a humidity chamber for 30 minutes at room temperature. Cells were then washed in 1X TBS, before incubation with 100 μl DAB solution (1 DAB (0.7 mg/ml) tablet and 1 H₂O₂/urea (0.6 mg/ml) tablet dissolved in 1 ml of tap water) per sample, for 15 minutes, and finally rinsed in RO-H₂O. DAB reacts with the labelled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. The glass coverslips were then gently transferred to a coverslip rack, making sure they did not dry out by keeping them submerged in RO-H₂O. Cells were then counterstained by incubation in 0.3 % methyl green for 5 minutes, to aid the morphological evaluation and characterisation of normal and apoptotic cells. To rinse off excess counterstain, the coverslips were dipped (1-2 times) in 100 % ethanol, which was repeated using fresh 100 % ethanol, followed by 1-2 dips in xylene, which was repeated using fresh xylene, before being mounted onto clean glass microscope slides using a drop of the mounting media DPX. Slides were left to dry overnight before observation via light microscopy, where a dark brown DAB stain indicates TUNEL positive staining, whereas shades of blue-green to greenish tan signify a non-reactive, TUNEL negative stain. Morphological changes characteristic of apoptotic cells were also used as verification of TUNEL positive cells.
2.2.6 Flow Cytometry.

Materials and instrumentation.

- PBS and (HCHO)ₚ: VWR International Ltd, Poole, UK.
- Ethanol: Hayman Ltd, Witham, UK.
- PI and RNase: Sigma-Aldrich Company Ltd, Poole, UK.
- Apoptag fluorescein *in situ* apoptosis detection kit for direct immunofluorescence staining, containing: equilibration buffer, TdT enzyme, reaction buffer and stop/wash buffer: Chemicon International Inc, Temecula, CA, USA.
- DNA-prep reagents kit, containing: DNA-prep stain (50 μg/ml PI, 4 KU/ml RNase & <0.1 % sodium azide) and DNA-prep LPR (<0.1 % potassium cyanide, <0.1 % sodium azide & non-ionic detergents), Epics Altra and Epics XL flow cytometers: Beckman Coulter (UK) Ltd, High Wycombe, UK.

Methods.

Staining cells with PI.

HCT-8 cells were grown in 25 cm² flasks until 80 % confluent, and then incubated with polyphenols in FBS-free media for 24 hours. Cell cultures were gently washed in PBS and incubated with trypsin-EDTA for 5-10 minutes before being harvested as cell suspensions. Following centrifugation at 800 g for 5 minutes, cells were fixed in 70 % ice-cold ethanol, added drop-by-drop while the cells were being vortexed to prevent cell clumping, and kept at 4 °C until transported to IFR, Norwich on ice. At IFR cells were centrifuged for 5 minutes at 1000 rpm, then resuspended in 100 μl of DNA-prep LPR and vortexed for 7 seconds; after which 1 ml of DNA-prep stain was added to each sample, and incubated for 30 minutes at room temperature, in the dark. Samples were then analysed by a Beckman Coulter Epics Altra flow cytometer, containing an argon-
ion laser, for forward and orthogonal light scatter and red (PI) fluorescence. DNA histograms were produced from acquired data using Expo32 computer software, in order to determine the presence of an apoptotic subG1 peak.

Staining cells by TUNEL.
HCT-8 cells were grown, treated and harvested as for PI staining, subsequently centrifuged at 800 g for 5 minutes and resuspended in 500 µl of PBS, prior to fixation with 5 ml (HCHO)₅, added drop-by-drop while being vortexed to prevent cell clumping. After a 15-minute incubation, on ice, cells were centrifuged as above, and the precipitated cells were resuspended in 2 ml 70 % (v/v) ethanol, added drop-by-drop. Fixed cells were then centrifuged once again at 800 g for 5 minutes and washed twice in PBS, resuspended in 75µl of equilibration buffer, recentrifuged, resuspended in 50 µl of working strength TdT (reaction buffer and TdT enzyme, ratio 7:3) and finally, wrapped in foil and incubated for 30 minutes at 37 °C. Half way through the incubation, after 15 minutes, samples were vortexed to resuspend settled cells, and following completion of the 30-minute incubation, 500 µl of working strength stop/wash buffer (stop/wash buffer and RO-H₂O, ratio 1:34), was added. Subsequently, cells were centrifuged at 800 g for 5 minutes, resuspended in 500 µl working strength stop/wash buffer, prior to the addition of 500 µl working strength PI solution (164 ng/ml PI and 8.3 µg/ml RNase in PBS), and finally, incubated for at least 15 minutes at 37 °C. Samples were analysed by a Beckman Coulter Epics XL flow cytometer containing an argon-ion laser, for forward and right angle light scatter, red PI and green TdT-fluorescin isothiocyanate (FITC) fluorescence. Dual-parameter correlated plots (cytograms) were produced from acquired data using Expo32 computer software to determine the presence of an apoptotic cell population.
2.2.7 **DNA gel electrophoresis.**

**Materials.**

- Boric acid: VWR International Ltd, Poole, UK.
- EDTA, Tris, agarose (low gelling temperature), EDTA-disodium salt, proteinase K, RNase A and ethidium bromide: Sigma-Aldrich Company Ltd, Poole, UK.
- SDS (sodium dodecyl sulphate): BioRad Laboratories Ltd, Merseyside, UK.
- Lambda DNA EcoR I Hind III Digest marker and DNA loading buffer (blue/orange 6X loading dye): Promega UK Ltd, Southampton, UK.

**Method.**

Following polyphenol treatment in FBS-free media for 24 hours, aliquots containing $10^6$ HCT-8 cells were fixed in 100 % ice-cold ethanol and stored at -20 °C. An 1.8 % agarose gel, in 150 ml of 1X TBE buffer (diluted 1:10 from 10X TBE buffer; 890 mM Tris, 890 mM boric acid and 25 mM EDTA, pH 8), containing 0.4 μg/ml ethidium bromide was prepared. Once set, a 1 cm strip of gel was cut away and replaced with a 0.8 % agarose digestion gel (in 10 ml 1X TBE buffer containing 2 % SDS and 1.25 mg/ml proteinase K), to which a comb was added before leaving the gel to set.

For analysis, cells were collected by centrifugation at 800 g for 10 minutes and resuspended in 10 μl PBS and 6 μl of 50 mg/ml RNase A, and left at room temperature for 1-5 minutes. Subsequently, 20 μl of DNA loading buffer was added to each sample before loading 20 μl into each well. λ DNA EcoR I Hind III Digest was used as a molecular weight marker. The gel was electrophoresed overnight at 10 V followed by 4 hours at 70 V, then washed in milliQ-H₂O, before being washed overnight on a shaker in TE8 buffer (10 mM Tris, 1 mM EDTA-disodium salt), containing 20 μg/ml RNase A. Finally the ethidium bromide stained bands were visualised and photographed using a UV transilluminator.
2.2.8 Determination of polyphenol hydrophobicity.

Materials.

- PrologP v5.1 Pallas software: CompuDrug International Inc, Budapest.

Method.

Pallas software is a collection of powerful tools for making predictions based on the structural formulae of compounds and, considering that no measured values were available, was employed to obtain partition coefficient (octanol/water) log$P$ values for all investigated polyphenols with known chemical structure. The PrologP v5.1 program predicts log$P$ values based on the formal fragmentation of the molecule into suitable structures, for which dependable log$P$ increments are known, and then log$P$ of the whole molecule is expressed as the sum of its corresponding fragments, corrected for any intramolecular interactions. In general, log$P$ values predicted by calculation are reliable, however, reliability decreases with increasing compound complexity. Log$P$ data obtained via PrologP determination was plotted against log % apoptosis for each compound to uncover a possible structure-function correlation.

2.3 RESULTS.

2.3.1 Evaluation of polyphenol-induced apoptosis.

A variety of cancer cell lines have been used to demonstrate apoptotic activity by polyphenols, including theaflavin, EGC, EGCG and theasinensin D, at concentrations ranging from 100-650 µM (Hibasami et al., 1998b; Okabe et al., 1999; Saeki et al., 1999a; 1999b; Tan et al., 2000), therefore concentrations within this range were chosen for this study. Commonly, carcinoma cell lines of intestinal origin used for in vitro studies include human colon HT-29 and Caco-2 cells, but these cell lines were being employed elsewhere on the POLYBIND project and thus another line was chosen to
complement findings from the project. Using HCT-8 cells, a selection of polyphenolic compounds representing major chemical classes (see below) were evaluated, initially in media supplemented with 10 % FBS, to determine their apoptotic potential.

- Flavan-3-ols (FAL): (+)-catechin, (-)-epicatechin, EGCG, ECG, GCG and EGC, black tea polyphenol mixtures, of either theaflavins, theafulvins or thearubigins, and proanthocyanidin B1 dimer.
- Flavonols (FOL): myricetin, kaempferol, quercetin and its glycoside rutin.
- Dihydrochalcones (DHC): phloridzin.
- Phenolic acids and derivatives (PAD): ellagic acid, gallic acid and curcumin.
- Hydroxystilbenes (HSB): resveratrol.
- Isoflavones (IFV): genistein.
- Indoles (IND): indole-3-carbinol.
- Hydroxycinnamic acids (HCA): caffeic, ferulic and chlorogenic acid.
- Synthetic flavonoids (SF): α- and β-naphthoflavone.
- Flavanones (FAN): naringenin and its glycoside naringin.

Staurosporine, a potent inhibitor of many kinases including protein kinase C, is also a known apoptotic inducer, reported to induce apoptosis through an intrinsic caspase pathway (Ahlemeyer et al., 2002; Caballero-Benitez & Moran, 2003; Gil et al., 2003), was used throughout this study as a positive control. Apoptotic activity, measured in the attached cell fraction, varied considerably among polyphenols, with some, including (+)-catechin, (-)-epicatechin and the B1 dimer, being devoid of apoptotic activity, whereas others induced over 20 % apoptosis. Apoptotic activity was also determined in the detached cell fraction, but data was not normally presented, as in general only a small proportion of cells (<5 %) became detached. The polyphenols listed in Table 2.1
only includes those which induced >20% apoptosis. Apoptosis in corresponding detached cell fractions was 86-98% (results not shown), when there were more than 200 cells to assess. Since concentrations used were considerably higher than those expected in tissues or plasma, after normal dietary intake, the possibility of using lower concentrations of polyphenols was considered.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Apoptosis</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N/A</td>
<td>0.5-2.0</td>
<td>93.5-99.0</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>1 μM</td>
<td>68.5-82.5</td>
<td>95.0-97.0</td>
</tr>
<tr>
<td>EGCG (FAL)</td>
<td>400 μM</td>
<td>25.0</td>
<td>97.5</td>
</tr>
<tr>
<td>Gallic Acid (PAD)</td>
<td>400 μM</td>
<td>22.75</td>
<td>97.0</td>
</tr>
<tr>
<td>Theaflavins (FAL)</td>
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<td>97.8</td>
</tr>
<tr>
<td>Thearubigins (FAL)</td>
<td>25 μg/ml</td>
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<td>97.5</td>
</tr>
<tr>
<td>Thearubigins (FAL)</td>
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<td>28.25</td>
<td>96.5</td>
</tr>
<tr>
<td>Rutin (FOL)</td>
<td>400 μM</td>
<td>33.5</td>
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<td>Naringin (FAN)</td>
<td>400 μM</td>
<td>24.75</td>
<td>98.5</td>
</tr>
<tr>
<td>Ellagic Acid (PAD)</td>
<td>200 μM</td>
<td>23.0</td>
<td>98.0</td>
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<tr>
<td>Ellagic Acid (PAD)</td>
<td>400 μM</td>
<td>37.75</td>
<td>97.5</td>
</tr>
<tr>
<td>Curcumin (PAD)</td>
<td>200 μM</td>
<td>21.0</td>
<td>98.0</td>
</tr>
<tr>
<td>Curcumin (PAD)</td>
<td>400 μM</td>
<td>24.0</td>
<td>97.5</td>
</tr>
<tr>
<td>Ferulic Acid (HCA)</td>
<td>400 μM</td>
<td>20.75</td>
<td>96.5</td>
</tr>
<tr>
<td>Resveratrol (HSB)</td>
<td>500 μM</td>
<td>20.0</td>
<td>98.0</td>
</tr>
</tbody>
</table>

Table 2.1: Polyphenols inducing more than 20% apoptosis in HCT-8 cells, incubated in media supplemented with 10% FBS. Cells were incubated for 24 hours with polyphenols, staurosporine or vehicle control, in 10% FBS media. Percentage apoptosis was determined using Hoescht 33342 staining and cell viability was assessed via trypan blue exclusion. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12 and 2% for apoptosis and viability, respectively.

2.3.2 Development of the method used to study apoptotic activity of polyphenols.

In the light of reports that polyphenols have a high binding affinity for proteins, most notably, proline rich-proteins (Haslam, 1974; Hagerman & Butler, 1981; Luck et al., 1994), preliminary experiments were undertaken to adapt a method for use in evaluating
the apoptotic activity of polyphenols at lower concentrations, in the absence of FBS, so as to prevent interactions of the polyphenols with proteins. Initially, cells were incubated with 400 μM EGCG in FBS-free media, for various incubation periods ranging from 30 minutes to 24 hours (Figure 2.3). This concentration of EGCG was chosen, since it was shown to induce apoptosis in the presence of FBS and, thus, would be expected to have an effect in its absence. EGCG induced 25 and 100 % apoptosis when incubated for 6 and 24 hours, respectively, however, viability also fell to 87.5 and 48.5 %, respectively. It is important to point out that cell viability is used as a guide of cell cytotoxicity, and percentage viability of 90 % or above is considered acceptable.

Although control sample apoptosis and cell viability data was available for all the time points tested (data not shown), all the control data was equivalent, therefore, only data obtained at the 30-minute time point was shown. Since the high polyphenolic concentrations investigated thus far are highly unlikely to be reached in vivo, and maybe cytotoxic, it was therefore pertinent to evaluate the apoptotic activity of a lower range of EGCG concentrations. Consequently, instead of adjusting incubation time, EGCG concentrations were reduced and cells were incubated for 24 hours (Figure 2.4). Results indicated that above 100 μM EGCG was cytotoxic to HCT-8 cells. At concentrations of 5-50 μM, EGCG induced apoptosis at 20-25 %, while maintaining viability above 95 %.

A direct comparison between EGCG-induced apoptosis (5-100 μM) in 10 % and FBS-free media was carried out, to determine to what extent FBS masks the apoptotic activity of EGCG, particularly at low concentrations. Figure 2.5 illustrates that EGCG is markedly more active in the absence of FBS, at the concentrations studied. Cell viability, in general, was high, except for 100 μM EGCG in FBS-free media, which
appears slightly cytotoxic. Since cell viability remains high (>95 %) with 5, 25 and 50 μM EGCG in the absence of FBS, these concentrations were selected to investigate the apoptotic activity of a range of polyphenolic compounds.

**Figure 2.3:** The effect of 400 μM EGCG on apoptosis and viability in HCT-8 cells following different incubation periods in FBS-free media. Results for percentage apoptosis were obtained using the Hoescht 33342 stain and viability via trypan blue exclusion. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 % for apoptosis and viability.

**Figure 2.4:** The effect of a range of EGCG concentrations upon apoptosis and viability in HCT-8 cells. Cells were incubated with EGCG for 24 hours in FBS-free media. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 and 3 % for apoptosis and viability, respectively.

Figure 2.5: Comparison of the apoptotic activity induced by EGCG in HCT-8 cells incubated in the presence and absence of 10 % FBS. Cells were incubated for 24 hours with EGCG. Percentage viability was >93 %, except at 100 µM EGCG in FBS-free media, when it dropped to 88 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 %.

2.3.3 Induction of apoptosis by polyphenols in cells cultured in FBS-free media.

Initially, compounds which previously induced >20 % apoptosis in the presence of FBS, were examined in FBS deficient media, along with a number of other compounds (see below), including proanthocyanidin dimers, trimer, oligomer and polymer, whole wine extract, quercetin sulphates and glucuronide, provided by collaborators involved in the EU-sponsored POLYBIND project.

- **Flavan-3-ols (FAL):** EGCG, the black tea polyphenol mixtures of theaflavins, theafulvins and thearubigins, the proanthocyanidin dimers B1, B2 and B7, B’2G dimer gallate, C1 trimer, whole red wine extract, grape seed oligomer, grape seed polymer and cocoa octamer.
- **Flavonols (FOL):** quercetin, its glycoside rutin, sulphates and glucuronide.
- **Phenolic acids and derivatives (PAD):** ellagic acid, gallic acid and curcumin.
- **Hydroxystilbenes (HSB):** resveratrol.
• Hydroxycinnamic acids (HCA): ferulic acid.
• Flavanones (FAN): eriodictyol and naringin (glycoside of naringenin).

Assessment of apoptosis was undertaken using the Hoechst 33342 dye and fluorescence microscopy (section 2.2.4). Although approximate outlines of the cytoplasm can be observed, predominate staining, by Hoechst 33342, occurs in the nuclei. HCT-8 cells displaying classical morphological changes, including distinctly fragmented and condensed nuclei, cell shrinkage, membrane blebbing, chromatin condensation (halo effect), or the presence of apoptotic bodies, were scored as apoptotic (Figure 2.6). Since EGCG-induced apoptosis has been reported on numerous occasions (Nakagawa et al., 2002; Ahn et al., 2003; Roy et al., 2003), and, in light of the positive results obtained from this study, it was considered pertinent to employ EGCG as a polyphenolic standard.

Flavan-3-ols.
Theaflavins were re-examined, initially at concentrations of 2.5-250 μg/ml, as with 10 % FBS media, but, as the highest concentration appeared to be slightly cytotoxic, theafulvins and thearubigins were studied at the lower concentrations of 2.5 and 25 μg/ml only. At 2.5 μg/ml, theaflavins induced 22 % apoptosis, while at the same concentration theafulvins and thearubigins induced 15 and 19.5 % apoptosis, respectively (Figure 2.7). No dose-dependent effect for apoptosis was evident, even though 10-fold differences in the concentrations of the black tea polyphenols were used. The apoptotic response from the proanthocyanidin dimers (Figure 2.8) is particularly low. Even though B'2G, the dimer gallate, exhibits slightly more activity, in a concentration-dependent manner, it is still below 18 %. Cell viability was unaffected, remaining above 94.5 %.
Figure 2.6: Intact and fragmented nuclei of HCT-8 cells visualised under fluorescence microscopy. Typical apoptotic morphology (Ap) and non-fragmented/normal nuclei (N) are observed in fixed cells following use of the Hoechst 33342 stain in HCT-8 cells incubated for 24 hours with DMSO (A), 1 μM staurosporine (B) or 25 μM EGCG (C). Overall magnification: x630.

Figure 2.7: Apoptotic potential of black tea polyphenols in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with theaflavins (TF), theafulvins (TFu) and thearubigins (TR). Results for percentage apoptosis were obtained using the Hoescht 33342 stain and viability via trypan blue exclusion. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10 and 7 % for apoptosis and viability, respectively.

Figure 2.8: Apoptotic activity of proanthocyanidin dimers in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with epicatechin-4,8-catechin (B1), epicatechin-4,8–epicatechin (B2), epicatechin-4,6–catechin (B7), and epicatechin gallate–4,8–epicatechin (B'2G). Percentage viability was >94.5 %. Apoptotic activity for 1 μM staurosporine was 67.75 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10 %.
Figure 2.9 illustrates low, but apparently concentration-dependent apoptotic activity for
the C1 trimer, whilst the grape seed oligomer, composed of (+)-catechin, (-)-epicatechin
and ECG monomers, with an average degree of polymerisation of 3.3 and 11 %
galloylated units, was clearly cytotoxic to HCT-8 cells at 25 and 50 µM, with little
evidence of apoptosis at 5 µM (7.25 %). With an average degree of polymerisation of
8.6 and 22 % galloylated units, the grape seed polymer, also composed of (+)-catechin,
(-)-epicatechin and ECG monomers, was extremely cytotoxic to HCT-8 cells, even at
the low concentration of 5 µM. Moreover, following incubation with 50 µM of the
grape seed polymer (data not shown), cell viability was so low that the vast majority of
cells detached, leaving too few cells in the attached cell fraction to comply with the
counting criteria (>200 cells). Subsequently, a further evaluation of apoptotic potential
of the polymer, at the lower concentrations of 0.5-5.0 µM, was undertaken. Figure 2.10
demonstrates a concentration-dependent response by the polymer, both for apoptosis
and cell viability. Polymer cytotoxicity was evident at concentrations higher than 2 µM.
At concentrations of 1 and 1.5 µM, cells were viable and a higher degree of apoptosis
was evident (Figure 2.10).

The cocoa octamer, primarily composed of (-)-epicatechin, with little or no (+)-catechin,
was investigated for apoptotic activity (Figure 2.11), at similar concentrations to those
of the grape seed polymer. Although the $M_r$ (relative molecular mass) of the octamer is
unknown, the average degree of polymerisation is similar to that of the grape seed
polymer. In the presence of the cocoa octamer, apoptosis increased in a concentration-
dependent manner, but at the highest concentration (25 µg/ml) cell viability was
unacceptable (72.5 %).
Figure 2.9: Effect of the proanthocyanidin trimer (C1), grape seed oligomer and polymer upon apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 and 13 % for apoptosis and viability, respectively.

Figure 2.10: Effect of low concentrations of the grape seed polymer on apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with the polymer. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 and 4 % for apoptosis and viability, respectively.
Figure 2.11: Apoptotic activity of the cocoa octamer in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media in the presence of cocoa octamer (1.25-25 μg/ml). Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 and 3 % for apoptosis and viability, respectively.

Figure 2.12: The effect of whole red wine extract upon apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media in the presence of whole red wine extract (5-100 μg/ml). Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 and 3 % for apoptosis and viability, respectively.
A whole red wine extract, largely containing a mixture of proanthocyanidin oligomers and polymers of (+)-catechin, (-)-epicatechin, EGC and ECG, and their derivatives, was assessed for apoptotic activity at a range of concentrations (Figure 2.12). Even though the whole red wine extract induced a concentration-dependent apoptotic effect, at the two highest concentrations studied, cytotoxicity was clearly evident.

**Flavanones, hydroxystilbenes and hydroxycinnamic acids.**

Induction of apoptosis by resveratrol (HSB), ferulic acid (HCA) and eriodictyol (FAN) was poor, with no apparent concentration-dependent effect (Figure 2.13). Eriodictyol exhibited the lowest apoptotic activity, while ferulic acid and resveratrol induced less than 15.5 % apoptosis, at the concentrations studied. Cell viability was unaffected, remaining above 94.5 %.

![Figure 2.13: Apoptotic effect of resveratrol, ferulic acid and eriodictyol in HCT-8 cells.](image)

Cells were incubated for 24 hours in FBS-free media with resveratrol, ferulic acid and eriodictyol. Percentage viability was >94.5 %. Apoptotic activity for 1 μM staurosporine was 59.75 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 %.
Phenolic acids and derivatives.

Even though their apoptotic activity was low, both gallic acid and ellagic acid appeared to induce apoptosis in a dose-dependent manner. Cell viability was unaffected, at the concentrations tested, remaining above 94% (Figure 2.14). Curcumin was clearly cytotoxic at concentrations above 25 μM, but at the lower concentration of 5 μM it induced 22% apoptosis (Figure 2.14). To evaluate whether curcumin-induced apoptosis, in HCT-8 cells, occurs in a concentration-dependent fashion, a further experiment was carried out, employing a range of lower concentrations (Figure 2.15). Once again, curcumin exhibited some cytotoxic activity at 25 μM (87%), but again no concentration-dependent apoptotic effect was evident.

![Graph showing apoptotic response of phenolic acids and derivatives in HCT-8 cells](image)

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Figure 2.14: Apoptotic response of phenolic acids and derivatives in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with ellagic acid, gallic acid and curcumin. Apoptotic activity for 1 μM staurosporine was 57.25%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 and 12% for apoptosis and viability, respectively.
Figure 2.15: Curcumin-induced apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with curcumin. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >14 and 1% for apoptosis and viability, respectively.

2.3.4 Assessment of apoptosis induced by flavonoid glycosides and conjugates.

Figure 2.16 illustrates the apoptotic response of rutin and naringin, flavonoid glycosides of quercetin and naringenin, respectively, which contain a disaccharide moiety. Both rutin and naringin induced apoptosis in a dose-dependent manner, although were less active than EGCG. Figure 2.17 shows the apoptotic activity of quercetin conjugates, with conjugation to either glucuronic acid at the 3 position or sulphate at the 3' or 7 position, in comparison to the parent compound. Although quercetin conjugates exhibited less apoptotic activity than the parent compound, a concentration-dependent apoptotic effect was observed.

After the evaluation of a wide range of polyphenols for their apoptotic activity, EGCG, theaflavins, curcumin and the grape seed polymer exhibited the greatest apoptotic activity, at lower concentrations, without inducing cytotoxicity. Consequently, these...
polyphenols were selected as "active" compounds, which would be employed in subsequent investigations.

**Figure 2.16**: Induction of apoptosis by the flavonoid glycosides rutin and naringin in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with rutin and naringin. Percentage viability was >91%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11%.

**Figure 2.17**: Influence of quercetin glucuronide and sulphate conjugates upon apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with quercetin, quercetin-3-glucuronide (Q3G), quercetin-3'-sulphate (Q3'S) and quercetin-7-sulphate (Q7S). Percentage viability was >93%. Apoptotic activity for 1μM staurosporine was 73.25%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >13%.
2.3.5 Cell permeability of polyphenolic compounds assessed for apoptosis.

LogP (octanol/water) partition coefficient values, which indicate a compound’s relative affinity for aqueous and organic phases, and to a large extent determine whether a compound will passively diffuse across a biological membrane, were calculated for all polyphenolic compounds with known chemical structures, using PrologP Pallas software (CompuDrug International Inc, Budapest). Partition coefficients were plotted against log % apoptosis to uncover a structure-activity correlation, whereby an increasing coefficient denotes increasing hydrophobicity (Figure 2.18). As expected, glycosylation and glucuronidation influence the logP value of a compound, as seen by quercetin-3-glucuronide and rutin, that appear clearly more hydrophilic than quercetin. In addition, curcumin, which induced high levels of apoptosis, was also characterised by the highest logP value, indicating that curcumin is highly hydrophobic. However, it is noteworthy that the logP value of a compound, and hence its ability to access the intracellular compartment passively, is not the only factor involved in cell membrane transport. For example, polyphenolic compounds, too polar to enter the cell passively, may be actively transported into a cell by carrier molecules.

Figure 2.18: Graph of log percentage apoptosis against logP. Partition coefficient data for EGCG, gallic acid (GA), rutin (Rut), naringin (Nar), ellagic acid (EA), curcumin (Curc), resveratrol (Res), eriodictyol (Erio), procyanidin dimers, B1, B2, and B7, dimer gallate (B'2G) and trimer (C1), quercetin (Q), quercetin-3-glucuronide (Q3G), quercetin-3'-sulphate (Q3'S), quercetin-7-sulphate (Q7S) and ferulic acid (FA) was obtained via Pallas software computational analysis of polyphenol chemical structures.
2.3.6 Evaluation of polyphenol-induced apoptosis by flow cytometry and TUNEL analysis.

Induction of apoptosis was further investigated with the use of flow cytometry, which measures the fluorescence intensity of individual cells stained with a fluorescent dye. This approach was taken to confirm the relative apoptotic activities of EGCG, theaflavins, curcumin and the grape seed polymer, which had previously induced >20% apoptosis when assessed with Hoechst 33342 staining (section 2.3.3).

Figure 2.19: Flow cytometry DNA histograms of HCT-8 cells treated with EGCG, curcumin and the grape seed polymer. HCT-8 cells were treated with DMSO as a control, 1 μM staurosporine, 5 μM EGCG, 15 μM curcumin or 2 μM polymer, for 24 hours in FBS-free media prior to fixation and PI staining. DNA histograms illustrate cell cycle progression through phases G1, S and G2/M, together with the apoptotic subG1 region, in which no distinct peaks were observed.
Following a 24-hour incubation with the polyphenols, HCT-8 cells underwent ethanol fixation and PI staining. Cells were then assessed for forward and orthogonal light scatter and red fluorescence, and DNA histograms, which represent nuclear shape and size, were constructed, illustrating different phases of the cell cycle together with the subG1 apoptotic region (Figure 2.19).

The polymer and EGCG caused no change to the cell cycle, while staurosporine appears to shift cells from G1 into G2/M. However, the effects generated by curcumin appear generally distorted, with more cells in S phase than the control. A very slight increase was observed in cells in the subG1 region following incubation with curcumin and the known apoptotic inducer, staurosporine (Figure 2.19). This increase, however, was not substantial enough to be considered a “distinct” subG1 apoptotic peak (Ormerod, 1998; 2001), a typical example of which is illustrated in Figure 2.20 (Saeki et al., 2002).

![Figure 2.20: Example of flow cytometry data demonstrating a distinct subG1 peak. Saeki et al. (2002) incubated U937 human leukemia cells in the presence and absence of 400 µM EGCG for 4 hours at 37°C. Following ethanol fixation and PI staining, cells were assessed with a FACS Calibur flow cytometer and analysed using Cell Quest software (Nippon Becton Dickinson), indicating the percentage of cells in the apoptotic subG1 region. In this case “Counts” equates to “events” or nuclei number counted.](image-url)
Cell cycle analysis was undertaken using Expo32 computer software, to ascertain the percentage of cells in each phase of the cell cycle, along with the small proportion of cells in the apoptotic region, where a subG1 peak (>6%) was expected to appear. Incubation of HCT-8 cells with EGCG or the polymer revealed a cell cycle comparable to the control, and even though there was a slight increase in the number of cells present in S phase, cell cycle progression seems uninhibited (Figure 2.21). Cells treated with the known apoptotic inducer, staurosporine, displayed a reduced G1 (from 90 to 48%) and extended G2/M phase (from 3 to 44%), compared with the control. A similar response was observed with curcumin, where the proportion of cells in G1 fell from 90 to 54%, whilst the percentage of cells in G2/M increased from 3 to 26% (Figure 2.21), indicating the presence of cell cycle delay with both staurosporine and curcumin. This may subsequently initiate cell cycle arrest, during a defined phase of the cycle, and potentially initiate apoptosis induction (Wenzel et al., 2000; Ahn et al., 2003).

Figure 2.21: Percentage of HCT-8 cells in different phases of the cell cycle. Data obtained by analysis of DNA histograms with Expo32 software, illustrating the percentage of HCT-8 cells in G1, S and G2/M phase together with those present in the apoptotic subG1 region, after incubation of cells with EGCG, curcumin or the grape seed polymer for 24 hours in FBS-free media.
As an alternative to the preceding cell cycle analysis methodology, apoptosis can also be assessed by the TUNEL method, in which free 3'-OH ends of broken strands of DNA can be labelled enzymatically, with TdT, catalysing the addition of biotin-labelled deoxynucleotides to the exposed DNA. The TUNEL assay was therefore employed to detect the apoptotic activity in HCT-8 cells incubated with EGCG, theaflavins, curcumin and the polymer, which all previously induced >20 % apoptosis via Hoechst 33342 staining, either by colorimetric staining and light microscopy, or fluorescent staining flow cytometry analysis. Flow cytometry cells were fixed in paraformaldehyde, a cross-linking fixative, believed to tether small chromatin fragments to the cell, preventing their extraction during subsequent processing steps. Following fixing with ethanol, and subsequent incubation with FITC-labelled TdT and counterstain with PI, HCT-8 cells were assessed for forward and right angle light scatter, red (PI) and green (FITC) fluorescence. Dual-parameter correlated plots (cytograms) of PI against TdT-FITC fluorescence were produced from acquired data and a quadrant gate set (Figure 2.22). This showed the majority of cells for each compound tested in region B3 (normal cells), with apoptotic cells emitting green fluorescence (B4) hardly visible. Therefore, further analysis was undertaken using Expo32 software, to calculate the percentage of green fluorescent apoptotic cells in region B4 (Figure 2.23). EGCG, curcumin and the polymer showed no evidence of apoptotic activity, producing a similar amount of green fluorescent cells to that of the control, whereas theaflavins produced a higher apoptotic activity than staurosporine (16.66 and 13.25 %, respectively).
Figure 2.22: Assessment of apoptosis by flow cytometry of cells labelled by the TUNEL assay. HCT-8 cells were treated with DMSO as a control, 0.5 μM staurosporine, 25 μM EGCG, 25 μg/ml theaflavins, 10 μM curcumin or 2 μM the grape seed polymer for 24 hours in FBS-free media prior to fixation and staining. Quadrants display normal (B3), apoptotic FITC stained (B4) and PI stained nuclei (B1).

For assessment of apoptosis via staining with a colorimetric TUNEL assay, HCT-8 cells were grown on poly-L-lysine coated glass coverslips, incubated with polyphenols, and stained. Cells displaying a dark brown DAB signal indicate TUNEL positive staining, while shades of blue-green to greenish tan signify a non-reactive, healthy cell. The classical morphological changes of fragmented and condensed nuclei, cell shrinkage and apoptotic bodies were also used for apoptotic characterisation, while non-apoptotic cells should be predominately rounded and appear counterstained with methyl green. Polyphenols exhibiting more than 5% TUNEL positive cells were classed as apoptotic, since a small proportion of cells can die, or are damaged, during both the cell culture and staining process. Following successive replications to improve staining, Figure 2.24 illustrates the only acceptable examples of HCT-8 stained cell samples, treated with DMSO, 0.5 μM staurosporine or 2 μM polymer; unfortunately the methyl green counterstain was somewhat faint. In consequence, results were not readily quantifiable, as in many cases cells were indistinguishable as TUNEL positive or negative. The
micrographs shown, represent cells incubated with staurosporine which appear apoptotic by staining primarily dark brown, while those of the DMSO control seem healthy, appearing predominately pale green. Cells incubated with the grape seed polymer displayed a presence of TUNEL positive cells, higher than in the control, indicating the presence of more apoptotic cells.

Figure 2.24: Colorimetric TUNEL staining of HCT-8 cells following treatment with DMSO, staurosporine and the grape seed polymer. Cells were grown on poly-L-lysine coated glass cover slips and incubated with DMSO as control, 1 μM staurosporine and 2 μM polymer for 24 hours in FBS-free media, prior to TUNEL staining. Apoptotic TUNEL positive cells (+ve) stained brown, while non-apoptotic TUNEL negative (-ve) cells appear pale green. Overall magnification of x400.
2.3.7 DNA fragmentation induced by polyphenol treatment.

Another distinguishing feature of apoptosis is cleavage of DNA into oligonucleosomal-sized fragments of 180 bp, which are observed in characteristic DNA laddering, following separation by agarose gel electrophoresis. In contrast, intact non-fragmented nuclei do not display a DNA ladder, and necrotic cells only produce a smear of randomly degraded DNA. Isolated DNA from HCT-8 cells, following incubation with EGCG, theaflavins, curcumin, the grape seed polymer and staurosporine, were separated by agarose gel electrophoresis containing 0.4 μg/ml ethidium bromide and observed under UV light. DNA from untreated control cells and cells incubated with the polyphenolics, EGCG, polymer (Figure 2.25), curcumin and theaflavins (Figure 2.26) remained intact. Only DNA from HCT-8 cells, incubated with staurosporine (Figure 2.26) or EGCG plus staurosporine (Figure 2.25) (see section 4.3.4), exhibited a smear of fragmented DNA, faintly covering the range of fragments expected, although clear DNA laddering was not evident.

![Figure 2.25: Agarose gel electrophoresis of isolated DNA from HCT-8 cells treated with EGCG, EGCG with staurosporine or the polymer for 24 hours in FBS-free media. Lane M, DNA marker; 1 & 7, DMSO treated control; 2 & 8, 1 μM staurosporine; 3, 5 μM EGCG; 4, 25 μM EGCG; 5, 5 μM EGCG and 1 μM staurosporine; 6, 25 μM EGCG and 1 μM staurosporine; 9, 2 μM polymer; 10, 1.5 μM polymer; 11, 1 μM polymer; 12, 0.5 μM polymer.](image-url)

2.3.8 Polyphenol-induced apoptosis in cancerous and non-cancerous cells.

A number of different polyphenols, such as EGCG, are reported to modulate both cell growth and apoptosis in a variety of tumour cell lines, with little or no effect in their normal counterparts (Huynh & Teel, 2000; Lu et al., 2000, 2001; Hsu et al., 2002). Consequently, EGCG, theaflavins, curcumin and the grape seed polymer, which all demonstrated apoptotic activity at low concentrations, when assessed via Hoechst 33342 staining in HCT-8 cells, were further investigated to ascertain the apoptotic effects of the polyphenols in ICE-6, a non-cancerous rat epithelial cell line. As observed in HCT-8 cells, 25 µM curcumin also proved cytotoxic to ICE-6 cells (Figure 2.27), and 100 µM EGCG (data not shown) was highly cytotoxic in both cell lines with nearly all cells becoming detached. Staurosporine induced a high level of apoptosis in HCT-8 cells (Figure 2.27), and, although a similar effect was observed in ICE-6 cells, viability was severely compromised (75.5 %). Figure 2.27 illustrates the apoptotic
response induced by 25 μM EGCG and 5 μM curcumin in HCT-8 cells, compared with the near endogenous response in ICE-6 cells. A similar outcome was evident with theaflavins and the polymer (Figure 2.28), suggesting that cancer cells are more susceptible to polyphenol-induced apoptosis.

![Graph showing apoptotic response](image1)

**Figure 2.27:** Apoptotic response of EGCG and curcumin in cancerous (HCT-8) and non-cancerous (ICE-6) cells. Cells were incubated for 24 hours in FBS-free media with EGCG and curcumin. Percentage viability was >90.5 %, except for 1 μM staurosporine (ICE-6) at 75.5 % and 25 μM curcumin (HCT-8 and ICE-6) at 6.5 and 23.5 % respectively. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12 %.

![Graph showing apoptotic response](image2)

**Figure 2.28:** Apoptotic response of theaflavins and the polymer in cancerous (HCT-8) and non-cancerous (ICE-6) cells. Cells were incubated for 24 hours in FBS-free media with polymer and theaflavins (TF). Percentage viability was >90.5 %, except for 2 μM polymer (ICE-6) at 75.5 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10 %.
2.3.9 Does caffeine induce apoptosis in HCT-8 cells?

In addition to a variety of polyphenolic compounds, tea also contains methyl xanthines. Theobromine accounts for approximately 0.3 % of the solid material extracted from tea leaves, with boiling water, while caffeine makes up a further 5-10 % (Marks, 1992). Although only constituting a small fraction of dried tea extracts, caffeine is held responsible for the majority of biological consequences of tea drinking (Lou et al., 1999; Lu et al., 2002; Yang et al., 2002). Therefore, the possibility that caffeine could contribute to the reported apoptotic capacity of tea was considered (Fernandez et al., 2003; He et al., 2003). Initially, studies were performed using a range of caffeine concentrations (5-100 μM), similar to those found in plasma following ingestion of tea (Marks, 1992), but no apoptotic activity was detected (Figure 2.29). Since the polyphenols found to be active in this study are either present in tea, or following consumption, may be present in plasma and tissues, potentially associating with caffeine, the influence of caffeine upon polyphenol-induced apoptosis was investigated, to establish whether caffeine can potentially induce apoptosis, either synergistically or additively, in combination with the polyphenols. Therefore, HCT-8 cells were incubated with EGCG, theaflavins (Figure 2.30), curcumin and the grape seed polymer (Figure 2.31), in the presence and absence of caffeine (5-50 μM). No change was observed in polyphenol-induced apoptosis, even at the highest caffeine concentration. It may be inferred that, in HCT-8 cells, caffeine has no apoptotic activity and, furthermore it does not interact with the polyphenols tested.

Figure 2.29: Apoptotic effect of caffeine in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media in the absence and presence of caffeine (5-100 μM). Percentage viability was >91%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12%.

Figure 2.30: Effect of caffeine upon EGCG- and theaflavins-induced apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with EGCG or theaflavins (TF) in the absence and presence of caffeine (5-50 μM). Percentage viability was >94%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10%.
Figure 2.31: Effect of caffeine upon curcumin- and polymer-induced apoptosis in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with curcumin or polymer in the absence and presence of caffeine (5-50 µM). Percentage viability was >94%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12%.

2.3.10 Comparison of apoptosis induced by whole tea and polyphenol-free tea.

Freeze-dried tea solubles (since they were obtained as a powder they are sometimes referred to as freeze-dried tea solids) derived from whole green and black tea infusions, along with decaffeinated polyphenol-free green and black tea, were used to corroborate the view that polyphenols are largely responsible for the apoptotic activity of tea (Ahmad et al., 1998; Fujiki & Suganuma, 2002; Wheeler & Wheeler, 2004). In addition, the possibility that, in whole tea, tea polyphenols induce apoptosis in an additive or synergistic manner, was examined. Known concentrations of EGCG in green tea were used to determine the equivalent amount of green tea solubles that may induce similar levels of apoptosis. The range of whole green tea containing final concentrations equivalent to 5, 10, 20, 25 and 50 µM EGCG was chosen for these studies. Consequently, apoptotic activity in HCT-8 cells, incubated with 2.5, 5, 10, 12.5
and 25 µg/ml of whole green tea, was compared with equivalent concentrations of polyphenol-free green tea. Figure 2.32 shows modest cytotoxicity, in HCT-8 cells, with whole green tea at concentrations over 10 µg/ml. At a concentration of 5 µg/ml, whole green tea (containing 10 µM EGCG) induced apoptosis at comparable levels to those of 25 µM EGCG, raising the possibility of an additive and/or synergistic response by EGCG and other green tea polyphenols. In contrast, no apoptotic effect was seen with the equivalent amount of polyphenol-free green tea.

Since apoptotic activity in HCT-8 cells was induced by theaflavins at concentrations of 2.5 and 25 µg/ml, whole black tea, containing equivalent concentrations of theaflavins, was used to investigate the apoptotic capacity of the black tea solubles. Consequently, the apoptotic activity in HCT-8 cells, incubated with 2.5, 10, 20, 25 and 50 µg/ml whole black tea, was compared with equivalent concentrations of polyphenol-free black tea (Figure 2.33). Results indicate an increase in apoptotic induction and a decline in cell viability as whole black tea concentration rises, causing cytotoxicity even at low concentrations of 10 µg/ml. This suggests a synergistic, or additive, apoptotic response by whole black tea. In comparison, theaflavins alone, at a concentration of 25 µg/ml, produced apoptosis without cytotoxicity. At a concentration of 2.5 µg/ml, where no cytotoxicity was evident with whole black tea, only a modest (12.5 %) degree of apoptosis was observed. Similar to polyphenol-free green tea, polyphenol-free black tea was devoid of apoptotic activity.
Figure 2.32: Induction of apoptosis by whole green tea compared to polyphenol-free green tea in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with whole green tea (WGT) or polyphenol-free green tea (FGT). Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 and 5% for apoptosis and viability, respectively.

Figure 2.33: Induction of apoptosis by whole black tea compared to polyphenol-free black tea in HCT-8 cells. Cells were incubated for 24 hours in FBS-free media with whole black tea (WBT) and polyphenol-free black tea (FBP). Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12% for apoptosis and viability.
2.4 Discussion.

Apoptotic activity of a large number of polyphenols was evaluated in the HCT-8 cell line, initially using RPMI 1640 media supplemented with 10 % FBS. Generally, >20 % apoptosis was obtained with 400 μM of the polyphenolic compounds, with a few compounds exhibiting >20 % apoptosis at 200 μM, concentrations which are considerably higher than those observed in human plasma (0.1-10 μM), following ingestion of a polyphenol-rich meal (Kroon et al., 2004). Indeed, the plasma concentration of an individual polyphenol rarely exceeds 1 μM, after consumption of 10-100 mg of the corresponding compound (Day et al., 2000; Scalbert & Williamson, 2000; Manach et al., 2004). However, plasma concentrations vary, depending upon the nature of the compound and food source, for example, plasma concentrations of 4 μM were obtained, after consumption of 50 mg of isoflavones (Manach et al., 2004). Therefore, the possibility of investigating the apoptotic response of low concentrations of polyphenols, similar to those found in vivo, was considered.

Polyphenol-protein interactions.

It has been reported that polyphenols have a high binding affinity for proteins, particularly hydrophobic, relatively large, proline-rich proteins, which possess open random-coil conformations (Haslam, 1974; Hagerman & Butler, 1981; Luck et al., 1994; Bennick, 2002). Consequently, protein-polyphenol interactions, that occur in media containing FBS, could potentially mask the polyphenols' apoptotic capacity, since binding would decrease their free concentration within the media (Arts et al., 2002). Therefore, it was decided to employ a method, initially using EGCG, whereby polyphenols could be incubated in FBS-free media, eliminating such interactions.
EGCG was noticeably more active at inducing apoptosis in the absence of FBS, at the concentrations studied, indicating that the presence of FBS masks the apoptotic activity of EGCG, particularly at lower concentrations. This is most likely because protein-polyphenol binding reduces the concentration of free EGCG in the media, thus reducing the concentration of EGCG accessible to the cells. Consequently, these lower concentrations were selected (5-50 μM), to evaluate the apoptotic activity of a number of polyphenols, which previously induced >20% apoptosis in HCT-8 cells, incubated in media containing 10% FBS.

Structure-activity relationships.

At a concentration of 5 μM, EGCG was one of the most active compounds at inducing apoptosis in HCT-8 cells, while other flavan-3-ols, including EGC, ECG and dimers of (+)-catechin and (-)-epicatechin, produced little or no apoptotic activity, suggesting a structure-activity relationship. This observation is similar to Chung et al. (2001) who reported that, in DU145 prostate cancer cells, (-)-epicatechin was inactive, however, apoptotic activity was observed with EGCG. Additionally, Saeki et al. (1999b; 2000) reported that, the presence of a 3',4',5'-trihydroxy (gallo) group on the B-ring of a flavan-3-ol is important, if not a minimum requirement, for the induction of apoptosis. Moreover, the addition of a 3-galloyl moiety is likely to enhance the apoptotic response. This implies that, in the case of flavan-3-ols, the greatest apoptotic activity appears to be conferred by the presence of both a gallo B-ring and a 3-galloyl moiety.

Black tea theaflavins, a mixture of theaflavin and its gallates, also produced a high percentage of apoptosis, at a concentration of 2.5 μg/ml, which is equivalent to 5 μM, based on the molecular weight of theaflavin. Since the proportion of the theaflavins in
the mixture was not known, it was thought pertinent to prepare concentrations in μg/ml. Surprisingly, the theaflavins’ apoptotic activity was not concentration-dependent, even though a 10-fold difference in concentrations was used. Lung et al. (2004), investigating black tea polyphenol-induced apoptosis in JCS leukaemia cells, reported that all the theaflavins tested induced apoptosis. However, theaflavin-3'-gallate and theaflavin-3,3'-digallate were the most potent, indicating that the presence of a 3-galloyl group at the 3' position may enhance the apoptotic activity of theaflavins (Lung et al., 2004). Moreover, Pan et al. (2000a) reported that theaflavin-3,3'-digallate, which contains the same number of OH groups as theasinensin A, a condensation product of two molecules of EGCG, inhibited tumour cell growth less effectively than theasinensin A itself. This indicates that if, indeed, it is the hydroxyl groups that are important, then the position of hydroxyl groups on phenolic compounds may determine their apoptotic activity.

The grape seed oligomer, composed of (+)-catechin, (-)-epicatechin and ECG monomers, with an average degree of polymerisation of 3.3 and 11 % galloylated units generated little apoptosis. In contrast, the grape seed polymer, with an average degree of polymerisation of 8.6 and 22 % galloylated units, induced apoptosis in a concentration dependent manner, even at low concentrations (<5 μM). A similar finding was reported by Matito et al. (2003), who studied the apoptotic effect of polyphenolic grape fractions in the murine hepatoma cell line Hepa-1c1c7, concluding that the grape fraction with the highest degree of polymerisation and percentage galloylation was the most effective apoptotic inducer.
The polyphenolics that were capable of activating apoptosis in HCT-8 cells were all flavan-3-ols; with the exception of curcumin, which induced apoptosis, even at the low concentration of 5 μM, however, no concentration-dependent effect was evident. This is in contrast to observations by Anto et al. (2002), who reported that curcumin induces apoptosis in a time and dose-dependent manner. Although, it is unclear whether this is always the case, since other studies investigating the apoptotic effect of curcumin fail to mention the presence of a concentration-dependent effect (Khar et al., 1999; Moragoda et al., 2001). Structure-activity relationships, as regards potential apoptotic pathways of the aforementioned “active” compounds, were further explored in chapter 4.

Cellular uptake of polyphenols.

LogP (octanol/water) partition coefficient values were calculated using PrologP Pallas computer software (section 2.3.5). Curcumin, which produced high levels of apoptosis, also has the highest logP value, implying that curcumin is hydrophobic (Khar et al., 1999), and would diffuse more readily into the cell than EGCG, which has a lower partition coefficient. This may be due to the 3-galloyl substitutions present in the structure of EGCG, which can result in a small increase in logP values, affecting the hydrophobicity of the compound, and thus, its ability to traverse cellular membranes passively (Scalbert & Williamson, 2000). Moreover, cellular absorption of EGCG may be enhanced, as demonstrated by Suganuma et al. (1999) who reported that (-)-epicatechin increased the incorporation of 3H-EGCG into PC-9 cells.

As expected, glycosylation affects the logP value of a compound, as shown by rutin, which appears clearly more hydrophilic than the parent compound quercetin. Consequently, rutin would be unable to traverse the cell membrane passively, unless the

hydophilic moieties are first enzymatically deglycosylated by LPH. Alternatively, it may be actively transported into the cell via a membrane transporter, such as SGLT1, and undergo hydrolysis intracellularly by CBG (Gee et al., 1998; Murota et al., 2000; Day et al., 2002). LogP observations from the current study are concordant with the work of Murota et al. (2000) who investigated the cellular uptake of quercetin using Caco-2 cell monolayers, and showed that quercetin glucosides were absorbed much less efficiently than the parent aglycone. Furthermore, hydrophobicity was an important determinant in the absorption of quercetin into Caco-2 cells, mainly due to the absence of a hydophilic sugar moiety.

The potential mechanisms through which the generally hydophilic, flavonoid glycosides are absorbed, were investigated by Gee et al. (1998), who reported that the glucose transporter SGLT1 partakes in the cellular uptake of quercetin glucosides. Subsequently, Day et al. (2003) demonstrated that the mechanism of absorption of quercetin-4'-glucoside involves active transport by SGLT1, with subsequent deglycosylation by CBG, as well as hydrolysis of the glucoside by LPH, with absorption by passive diffusion of the released aglycone. Quercetin-3-glucoside, in contrast, appears to be absorbed purely by passive diffusion following hydrolysis by LPH (Day et al., 2003). O’Leary et al. (2001; 2003) proposed that quercetin glucuronides, which are also highly hydophilic, may actively enter cells via transporters similar to OATP2, and subsequently undergo intracellular deglucuronidation by β-glucuronidase.
Confirmation of polyphenol-induced apoptosis.

In addition to morphological analysis, techniques, which assess the different stages of apoptosis, were used to confirm apoptotic activity of the “active” compounds. DNA degradation associated with apoptosis was investigated using DNA from HCT-8 cells treated with curcumin, EGCG, theaflavins and the polymer, whereby cells incubated with staurosporine produced a smear of fragmented DNA, faintly covering the range of fragments expected. However, there was no clear DNA laddering in response to any of the polyphenols tested, despite morphological evidence of apoptosis by Hoechst staining. Although it was hoped that DNA fragmentation would confirm apoptosis as the mode of cell death, the absence of clear DNA ladders is not altogether surprising, since apoptosis may also occur in the absence of 180 bp DNA oligonucleosomal fragments. This is because, during apoptosis, in association with chromatin condensation, DNA can initially fragment into larger 50 kbp fractions, which can be identified by field inversion gel electrophoresis (FIGE) (Cohen et al., 1994; Ormerod et al., 1994). The absence of DNA ladders may be related to the use of low concentrations of polyphenols, as in the present study, since many studies reporting the presence of DNA ladders, by agarose gel electrophoresis, employed higher polyphenol concentrations (Ahmad et al., 1997; Hibasami et al., 1998a; Okabe et al., 1999; Woo et al., 2003). For example, Okabe et al. (1999) observed ladders in DNA from KATO III stomach cancer cells incubated with 200 & 500 μM EGCG and 500 μM ECG, but not EGC, (-)-epicatechin or theaflavin, which were also tested at high concentrations. Similarly, Woo et al. (2003) reported that Caki renal carcinoma cells incubated with curcumin for 24 hours, at concentrations of 50-100 μM, induced DNA fragmentation. However, Wang et al. (1999) observed clear DNA ladders with only 60 μM of the polyphenols apigenin, quercetin, myricetin and kaempferol in HL-60 cells, after a 12-
hour incubation, although no such response was observed with the same concentration of EGCG.

TUNEL staining and flow cytometry analysis showed that only theaflavins appeared to induce apoptosis. However, when colorimetric TUNEL stains were evaluated, in many cases, cells were indistinguishable as TUNEL negative or positive; consequently, analysis was qualitative. Flow cytometric analysis of TUNEL stained A431 epidermoid carcinoma cells, following incubation with EGCG (80-320 μM), has been reported to induce apoptosis in a concentration-dependent manner, even if the corresponding viability data (<80 %) indicated cytotoxicity (Ahmad et al., 1997).

Flow cytometric analysis of HCT-8 cells stained with PI failed to exhibit distinct apoptotic hypodiploid or subG1 peaks (also associated with degradation of DNA during apoptosis), notwithstanding apoptotic morphology being observed after Hoechst 33342 staining. Previous studies also encountered problems with PI binding during flow cytometry studies (Bielak-Zmijewska et al., 2000; Larrosa et al., 2003). Contradictory results were reported by Larrosa et al. (2003), who investigated the apoptotic activity of resveratrol in Sk-Mel-28 melanoma cells, wherein no subG1 peaks were observed after PI staining of cells, incubated with 100-300 μM resveratrol for 24 hours, whereas, apoptosis was detected using the Annexin V assay. Even when the same compound was investigated, results obtained by different methods of apoptotic assessment appear to vary, depending on the cell type employed. For example, Bielak-Zmijewska et al. (2000), using morphological assessment, demonstrated curcumin-induced apoptosis in a variety of cancer cells, however, there was a lack of DNA fragmentation. Although
apoptotic subG1 peaks and caspase-3 activation were observed in HL-60 cells, these observations were not evident in Jurkat cells (Bielak-Zmijewska et al., 2000).

Cell cycle analysis was undertaken to determine the proportion of cells in each phase of the cell cycle, particularly those in the subG1 apoptotic region. HCT-8 cells, incubated with EGCG and the polymer, showed no change to that of the control cell cycle, whilst an increase in the proportion of cells in G2/M phase, and thus a decrease in cells in phase G1, was noted for curcumin. This appears to indicate a cell cycle arrest in phase G1, which has been previously reported for EGCG in CaSki cells (Ahn et al., 2003), and in MCF10A cells (Liberto & Cobrink, 2000), at concentrations higher than those employed in the present study. Hence cell cycle arrest may precede apoptosis; for example, HT-29 human colon carcinoma cells incubated with flavone (2-prebyl-4H-1-benzopyran-4-one) (150 µM) for 24 hours showed an increase in the proportion of cells at S and G2/M phases, thus causing cell cycle arrest, but following a 48-hour incubation apoptotic cells were observed in the subG1 region (Wenzel et al., 2000). In CaSki human cervical cancer cells incubated with EGCG (35 µM) for 24 hours, no apoptosis was apparent, although cell cycle arrest was observed at phase G1. In contrast, treatment with a higher concentration of EGCG (100 µM) resulted in a considerable increase of subG1 apoptotic cells (Ahn et al., 2003).

Since the presence of a subG1 peak is also associated with the degradation of DNA during apoptosis, it is possible that apoptosis occurred without the presence of concomitant internucleosomal DNA degradation (Ormerod et al., 1994). Moreover, polyphenol-treated cells in cell cycle arrest have the potential to undergo apoptosis. This was observed by Ahmad et al. (2000), who reported that EGCG causes induction
of G1 phase cki, in human epidermoid carcinoma A431 cells, which bind to, and inhibit, specific cyclin-cdk complexes, thereby inducing a G1 phase arrest, which is thought to be an irreversible process ultimately resulting in apoptosis. Consequently, pRb (retinoblastoma protein) is phosphorylated, which activates the E2F transcription factor, resulting in an artificial checkpoint at the G1-S transition, thereby causing G1 arrest and subsequent apoptotic death (Ahmad et al., 2002). Therefore, in the light of the work of Ormerod et al. (1994) and Ormerod (1998; 2001), it is imperative to always assess cells morphologically, as morphology remains the only means of unequivocally identifying an apoptotic cell, a fact reinforced by the current study. Although the present study has enabled identification of “active” polyphenols, it is unclear whether apoptosis is induced via an intrinsic or extrinsic pathway. This will be further investigated in chapter 3.

**Apoptotic activity of tea: potential interactions of polyphenols.**

A study investigating the potential apoptotic activity of caffeine in HCT-8 cells was carried out, following reports that this methyl xanthine may be responsible for the apoptotic effect conferred by black and green tea (Fernandez et al., 2003; He et al., 2003). The average plasma half-life of caffeine in normal healthy human adults is short, in the region of 4-6 hours, and so caffeine concentrations in the circulation may be relatively low. Hence, after tea consumption, the concentration of caffeine present in plasma is in the region of 21 μM, depending on the degree of absorption (Marks, 1992).

Caffeine was initially investigated using concentrations similar to those found in plasma after tea consumption (Marks, 1992), but no apoptotic activity was detected. To evaluate whether caffeine influences polyphenol-induced apoptosis, in an additive or synergistic manner, physiological concentrations of caffeine were incubated with active
polyphenols, previously shown to induce apoptosis in this study. However, at the concentrations tested, caffeine had no effect upon EGCG-, theaflavins-, curcumin- or polymer-induced apoptosis, inferring that, at least in HCT-8 cells, caffeine has no significant influence upon apoptosis induction, either alone or in the presence of polyphenols.

For the most part, studies reporting apoptotic induction by caffeine were dependent on the use of high caffeine concentrations, for example, He et al. (2003) observed apoptosis in JB6 C41 cells, after treatment with 450 μM caffeine, however, no apoptotic activity was observed at the lower concentration of 50 μM, a response comparable to that observed during the present study. In addition, by employing far higher concentrations of caffeine (10-16 mM), apoptosis was reported in Chinese hamster ovary cells (Fernandez et al., 2003), although no apoptotic activity was displayed when caffeine concentrations were reduced to 1 mM, which in itself is considerably higher than the concentrations employed during the current study. It therefore appears that caffeine-induced apoptosis is generally concentration-dependent, with activity most evident at low, non-physiological, mM concentrations. Moreover, such apoptosis results are also questionable, since high concentrations of caffeine can reduce cell viability (Fernandez et al., 2003; He et al., 2003; Ito et al., 2003).

To further assess the role of polyphenols in tea-induced apoptosis, studies were conducted in HCT-8 cells, where the apoptotic activity of whole tea was compared with tea from which the polyphenols were removed (polyphenol-free tea), at equivalent concentrations. Results clearly showed that whole green and black tea are active at inducing apoptosis, while polyphenol-free teas failed to induce apoptosis, indicating that
polyphenols make an important contribution to the apoptotic activity of tea. Whole black tea, at a concentration of 10 μg/ml (containing an equivalent concentration of theaflavins), induced apoptosis at higher levels than that of 25 μg/ml theaflavins. Similarly, at a concentration of 5 μg/ml, whole green tea (containing the equivalent of 10 μM EGCG) induced apoptosis at comparable levels to those of 25 μM EGCG. This indicates that an additive and/or synergistic response of tea polyphenols is likely to be responsible for apoptotic activity exhibited by tea, and may explain the high levels of cytotoxicity observed, particularly regarding whole black tea. This observation is similar to Suganuma et al. (1999) who, using the human lung cancer cell line, PC-9, investigated the apoptotic effects of (-)-epicatechin, an apparently inactive tea polyphenol. Initial results, indicated that green tea had stronger apoptotic activity than was expected from the polyphenol content. Subsequently, co-treatment of (-)-epicatechin with EGCG, EGC or ECG, synergistically induced apoptosis in PC-9 cells, mediated by enhanced incorporation of the green tea polyphenols into the cells, as demonstrated by a significant increase in intracellular [3H] EGCG, in the presence of (-)-epicatechin (Suganuma et al., 1999).

Conclusions.

After evaluation of a wide range of polyphenols for their apoptotic activity in the absence of FBS, at concentrations relevant to humans (Kroon et al., 2004), EGCG, theaflavins, curcumin and the grape seed polymer, were found to be the most active at inducing apoptosis in the ileocecal adenocarcinoma cell line HCT-8. Further assessment of apoptosis, using the TUNEL assay, agarose gel electrophoresis and flow cytometry, was unable to confirm activity, which, to a certain extent, appears to be due to the low, but more physiological, concentrations employed in the present study. For
the most part, other studies reporting the presence of DNA ladders, TUNEL positive cells and subG1 peaks, employed high polyphenol concentrations (Ahmad et al., 1997; Hibasami et al., 1998a; Okabe et al., 1999; Woo et al., 2003). Subsequently, caspase activation and inhibition studies were undertaken, using the aforesaid compounds, to confirm polyphenol-induced apoptosis and establish whether the caspase cascade is a potential mechanism behind their apoptotic activity (chapter 3).
CHAPTER 3.

Influence of Polyphenols upon the Caspase Cascade.
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3.1 INTRODUCTION.

Apoptosis is regarded as an integral physiological process of both normal development and tissue homeostasis in multicellular organisms. Conversely, uncontrolled apoptosis potentially contributes to a range of pathological diseases, with excessive apoptosis culminating in neurodegeneration or stroke, and insufficient apoptosis resulting in cancer. However, therapeutic modulation of apoptosis may be instrumental in the reduction of cancer cell incidence (Bratton & Cohen, 2001; Zimmermann et al., 2001; McCarthy, 2003).

The characteristic morphological and biochemical changes of apoptosis arise following activation of a family of intracellular cysteinyl aspartate-specific proteases, more commonly known as caspases (as discussed in section 1.4). The caspase cascade can be initiated via two main signalling pathways (Figure 1.7). One pathway involves extracellular binding of a cell surface death receptor, such as Fas or TNF, leading to subsequent oligomerisation and autocatalytic processing of procaspase-8 to its active form caspase-8 (Zimmermann et al., 2001; Zhivotovsky, 2003). Alternatively, intracellular chemical stimuli, downstream of the mitochondria, may trigger the release of cytochrome c into the cytosol. Cytochrome c binds to Apaf-1, which oligomerises in the presence of ATP, mediating a conformational change, thus enabling the recruitment of procaspase-9, and its subsequent autoproteolysis and activation to caspase-9. However, in some cell types, caspase-8 cleaves Bid, the only member of the Bcl-2 protein family whose C-terminal fragment translocates into the mitochondria, and through the oligomerisation of Bax and Bak, cytochrome c is released, engaging the intrinsic pathway and consequently activating caspase-9 (Bratton & Cohen, 2001; Zimmermann et al., 2001; Degterev et al., 2003). In the same way as with caspase-8,
once activated, caspase-9 propagates the death signal by proteolytically processing and activating downstream effector caspases in the cascade, culminating in the cleavage of essential enzymes and structural proteins, including DNA-dependent protein kinase, lamins, fodrin and protein kinase C (PKC), indicative of apoptotic cell death (Bratton et al., 2000; Cain, 2000; Zhivotovsky, 2003).

Polyphenol-induced apoptosis has been demonstrated in a variety of tumour cell lines, many reporting caspase-3 activation, the final step in the caspase cascade, which precedes apoptosis (Chen et al., 2000; Kumi-Diaka et al., 2000; Kennedy et al., 2001; Anto et al., 2002; Nakagawa et al., 2002; Woo et al., 2003). However, the mechanisms through which polyphenols activate upstream caspases, and subsequently caspase-3, remain to be clarified.

Studies investigating the effect of curcumin in a variety of human melanoma, leukemia and carcinoma cell lines, which all demonstrated caspase-3 activation (Bielak-Zmijewska et al., 2000; Bush et al., 2001; Anto et al., 2002; Woo et al., 2003), were reported to involve the upstream activation of caspase-8, or additional Bid cleavage and cytochrome c release. Moreover, curcumin-induced apoptosis of KATO III gastric and HCT-116 colon cancer cells was reportedly associated with both caspase-3 and -8 activation, which is indicative of the extrinsic apoptotic pathway (Moragoda et al., 2001). In contrast, similar studies undertaken with curcumin reported the activation of caspase-8 and -9 in K562 leukemia cells, implying that curcumin might induce apoptosis by involving both the extrinsic and intrinsic apoptotic pathways (Duvoix et al., 2003). Furthermore, in Ehrich’s ascites carcinoma cells, curcumin-induced apoptosis was associated with caspase-3 activation, cytochrome c release and
upregulation of the pro-apoptotic Bax protein (Pal et al., 2001). Similarly, EGC-induced apoptosis in breast cancer cells was reportedly associated with a decrease in anti-apoptotic Bcl-2 and an increase in pro-apoptotic Bax protein levels, with apoptosis efficiently suppressed by a caspase-8 inhibitor (Vergote et al., 2002).

Other polyphenols investigated, such as EGCG (Nakagawa et al., 2002; Saeki et al., 2002; Roy et al., 2003), quercetin (Wang et al., 1999; Mertens-Talcott et al., 2003), resveratrol (Park et al., 2001) and genistein (Kumi-Diaka et al., 2000), all demonstrated caspase-3 activation in a variety of cancer cell lines. However, contradictory responses to the activation of caspases -8 and -9 were observed, so that it remains unclear by which pathway polyphenols generally induce apoptosis. Furthermore, the involvement of caspase activation during polyphenol-induced apoptosis appears to vary, depending not only on the polyphenol, but also the cell type under investigation.

In the present study, the polyphenols EGCG, theaflavins, curcumin and the grape seed polymer, which were found to induce apoptosis in the human ileocecal adenocarcinoma cell line, HCT-8, by morphological assessment (see section 2.3.3), were further investigated to determine which caspases are activated during polyphenol-induced apoptosis. This was achieved by initially examining caspase-3 and -7, to confirm induction of the caspase cascade, and subsequently, focusing on caspase-8 and -9, to evaluate whether upstream activation occurs via an intrinsic or extrinsic pathway.
Chapter 3. Influence of Polyphenols upon the Caspase Cascade.

3.2 MATERIALS AND METHODS.

3.2.1 Caspase inhibition.

Materials.


Method.

The caspase-1 and -3 broad specificity inhibitor Z-VAD-FMK was initially dissolved in DMSO to prepare a 10 mM stock solution, which was stored at -20 °C. HCT-8 cells were cultured in 6-well plates until 80 % confluent (as detailed in 2.2.1 and 2.2.2), then washed gently in PBS, before undergoing a 30-minute pre-treatment with 10, 50 and 100 μM Z-VAD-FMK, in FBS-free media. Following this incubation, the polyphenols of interest were added to the FBS-free media, and HCT-8 cells were incubated for a further 24 hours under standard conditions of 37 °C and 5 % CO₂ in a humid environment. Cell viability (as detailed in 2.2.3) and percentage apoptosis (as detailed in 2.2.4) were determined to assess the extent of caspase inhibition in polyphenol-induced apoptosis during a 24-hour incubation period.

3.2.2 Caspase-3/7 activation assay.

Materials and instrumentation.

- Z-DEVD-AFC (Z-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin): Calbiochem, Merck Biosciences Ltd, Nottingham, UK.
- PerkinElmer LS 50B fluorimeter with FL WinLab software: PerkinElmer Instruments Inc, Beaconsfield, UK.
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- HEPES (N-[2-hydroxyethyl]piperazine-N'-2-ethansulphonic acid), EGTA (ethylenbis[oxyethylene-nitro]tetra acetic acid), β-glycerophosphate (disodium salt), igepal CA-630, dithiothreitol (DTT): Sigma-Aldrich Company Ltd, Poole, UK.

Method.

Activation of caspase-3 and -7 by polyphenols was investigated, as described by Nicholson et al. (1995) and Jones et al. (1998). HCT-8 cells were cultured in 25 cm² flasks until 80 % confluent, and incubated for 24 hours with polyphenols in FBS-free media, as detailed in 2.2.1 and 2.2.2. Following treatment, control and apoptotic cell pellets were washed in ice-cold PBS, resuspended in cell lysis buffer (40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl₂·6H₂O, 5 mM EGTA, 10 mM HEPES, pH 7.0) and subjected to cell membrane disruption by four freeze/thaw cycles in a dry ice/ethanol bath, after which they were centrifuged at 15,000 g for 40 minutes at 4 °C.

Using a black microtitre plate, a reaction mixture containing 186 μl caspase assay buffer (100 mM HEPES, 10% (v/v) sucrose, 0.1 % (v/v) igepal CA-630, 10 mM DTT, pH 7.25), 4 μl Z-DEVD-AFC (2 mM stock in DMSO), and 10 μl cell extract was added in each well. Samples were assayed for AFC (fluorogenic product) activity, every 10 minutes for 70 minutes, using a PerkinElmer LS 50B fluorimeter equipped with FL WinLab software (PerkinElmer Instruments Inc), capable of measuring excitation at 360 nm and emission at 530 nm. The microtitre plate was removed from the fluorimeter, covered in foil and incubated at 37 °C between each reading. Using an AFC calibration curve (0.5-20 μM) and the Bio-Rad protein assay (section 3.2.3), enzyme activity was expressed as nanomoles of AFC liberated per milligram of protein per minute.
3.2.3 **Bio-Rad protein assay.**

*Materials and instrumentation.*

- BSA: Sigma-Aldrich Company Ltd, Poole, UK.
- Labsystems Multiscan RC spectrophotometer with Genesis software: Labsystems, now Thermo Electron Corporation, Altrincham, UK.

*Method.*

The Bio-Rad protein assay is a colorimetric assay measuring total protein concentration, based on the Coomassie Brilliant Blue dye-binding procedure developed by Bradford (1976). After constructing a standard curve with BSA (5-200 µg/ml), 160 µl diluted sample/standard were added to a microtitre plate, followed by addition of 40 µl of Bio-Rad dye reagent concentrate, to give a final volume of 200 µl per well. The microtitre plate was gently shaken to mix the contents of the wells, then after a period of 5 minutes to 1 hour, absorbance was read at 595 nm using a Labsystems Multiscan RC spectrophotometer with Genesis software (Labsystems).

3.2.4 **Caspase-8 and caspase-9 activity assays.**

*Materials and instrumentation.*

- Caspase-8 activity assay fluorometric kit; containing microtitre plate, caspase-8 extraction buffer, assay buffer, recombinant caspase-8, caspase-8 substrate conjugate (Z-IETD-AFC), caspase-8 inhibitor (Z-IETD-FMK) and positive control cell pellets. Caspase-9 activity assay fluorometric kit; containing microtitre plate, caspase-9 extraction buffer, assay buffer, recombinant caspase-9, caspase-9 substrate conjugate (Z-LEHD-AFC), caspase-9 inhibitor (Z-LEHD-
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FMK) and positive control cell pellets: Oncogene Research Products, Merck Biosciences Ltd, Nottingham, UK.

• DTT: Sigma-Aldrich Company Ltd, Poole, UK.

• PerkinElmer LS 50B fluorimeter with FL WinLab software: PerkinElmer Instruments Inc, Beaconsfield, UK.

Methods.

The same method was employed for both caspase-8 and caspase-9 activity assays, with use of the relevant kit components, to determine the specific cleavage of AFC fluorescently labelled IETD and LEHD substrates, respectively. HCT-8 cells were grown in 25 cm² flasks until 80% confluent and then incubated with a range of polyphenols in FBS-free media for 24 hours, as detailed in 2.2.1, 2.2.2, 4.2.2 and 4.2.4. Culture media containing a few dead or dying cells were kept and pooled with the attached cells which, after the addition of PBS, were harvested by scraping and then centrifuged at 500 g for 5 minutes. Cell pellets were then stored at −20 °C until sufficient pellets to fill the 96-well assay plate were prepared. Sample cell pellets were resuspended in 60 µl of extraction buffer (10 µl of 1 M DTT stock solution per 1 ml of extraction buffer), while control pellets (HL-60 cells incubated with actinomycin D for 19 hours, provided with the kit) were resuspended in 50 µl of extraction buffer. All cell pellets were then incubated on ice for 10 minutes, before being centrifuged for 5 minutes at 500 g. Using the microtitre plate provided, 50 µl of cleared cell lysates were added per well, using 50 µl of extraction buffer in spare well, as a blank.

Both recombinant caspase-8 (or caspase-9) vials were defrosted by hand, and pulse spun, enabling collection of the small volume (2 µl) provided in the conical section of the vial. Then, 48 µl of extraction buffer was added to each vial, of which 50 µl was transferred to a well on the microtitre plate. Subsequently, 50 µl of assay buffer (10 µl
of 1 M DTT stock solution per 1 ml of assay buffer) was added to each well, followed by the addition of 1 μl of caspase-8 (or caspase-9) inhibitor to a specific set of wells. The microtitre plate was then covered in foil and incubated at 37 °C for 30 minutes. Finally, 10 μl of substrate conjugate was added to each well, and the microtitre plate was read on a PerkinElmer LS 50B fluorimeter with FL WinLab software (PerkinElmer Instruments Inc), at an excitation of 400 nm and emission of 505 nm, to obtain data for time zero. The microtitre plate was then covered in foil once again and incubated for a further 2 hours at 37 °C, before fluorescence was once again determined. Results of the relative fluorescence signal, measured in relative fluorescence units (RFU), were expressed as the difference between readings at the two time points, after subtraction of the appropriate buffer controls.

3.3 RESULTS.

3.3.1 Caspase-3 inhibition of polyphenol-induced apoptosis.

Polyphenols found to induce apoptosis in HCT-8 cells, by Hoechst 33342 staining, during the present study (section 2.3.3), were further investigated to ascertain if polyphenol-induced apoptosis occurs via the caspase cascade. Two approaches were employed during this study; firstly, an indirect measurement, which relies on a caspase inhibitor, but still assesses apoptosis by morphology. The second approach involves the direct measurement of caspase activity using specific fluorogenic substrates. The polyphenols EGCG, theaflavins, curcumin and the grape seed polymer were incubated in the presence and absence of 10, 50 and 100 μM of the broad specificity pan reactive caspase-3 inhibitor Z-VAD-FMK, and assessed for apoptosis using the Hoechst 33342 dye and fluorescence microscopy.
Results show that 100 μM Z-VAD-FMK alone had no effect upon apoptosis of HCT-8 cells. However, Z-VAD-FMK appears to inhibit apoptosis induced by 25 μM EGCG (Figure 3.1), 25 μg/ml theaflavins (Figure 3.2), 10 μM curcumin (Figure 3.3) and 2 μM polymer (Figure 3.4) in a concentration-dependent manner. These observations suggest that caspases are involved in polyphenol-induced apoptosis, most probably by activation of the caspase cascade.

Figure 3.1: Inhibition of EGCG-induced apoptosis by Z-VAD-FMK. HCT-8 cells were pre-incubated for 30 minutes with the pan-reactive caspase-3 inhibitor Z-VAD-FMK (FMK), and then incubated for a further 24 hours, following the addition of 25 μM EGCG to the FBS-free media. Results for percentage apoptosis were obtained using the Hoechst 33342 stain and percentage viability of >93.5 % was obtained by trypan blue exclusion. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >14 %.
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Figure 3.2: Inhibition of theaflavins-induced apoptosis by Z-VAD-FMK. HCT-8 cells were pre-incubated for 30 minutes with the pan-reactive caspase-3 inhibitor Z-VAD-FMK (FMK), and then incubated for a further 24 hours, following the addition of 25 µg/ml theaflavins to the FBS-free media. Percentage viability was >97.5%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11%.

Figure 3.3: Inhibition of curcumin-induced apoptosis by Z-VAD-FMK. HCT-8 cells were pre-incubated for 30 minutes with the pan-reactive caspase-3 inhibitor Z-VAD-FMK (FMK), and then incubated for a further 24 hours, following the addition of 10 µM curcumin to the FBS-free media. Percentage viability was >91%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >14%.
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3.3.2 Caspase-3/7 activation in HCT-8 cells treated with polyphenols.

Following on from inhibition studies, indicating that caspase-3 is involved in polyphenol-induced apoptosis, further studies were undertaken to ascertain the extent to which caspase-3 was activated during polyphenol-induced apoptosis. This was achieved by investigating the caspase activity of HCT-8 cells incubated with EGCG or curcumin, and employing the known apoptotic inducer, staurosporine, as a positive control. Using a specific caspase-3 and -7 fluorogenic substrate, known as Z-DEVD-AFC, HCT-8 cell extracts were assayed for caspase-3 and -7 activity by microfluorimetry. Results show a clear induction of caspase-3 and -7 activity by the positive control staurosporine (Figures 3.5 and 3.6). In addition, EGCG (Figure 3.5) and curcumin (Figure 3.6) both demonstrate a concentration-dependent induction of caspase-3 and -7 activity, within a concentration range that has previously exhibited optimal apoptotic induction without cytotoxicity (sections 2.3.2 and 2.3.3). These
observations, which are consistent with those from caspase inhibition studies (section 3.3.1), imply that the caspase cascade is activated during polyphenol-induced apoptosis, since caspase-3 and -7 are the final caspases in the cascade.

![Graph](image1)

**Figure 3.5:** Caspase activation of HCT-8 cells incubated with EGCG. Z-DEVD-AFC cleavage activity in cell extracts treated with EGCG for 24 hours in FBS-free media was assessed by microfluorimetry. Results are expressed as the average of duplicate determinations, which did not differ between them by >8 %.

![Graph](image2)

**Figure 3.6:** Caspase activation of HCT-8 cells incubated with curcumin. Z-DEVD-AFC cleavage activity in cell extracts treated with curcumin for 24 hours in FBS-free media was assessed by microfluorimetry. Results are expressed as the average of duplicate determinations, which did not differ between them by >11 %.
3.3.3 *Activity of caspase-8 and caspase-9 in HCT-8 cells treated with polyphenols.*

Subsequent to the realisation that EGCG, theaflavins, curcumin and the polymer are likely to induce apoptosis through the caspase cascade, further studies were embarked on to determine the pathways involved. This was achieved by investigating the effect of the aforementioned polyphenols upon the activity of caspase-8, which is associated with death-receptor activated apoptosis, and caspase-9, that is activated during chemical/stress-induced apoptosis. Therefore, caspase-8 and caspase-9 activity assays were employed to ascertain whether extracellular or intracellular triggers are involved in the apoptotic pathways.

*Caspase-8 activity.*

Recombinant caspase-8 was used as a positive standard for the assay and produced a substantial amount caspase-8 activity. An additional positive control, supplied with the assay kit, was HL-60 cells previously treated with 0.5 μg/ml of the apoptotic inducer actinomycin D, for 19-24 hours. These induced a similar relative fluorescent signal to the regular positive control of 0.5 μM staurosporine-treated HCT-8 cells (Figure 3.7). Results showed that the polyphenols EGCG, curcumin and the grape seed polymer exhibited activity at about 10 % of recombinant caspase-8 standard. However, in both caspase-8 and -9 assays, the relative fluorescent signal expressed by cells, incubated solely with the vehicle DMSO, appears somewhat high (Figures 3.7 and 3.8) and it remains unclear as to whether this is due to endogenous caspase activity, or as a result of the HCT-8 cells being incubated for 24 hours in FBS-free media. Thus, in comparison with the DMSO control, induction of caspase-8 activity displayed by the polyphenols was negligible. Furthermore, theaflavins exhibited no effect upon capase-8 activity, producing <43 RFU than that of the DMSO control (Figure 3.7). The prospect
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that a higher concentration of theaflavins may trigger caspase-8 activity was ruled out, seeing as concentrations of 100 and 250 µg/ml exhibited extensive cytotoxicity (sections 2.3.3 and 4.3.3). In addition, to determine the specificity of caspase-8 activity, EGCG, theaflavins, curcumin and the polymer were investigated using the specific, cell permeable and irreversible caspase-8 inhibitor, Z-IETD-FMK. All compounds displayed 90 % or greater inhibition of caspase-8 activity, which was considered to be specific (Figure 3.7).

Figure 3.7: Caspase-8 activity and inhibition in HCT-8 cells treated with polyphenols. Cell lysates were treated with EGCG, theaflavins (TF), curcumin, polymer, staurosporine (Stauro) or DMSO (vehicle control) for 24 hours in FBS-free media, before being assessed for caspase-8 activity by microfluorimetry. In addition, recombinant caspase-8 and HL-60 cells incubated with 0.5 µg/ml actinomycin D for 19-24 hours (HL-60 positive control) were assayed, and samples were incubated with the caspase-8 inhibitor Z-IETD-FMK. Results are expressed as mean RFU (relative fluorescence units) ± SEM of triplicate determinations for caspase-8 activity of HCT-8 cells, and as single values for caspase-8 inhibition and caspase-8 activity of recombinant caspase-8 and HL-60 positive control.

Caspase-9 activity.

The recombinant caspase-9 protein induced a high level of caspase-9 activity, with actinomycin D-treated HL-60 cells displaying comparable caspase-9 activity to staurosporine-treated HCT-8 cells (Figure 3.8), although activity appeared slightly lower than that observed for caspase-8 in the previous assay (Figure 3.7).
polyphenolic compounds investigated produced a variety of responses, exhibiting
activity at 6-33 % of the recombinant caspase-9 standard. Thus, when compared with
the DMSO control, curcumin induced a relatively high caspase-9 activity, equivalent to
>68 RFU of the DMSO control, whereas the polymer and EGCG produced a slight
increase in caspase-9 activity. Once again, no effect upon caspase activity was
observed by theaflavins (producing <36 RFU than that of the DMSO control), although
induction of caspase-9 activity was more pronounced than that of caspase-8 (Figure 3.8
and Figure 3.9). Additionally, to determine the specificity of positive caspase-9
activity, EGCG, theaflavins, curcumin and the polymer were examined using the
specific, cell permeable and irreversible caspase-9 inhibitor, Z-LEHD-FMK. The
polymer displayed 80 % inhibition of caspase-9 activity, while 90 % or greater
inhibition was obtained for the other polyphenols tested, theaflavins almost attaining
complete inhibition (97 %) (Figure 3.8).

![Caspase-9 activity and inhibition in HCT-8 cells treated with polyphenols](image)

Figure 3.8: Caspase-9 activity and inhibition in HCT-8 cells treated with polyphenols. Cell lysates
were treated with EGCG, theaflavins (TF), curcumin, polymer, staurosporine (Stauro) or DMSO (vehicle
control) for 24 hours in FBS-free media, before being assessed for caspase-9 activity by
microfluorimetry. In addition, recombinant caspase-9 and HL-60 cells incubated with 0.5 μg/ml
actinomycin D for 19 hours (HL-60 positive control) were assayed, and samples were incubated with the
caspase-9 inhibitor Z-LEHD-FMK. Results are expressed as mean RFU (relative fluorescence units) ±
SEM of triplicate determinations for caspase-9 activity of HCT-8 cells, and as single values for caspase-9
inhibition and caspase-9 activity of recombinant caspase-9 and HL-60 positive control.
Data obtained from both enzyme assays (Figures 3.7 and 3.8) demonstrates that all of the polyphenols tested appear to induce considerably greater caspase-9 activity than caspase-8 activity, which is particularly pronounced in the case of curcumin. These observations suggest that EGCG, theaflavins, curcumin and the grape seed polymer may therefore induce apoptosis independently of death-receptor activation (section 4.3.6), via an intrinsic pathway.

Caspase-9 activity: whole tea, polyphenol-free tea and whole wine extract.

Freeze-dried tea solubles, derived from whole green and black tea, and polyphenol-free green and black tea were used to corroborate the view that it is the polyphenols in tea, which are responsible for its apoptotic activity. In addition, a whole red wine extract was also investigated to determine whether, in combination, polyphenols are able to induce apoptosis in an additive or synergistic manner. In the light of preceding caspase assay results, indicating that caspase-9 activity induced by the polyphenolic compounds was more pronounced than caspase-8 activity, the caspase-9 activity of whole green and black tea, polyphenol-free green and black tea as well as a whole red wine extract were investigated. The whole red wine extract, investigated previously for apoptotic activity by Hoescht 33342 staining, induced >20 % apoptosis (section 2.3.3). Results from the caspase-9 activity assay indicate that the whole red wine extract stimulated caspase-9 activity, producing >24 RFU of the DMSO control, with 81 % inhibition, following addition of the specific caspase-9 inhibitor, Z-LEHD-FMK (Figure 3.9).

In the previous studies assessing apoptotic morphology, by Hoescht 33342 staining, both whole green and black tea produced >20 % apoptosis in HCT-8 cells (section 2.3.10), in comparison, little or no apoptosis seen in cells incubated with polyphenol-
free green and black teas. When assayed for caspase-9 activity, whole green and black tea produced <28 and <31 RFU of the DMSO control, respectively. However, in the presence of polyphenol-free green and black tea, caspase-9 activity increased to >71 and >26 RFU of the DMSO control, respectively. In the light of complete inhibition with Z-LEHD-FMK, although caspase-9 activity induced by whole green and black tea was generally low, it appears specific, implying the potential involvement of caspase-9. However, in polyphenol-free green and black tea, 53 and 77 % caspase-9 inhibition was observed, respectively (Figure 3.9), indicating that caspase-9 activity induced by polyphenol-free green tea is non-specific, whilst the specificity of polyphenol-free black tea remains unclear.

![Figure 3.9: Caspase-9 activity and inhibition in HCT-8 cells treated with tea and wine.](image)

Cell lysates were treated with whole green tea (WGT), whole black tea (WBT), polyphenol-free green tea (FGT) and polyphenol-free black tea (FBT), as well as a whole red wine extract, staurosporine (Stauro) or DMSO (vehicle control) for 24 hours in FBS-free media, before being assessed for caspase-9 activity by microfluorimetry. In addition, recombinant caspase-9 and HL-60 cells incubated with 0.5 μg/ml actinomycin D for 19 hours (HL-60 positive control), were assayed and samples were incubated with the caspase-9 inhibitor Z-LEHD-FMK. Results are expressed as mean RFU (relative fluorescence units) ± SEM of triplicate determinations for caspase-9 activity of HCT-8 cells, and as single values for caspase-9 inhibition and caspase-9 activity of recombinant caspase-9 and HL-60 positive control.
3.4 DISCUSSION.

The polyphenols, EGCG, theaflavins, curcumin and grape seed polymer, previously established to induce apoptotic morphology in HCT-8 cells, as assessed by Hoechst 33342 staining (section 2.3.3), were further investigated to ascertain whether polyphenol-induced apoptosis occurs via the caspase cascade. This was achieved by employing a variety of enzyme activation and inhibition assays, measuring the induction of either downstream caspases, to include caspase-3, signifying an involvement of the caspase cascade, or the induction of upstream caspases, such as caspase-8 and -9, to determine which particular apoptotic pathway was activated.

Influence of polyphenols upon caspase-3 induction.

The involvement of caspases, in polyphenol-induced apoptosis, was initially assessed by morphology, following pre-incubation of HCT-8 cells with the pan-reactive caspase-3 inhibitor Z-VAD-FMK. This inhibited EGCG-, theaflavins-, curcumin- and polymer-induced apoptosis in a concentration-dependent manner, suggesting that caspases are involved in polyphenol-induced apoptosis, most probably by activation of the caspase cascade. However, even at the highest Z-VAD-FMK concentration employed, inhibition of polyphenol-induced apoptosis was incomplete. A similar observation of incomplete suppression of polyphenol-induced apoptosis was reported for EGCG and genistein incubated with Z-VAD-FMK (Kumi-Diaka et al., 2000; Nakagawa et al., 2002). Since polyphenol-induced apoptosis was obtained in the absence of any obvious DNA laddering (section 2.3.7), it is perfectly possible that in such a complex pathway, which is not yet fully understood, other factors and alternative pathways, such as those involving H$_2$O$_2$ and inhibition of NF-κB, may contribute (Ahmad et al., 2000; Yang et
al., 2000). Thus, it is possible that caspase 3, assessed in this manner, may not always be a reliable indicator of caspase activity.

Direct activation of caspase-3 was confirmed by employing an activation assay that determines the cleavage of AFC, fluorescently labelled with the specific caspase-3 and -7 substrate Z-DEVD. EGCG and curcumin both induced caspase-3 and -7 activity in a concentration-dependent manner, indicating that the caspase cascade is activated during polyphenol-induced apoptosis. These observations are in agreement with previous studies investigating the molecular mechanisms of apoptotic induction by polyphenols, wherein EGCG- and curcumin-induced caspase-3 activation was reported in a variety of different cancer cell lines (Bielak-Zmijewska et al., 2000; Bush et al., 2001; Pal et al., 2001; Anto et al., 2002; Hsu et al., 2003; Roy et al., 2003; Woo et al., 2003). In addition, Hsu et al. (2003), reported that, after treatment with green tea polyphenols, apoptosis only occurred in tumour cells expressing wild type caspase-3, in comparison with caspase-3 null cells, as observed by morphological analysis and caspase-3 activity assay. Moreover, another contributory factor reported to be associated with elevated caspase-3 activity, during curcumin-induced apoptosis, involves the down-regulation of IAP (inhibitor of apoptosis protein), a family of proteins which appear to act as direct inhibitors to activated effector caspases, -3 and -7 (Roy et al., 1997; Curtin and Cotter, 2003; Woo et al., 2003).

Caspase-8 and -9 activity induced by polyphenols.

Since caspase-3 and -7 activation occurs downstream of the trigger, further studies were undertaken to investigate the effect of caspase-8 activity, which is implicated in death-receptor-induced (extrinsic) apoptosis and caspase-9 activity, that is activated during
chemical/stress-induced (intrinsic) apoptosis. Endogenous levels of caspase-8 and -9 activity obtained for HCT-8 control cells, treated with the vehicle DMSO, were somewhat higher than expected. It is unclear whether this is solely due to possible cell specific, endogenous caspase activity, such as that observed by Roy et al. (2003), reporting on the dose-dependent EGCG-induced caspase-3 and -8 activation in K562 cells. Moreover, it is unlikely to be concentration-dependent, seeing as, during morphology studies, <1 % DMSO was used as a solvent vehicle, yielding apoptosis comparable to non-treated cells. In addition, published work indicates that a concentration >2.5 % DMSO is required to induce apoptosis in a variety of carcinoma cell lines, as observed by flow cytometric and morphological assessment (Koike et al., 1996; Liu et al., 2001).

During the present study, theaflavins exhibited apoptotic activity, as observed by Hoescht 33342 morphological assessment (section 2.3.3) and caspase-3 inhibition studies (section 3.3.1). This is concurred by previous studies investigating the apoptotic activity of individual theaflavins (Pan et al., 2000a), wherein theaflavin, theaflavin-3-gallate and theaflavin-3,3′-digallate all induced apoptosis, in U937 cells, as observed by morphological assessment, DNA fragmentation and caspase-3 activation. However, in the current study the caspase-8 and -9 activity induced by theaflavins was, in general, extremely low, implying the potential contribution of caspase-independent factors in theaflavins-induced apoptosis. It is, therefore, possible that, by suppressing the cell survival, proliferative and differentiating responses of MAPK and/or NIK signal transduction pathways, theaflavins may enhance their apoptotic response via potential interaction with inhibitory κB (IκB) kinase (IKK), NF-κB and PKC (Cross et al., 2000; Pan et al., 2000b; Lin, 2002; Lin et al., 2003). However, in such a complex pathway,
the mechanism(s) through which theaflavins stimulate apoptosis, remains unclear; to a certain extent this may be due to lack of studies investigating the apoptotic mechanisms of black tea polyphenols, which clearly need further work to be elucidated.

Induction of caspase-9 activity by EGCG appears to suggest intracellular triggering of apoptosis, at least in HCT-8 cells. Results obtained from polyphenol cell permeability (section 2.3.5), and EGCG uptake studies undertaken in HT-29 cells (Hong et al., 2002), imply that EGCG is sufficiently hydrophobic to passively diffuse across the cell membrane. This, in association with morphological assessment (section 2.3.2), caspase-3 and -9 data, appears consistent with intracellularly induced apoptosis. It concords with the work of Chen et al. (2003a) who reported that, after incubation with EGCG, caspase-3 and -9 activation was detected in HT-29 human colon cancer cells, accompanied by a decrease in mitochondrial membrane potential and cytochrome c release, with levels of caspase-8 remaining unchanged, implying involvement of an intrinsic pathway. Moreover, although caspase-8 and -9 were not investigated, Kennedy et al. (2001), studying the apoptotic mechanisms of EGCG, reported a dose-dependent activation of caspase-3, concomitant with concentration-dependent release of cytochrome c in EGCG-treated cell cytosol, indicating that the subsequent activation of caspase-9 is highly likely. In contrast to the above observations, extracellularly triggered EGCG-induced apoptosis has also been reported, mediated by caspase-3 and caspase-8 activation (Hayakawa et al., 2001; Roy et al., 2003). In these studies, agonistic anti-Fas antibodies independently induced apoptosis, implicating the expression of Fas in U937 cells. In addition, affinity chromatography studies revealed binding between EGCG and Fas, indicating that, in U937 cells at least, EGCG induces apoptosis via the death receptor Fas (Hayakawa et al., 2001). However, Hayakawa et
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al. (2001) does allude to the possibility that other mechanisms may be operative concurrently, as observed during the current study (section 2.3.5), for example, Fas expression in HCT-8 cells appeared low (section 4.3.6), and thus, EGCG might also diffuse into the cell and act intracellularly.

The grape seed polymer also induced caspase-9 activity during apoptosis, indicating the involvement of an intracellularly induced caspase cascade. This is in agreement with Agarwal et al. (2002), who reported an increase in caspase-9, -3 and -7 activity in proanthocyanidin grape seed extract-treated carcinoma cells. However, the polymer has an appreciably large molecular weight, and low bioavailability, explained in part by Lipinski's Rule of 5 (Lambert & Yang, 2003), indicating that the polymer is highly unlikely to have entered the cell by passive diffusion. Similar observations were made in a study investigating proanthocyanidin transport across Caco-2 cell monolayers (Deprez et al., 2001). In this study, (+)-catechin, the proanthocyanidin dimer and trimer were all reported to have similar derived apparent permeability coefficients, indicating cellular absorption in vivo. In contrast, the permeability coefficients of a proanthocyanidin polymer (molecular weight of 1,740) was approximately 10 times lower, implying that bioavailability is limited to the gut lumen (Deprez et al., 2001). It may be possible that the polymer entered the cell by fluid phase endocytosis, but that seems unlikely since it would still be encompassed by a membrane bound vesicle (Mukherjee et al., 1997; Conner & Schmid, 2003). Accessibility to the intracellular cytosolic compartment may have occurred actively, via a membrane bound transporter, such as SGLT1 or OATP2 (Gee et al., 1998; Murota et al., 2000; O'Leary et al., 2003), but, again, this appears unlikely, due to the large molecular weight of the polymer.
The polymer may, however, be degraded into smaller components, before entering the cell. Similar observations have been made in studies investigating proanthocyanidin metabolism, wherein workers reported that purified polyphenolic polymers, once in the colon, were degraded into low molecular weight aromatic compounds, by human colonic microflora. Being considerably smaller molecules, such microbial metabolites are more likely to traverse biological membranes, although little is known about their biological activities (Deprez et al., 2000; Scalbert et al., 2000; Gonthier et al., 2003), but no colonic microflora were present, in vitro, during the current study. Alternatively, the polymer may not traverse the cell membrane at all, but stimulate apoptosis extracellularly, via caspase-8 activation. However, a weak caspase-8 signal is reported to be insufficient to induce caspase-3. Under such circumstances, cleavage of Bid may well occur, resulting in the release of mitochondrial components, inducing caspase-9 activation and subsequently apoptosis (Kuwana & Newmayer, 2003; Sprick & Walczak, 2004). However, in the present study, the abovementioned scenarios seem unlikely, due to the absence of colonic microflora in culture media and the low expression of Fas at the HCT-8 cell surface (section 4.3.6).

The highest caspase-9 activity, of all the polyphenols tested in HCT-8 cells, was induced by curcumin, indicating a possible activation of apoptosis via the mitochondrial pathway. It concords with the findings of Bharti et al. (2003), who reported that curcumin induced caspase-9 activation in human multiple myeloma cells. In contrast to the above observations of curcumin-induced caspase-9 activation, Duvoix et al. (2003), reported parallel activation of caspase-8 and -9 by curcumin in K562 human chronic myelogenous leukemia cells, inferring dual activation of both intrinsic and extrinsic pathways by curcumin. In addition, curcumin is reported to induce apoptosis via
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activation of caspase-8, Bid cleavage, cytochrome c release and caspase-3 activation, in HL-60, but not in Bcl-2 and Bcl-X\textsubscript{L}-transfected cells (Anto \textit{et al.}, 2002). The same workers reported that Bcl-2 and Bcl-X\textsubscript{L} are therefore likely to act as critical negative regulators of curcumin, suppressing mitochondrial cytochrome c release and subsequently caspase-9 activation. Furthermore, suppression of Bcl-X\textsubscript{L} is reportedly due to a down regulation of hyperphosphorylated Bcl-X\textsubscript{L} protein expression, which is thought to contribute towards the demonstrated chemopreventive properties of polyphenols (Kazi \textit{et al.}, 2002). It may, therefore, be possible that curcumin-induced apoptosis occurred in HCT-8 cells via Bid cleavage, even though caspase-8 activity was negligible. However, in view of the fact that neither Bid nor cytochrome c expression were investigated, the mechanism by which curcumin induces apoptosis in HCT-8 cells remains to be elucidated. Conversely, studies investigating the pathways involved in curcumin-induced apoptosis, using a number of melanoma cell lines, reported that curcumin appears to induce apoptosis via the Fas death receptor pathway, following caspase-3 and -8 activation (Bush \textit{et al.}, 2001). In these studies, curcumin-induced Fas aggregation was shown using FITC-conjugated anti-Fas antibody in MMRU and PMWK melanoma cell lines (Bush \textit{et al.}, 2001), suggesting that Fas activation is dependent upon the expression of the Fas receptor on the surface of a particular cell type. Nevertheless, expression of Fas at the HCT-8 cells surface appears to be low (section 4.3.6), which may explain the lack of caspase-8 activity observed during the present study.

The specificity of caspase-8 and -9 activity induced by the polyphenols was confirmed by employing the specific, cell permeable and irreversible caspase-8 inhibitor, Z-IETD-FMK or caspase-9 inhibitor, Z-LEHD-FMK. In general, inhibition of polyphenol-
induced caspase activity was considerable, with the exception of the polymer and theaflavins, which demonstrated almost complete inhibition of caspase-8 and -9 activities, respectively. These observations suggest that the compounds tested have a comparatively high efficacy for upstream caspase-8 and -9 and, from the caspase activity data, are likely to induce apoptosis via involvement of an intrinsic caspase-dependent pathway.

Influence of tea and red wine upon caspase-9 activation.

In view of the previous caspase assay data, demonstrating a more pronounced caspase-9 activity by the polyphenols, caspase-9 activity was assessed in the presence of freeze-dried tea solubles, derived from whole green and black tea, and polyphenol-free green and black tea, as well as a whole red wine extract. Green and black polyphenol-free teas appeared to induce relatively high levels of caspase-9 activity, whilst whole green and black tea had little effect on caspase-9 activity. Data contradicted those previously obtained for the teas, by assessment of apoptotic morphology using the Hoescht 33342 dye (section 2.3.10). Moreover, following the addition of the specific caspase-9 inhibitor, Z-LEHD-FMK, this low level of activity was almost completely inhibited by whole green and black tea, whereas, polyphenol-free green and black tea displayed a less effective suppression of caspase-9 activity, implying that in HCT-8 cells, polyphenol-free green and black tea caspase activity may be non-specific, since only partial inhibition was observed. Nonetheless, the point still remains, that no significant caspase-9 activity was observed in the presence of whole green or black tea. It is possible that caspase-8 activity may have been induced by the whole teas, leading to apoptotic morphology, as observed via Hoescht 33342 staining (section 2.3.10), and, thus, whole green and black tea-induced apoptosis may be the result of an
extracellularly triggered pathway. However, due to time restraints, limited resources, and promising caspase-9 activity data by individual compounds, the teas and wine extract were not assessed for caspase-8 activity. Alternatively, whole green and black tea may well have induced apoptosis via the involvement of additional mechanisms, such as MAPK or NF-κB (Cross et al., 2000; Lin et al., 2003).

The whole red wine extract induced caspase-9 activity, as confirmed by suppression, following the addition of the specific inhibitor Z-LEHD-FMK. It is, therefore, possible that the whole red wine extract activates apoptosis via an intrinsic pathway, although its effect upon caspase-8 activity is unclear. Similar observations of caspase-9 activation have been made in studies investigating the effect of red wine components upon apoptosis in a variety of cancer cell lines (Dorrie et al., 2001; Agarwal et al., 2002; Mahyar-Roemer et al., 2002; Delmas et al., 2003; Kim et al., 2004). In these studies, resveratrol and proanthocyanidin grape extract induced caspase-3 and -9 activity, with subsequent inhibition of caspase-9, a reduction in Bcl-2 expression, changes in mitochondrial membrane permeability, and cytochrome c release, leading to apoptosis (Dorrie et al., 2001; Agarwal et al., 2002; Mahyar-Roemer et al., 2002; Delmas et al., 2003; Kim et al., 2004). Furthermore, bioavailability data, obtained during the present study (section 2.3.5), implies that resveratrol is highly hydrophobic and is, therefore, likely to enter the cell via passive diffusion. This compares well to reports from in vivo studies investigating [14C]-trans-resveratrol bioavailability and tissue distribution following oral administration, as demonstrated by the presence of [14C]-trans-resveratrol in organs and biological fluids involved in absorption and elimination, which included the stomach, liver, kidney, intestine, bile and urine (Vitrac et al., 2003). Taken
together, these results indicate that red wine components, such as resveratrol, and possibly the whole red wine extract, may induce apoptosis via an intrinsic pathway.

**Conclusions.**

In conclusion, the involvement of the caspase cascade in polyphenol-induced apoptosis was established by assessing caspase-3 and -7 activity. This was achieved directly by caspase-3 and -7 activation assays, and indirectly by the caspase-3 inhibitor Z-VAD-FMK and morphological analysis, as well as enabling the substantiation of apoptotic morphology data obtained previously (chapter 2). Following studies to elucidate which pathway is taken through the cascade; it appears that all the polyphenols tested may activate apoptosis via an intrinsic mitochondrial pathway. Since, mitochondrial cytochrome c release and Bid cleavage were not investigated during the current study, definitive clarification of a polyphenol-activated caspase pathway, in HCT-8 cells, at present, cannot be made. Therefore, additional studies were embarked on to elucidate possible mechanisms, including the influence of reactive oxygen species, hydrogen peroxide generation and Fas expression, which may potentially contribute to the apoptotic activity of polyphenols (chapter 4).
CHAPTER 4.

Polyphenol-Induced Apoptosis: Potential Mechanisms of Action.
4.1 INTRODUCTION.

Polyphenolic compounds, including tea polyphenols and curcumin, have been shown to suppress colon carcinogenesis in animal models and human epidemiological studies (Azuine & Bhide, 1992; Franceschi et al., 1997; Huang et al., 1997; Ji et al., 1997; Kawamori et al., 1999; Caderni et al., 2000; Il’Yasova et al., 2003). Numerous studies corroborate that polyphenols display profound antioxidant activity, as well as being strong scavengers against ROS, both of which appear to contribute to their anticarcinogenic effects (Ishige et al., 2001; Nakagawa & Yokozawa, 2002). In addition, several studies have established that polyphenols inhibit cell proliferation of cancer cells, by initiating cell cycle arrest and apoptosis, which is reportedly mediated through the inhibition of NF-κB and MAPK, binding to Fas at the cell surface, mitochondrial depolarisation and activation of caspase-3, -8 and -9 (Kumi-Diaka et al., 2000; Bush et al., 2001; Chung et al., 2001; Hayakawa et al., 2001; Agarwal et al., 2002; Lin, 2002; Chen et al., 2003a). However, at present, the exact mechanism through which polyphenols exert their chemopreventive activity is unknown.

Cancer cells are reported to be reliant upon basal levels of ROS, particularly $\text{H}_2\text{O}_2$, which are produced constitutively by the cells, and appear to function as secondary messengers in signalling pathways, that are involved in cell survival and proliferation (Loo, 2003). The use of polyphenols, functioning as antioxidants, with ROS scavenging properties, are reported to interrupt proliferative signalling pathways, inducing cell cycle arrest and apoptosis. On the other hand, under certain experimental conditions, polyphenolic compounds paradoxically exhibit their pro-oxidant properties by auto-oxidation, generating ROS, such as $\text{H}_2\text{O}_2$, inducing an intolerably high level of oxidative stress, above and beyond that already present under endogenous conditions,
which are lethal to cancer cells (Li & Xie 2000; Long et al., 2000; Lapidot et al., 2002; Kessler et al., 2003; Loo, 2003). In addition, it is possible that the Fenton reaction may be involved in pro-oxidant mechanisms induced by polyphenols (Nakagawa et al., 2002; Ligeret et al., 2004).

Since the advent of oxygen on earth, cells have developed antioxidant defence systems, which have evolved, to contend with oxidative stress. These defences include the presence of large quantities of SOD, catalase and various peroxides, intracellularly, and possibly extracellularly (as described in 1.2.4), all designed to cope with oxygen toxicity, including O$_2^-$ and H$_2$O$_2$ (Halliwell & Gutteridge, 1999; Chandra et al., 2000). Several studies have outlined the possibility that apoptosis, induced by polyphenols, may be a consequence of rapid generation of H$_2$O$_2$ (as described in 1.2.4) (Long et al., 2000; Sakagami et al., 2001; Lapidot et al., 2002). In addition, H$_2$O$_2$ generation has been demonstrated in a number of studies, in which the death of cancer cells was attributed to H$_2$O$_2$; where polyphenol-induced H$_2$O$_2$ generation and apoptosis were inhibited by exogenously added catalase (Yang et al., 1998; Nakagawa et al., 2002; Loo, 2003). Therefore, it remains unclear how H$_2$O$_2$, if generated, can significantly affect cellular apoptosis, in the light of this battery of antioxidant defences.

Sakagami and Satoh (1997) reported that ascorbic acid may potentially induce apoptosis via its pro-oxidant, rather than antioxidant, activities, by dose-dependently increasing the oxidation potential in culture media. In addition, these pro-oxidant effects have been demonstrated, in vitro, with ascorbic acid and other reducing agents, including GSH, in the presence of transition metal ions (Halliwell & Gutteridge, 1999). Conversely, Chen et al. (1998) reported that ascorbic acid significantly increased the
stability of EGCG and EGC in culture media, possibly acting as an antioxidant, preventing oxidation of polyphenols (Chen et al., 1998). A number of similar investigations revealed that polyphenols, added to cell cultures, appeared to undergo chemical oxidation, to various extents, depending upon cellular conditions, leading to the generation of $\text{H}_2\text{O}_2$ and other oxidation products such as ortho-quinone and ortho-semiquinone (Kandaswami et al., 1993; Metodiewa et al., 1999; Hong et al., 2002; Roques et al., 2002; Boots et al., 2003; Halliwell, 2003). In light of these studies, it has been suggested that the addition of ascorbic acid to cell culture media might potentially prevent the oxidation and degradation of polyphenols (Kandaswami et al., 1993; Hong et al., 2002; Roques et al., 2002). However, it is noteworthy that the products of oxidation may be potentially more apoptotic than the parent compound. In comparison to the above observations, studies have indicated that oxidative coupling of polyphenols, in particular EGCG, resulted in a dramatic enhancement in antioxidant activity, potentially leading to an increase in apoptotic activity (Kurisawa et al., 2003; 2004). Since several studies propose that the addition of ascorbic acid to culture media may avert polyphenol oxidation (Kandaswami et al., 1993; Chen et al., 1998; Roques et al., 2002), and that the presence of GSH reacts with oxidised polyphenols, preventing thiol arylation, which is potentially toxic (Boots et al., 2003), the influence of ascorbic acid and GSH on polyphenol-induced apoptosis was investigated in the present study.

Originating in the liver, bile acids are transported, via the bile, to the intestine, where they perform an important role in the absorption of lipids and lipid-soluble nutrients. An enzymic cascade converts cholesterol to the primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA). However, following deconjugation, the highly hydrophobic secondary bile acids of deoxycholic acid (DCA) and lithocholic
acid (LCA) are formed, respectively. The main faecal bile acids, DCA and LCA are suspected to be the forms implicated in colon cancer (Nagengast et al., 1995; Agellon & Torchia, 2000; Debruyne et al., 2001; Kasbo et al., 2002). Due to their structural resemblance to carcinogenic PAHs, bile acids were investigated for their potential involvement in carcinogenesis (Debruyne et al., 2001). A vast array of animal studies have demonstrated a causal relationship between bile acids and colorectal carcinogenesis. Moreover, in view of the fact that bile acids administered in the absence of a carcinogen were unable to induce tumours, they are now believed to act as tumour promoters (Chomchai et al., 1974; Narisawa et al., 1974; 1978; Rafter et al., 1986; Sutherland & Bird, 1994; Reddy, 1999; Kasbo et al., 2002). In addition, the presence of colorectal cancer is reported to be associated with elevated levels of secondary bile acids (Debruyne et al., 2001; Qiao et al., 2001a).

At present, the detailed molecular mechanisms by which DCA, and other bile acids, function as tumour promoters are not clear. However, increasing evidence suggests that they may exert their activity by affecting intracellular signalling and gene expression, which ultimately influence cell proliferation and apoptosis (Martinez et al., 1998; Qiao et al., 2001a). Numerous in vitro studies, investigating the signalling induced by bile acids, have demonstrated that highly hydrophobic compounds, such as DCA, which have been implicated in the promotion of colorectal tumours, appear to induce apoptosis, possibly via activation of the caspase cascade (Haza et al., 2000; Schlottmann et al., 2000; Powell et al., 2001; Qiao et al., 2001b; Glinghammar et al., 2002; Milovic et al., 2002). Bile acids, including DCA, activate PKC signalling pathways that are involved in cell proliferation, as well as inducing COX2 promoter activity, which leads to the formation of the COX2 enzyme and consequent increases in
prostaglandin production. Moreover, DCA stimulated MAPK activity of both ERK and p38 pathways in human colon cancer cells (Martinez et al., 1998; Qiao et al., 2001a; Glinghammar et al., 2002; Kim et al., 2002; Im & Martinez, 2004). In addition, research by Lechner et al. (2002) suggests that hydrophobic bile acids can induce oxidative stress, wherein DCA reportedly stimulated ROS production, leading to the activation of the cell death machinery. In the light of accumulating evidence, suggesting that DCA and other bile acids may induce apoptosis and inhibit cell proliferation, which are indicative of a chemopreventive/anti-carcinogenic action, it becomes important to re-evaluate the underlying molecular mechanism involved, since the aforementioned mechanisms appear contradictory to that of tumour promotion.

In the present study, the polyphenols EGCG, theaflavins, curcumin and the grape seed polymer, found to induce apoptosis in the human HCT-8 cell line, by morphological assessment (section 2.3.3), were further investigated to unravel possible mechanisms of apoptosis induction. This was achieved by studying the influence of a range of chemicals, including bile acids, antioxidant enzymes and \( \text{H}_2\text{O}_2 \), which are all reported to modulate oxidative stress, in polyphenol-induced apoptosis. The objective was to uncover potential pro-oxidant or antioxidant mechanisms, and interactions through which polyphenols instigate apoptosis in HCT-8 cells.

4.2 MATERIALS AND METHODS.

4.2.1 Polyphenol and antioxidant treatment of HCT-8 cells.

Materials.

- Staurosporine, curcumin, GSH and sodium ascorbate (ascorbic acid): Sigma-Aldrich Company Ltd, Poole, UK.
EGCG and theaflavins: Donated by Dr. Conrad Astill, Unilever UK, Colworth, UK.

Grape seed polymer (polymer): Donated by Dr. Veronique Cheynier, INRA, Montpellier, France.

Method.

HCT-8 cells were cultured (as detailed in 2.2.1) in 6-well plates until 80 % confluent, then washed in PBS, before addition of fresh FBS-free media containing either ascorbic acid (10-200 μM) or GSH (5 mM). Cells underwent pre-treatment with the antioxidants for 5 minutes, at room temperature, before addition of EGCG (5-50 μM), curcumin (5-15 μM), polymer (1-2 μM) or theaflavins (2.5-25 μg/ml), and were then incubated for a further 24 hours under standard conditions of 37 °C and 5 % CO₂ in a humid environment. Negative control cells were incubated in FBS-free media and the vehicle only, and positive control cells were treated with 0.5-1 μM of staurosporine, an established inducer of apoptosis. Subsequently, cells were harvested and assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4).

4.2.2 Polyphenol and bile acid treatment of HCT-8 cells.

Materials.

- DCA and CA: Sigma-Aldrich Company Ltd, Poole, UK.

Method.

HCT-8 cells were cultured (as detailed in 2.2.1) in 6-well plates or 25 cm² culture flasks until 80 % confluent, then washed in PBS before the addition of fresh FBS-free media containing either DCA or CA (50-500 μM). After pre-treatment with bile acids for 5 minutes, at room temperature, cells were incubated for a further 24 hours (under
standard conditions) in the presence and absence of EGCG (25 μM), curcumin (10 μM), polymer (2 μM) or theaflavins (2.5-100 μg/ml). Subsequently, cells were either assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4), or assayed for caspase-9 activity (as detailed in 3.2.4).

4.2.3 EGCG and staurosporine treatment of HCT-8 cells.

**Materials.** (Detailed in 4.2.1).

**Method.**

HCT-8 cells were cultured (as detailed in 2.2.1) in 6-well plates until 80 % confluent, then washed in PBS before pre-incubation with fresh FBS-free media, containing EGCG, (5-25 μM) at room temperature for 5 minutes. Cells were then incubated for a further 24 hours (under standard conditions), in the presence and absence of staurosporine (0.5-1 μM), before being harvested and assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4).

4.2.4 Chemical treatment of HCT-8 cells: the role of iron and the Fenton reaction.

**Materials.**

- SOD, catalase, iron (III) chloride hexahydrate and H₂O₂ 30 % (“/w): Sigma-Aldrich Company Ltd, Poole, UK.
- Desferal® (desferrioxamine mesylate): Novartis UK Ltd, Horsham, UK.

**Methods.**

HCT-8 cells were cultured (as detailed in 2.2.1) in 6-well plates or 25 cm² culture flasks until 80 % confluent, then washed in PBS before addition of fresh FBS-free media containing either catalase (100 U/ml), SOD (50 U/ml) or desferrioxamine (100 μM). Following an 1-hour pre-incubation at 37 °C and 5 % CO₂, the cells were incubated in
the presence or absence of EGCG (25 μM) in FBS-free media, for 24 hours under standard conditions. Subsequently, cells were either assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4), or assayed for caspase-9 activity (as detailed in 3.2.4). In addition, incubations were undertaken to investigate the role of H2O2 upon polyphenol-induced apoptosis. In this case, following culture (in 6-well plates), cells were washed in PBS and incubated in FBS-free media containing H2O2 (0.2-1 mM) and incubated for 24 hours under standard conditions. Alternatively, after washing with PBS, cells were pre-incubated with FBS-free media containing EGCG (25 μM) or theaflavins (25 μg/ml) for 5 minutes, at room temperature. Cells were then incubated for a further 24 hours, under standard conditions in the presence or absence of H2O2 (50-100 μM). Cells were then assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4). To investigate the potential role of iron and the Fenton reaction to induce apoptosis, HCT-8 cells were cultured in 6-well plates, washed in PBS and incubated with FBS-free media containing H2O2, EGCG or Fe3+, either alone, altogether or in combination. The latter chemicals were added to the media consecutively, in the aforementioned sequence. Finally, cells were incubated for 24 hours, under standard conditions, after which they were assessed for cell viability and apoptosis (as detailed in 2.2.3 and 2.2.4).

4.2.5 Hydrogen peroxide/catalase assay.

Materials and instrumentation.

- Imidazole-HCl and Ti(SO₄)₂ (titanium sulphate): VWR International Ltd, Poole, UK.
- BSA, Triton X-100 (t-octoxynypolyethoxy ethanol), H₂O₂ 30 % (w/w) and iron (III) chloride hexahydrate: Sigma-Aldrich Company Ltd, Poole, UK.
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- RPMI media, with L-glutamine: Invitrogen Ltd, Paisley, UK.
- EGCG: Donated by Dr. Conrad Astill, Unilever UK, Colworth, UK.
- Ultrospec II 4050 spectrophotometer: Pharmacia LKB Biotechnology Inc, Piscataway, NJ, USA.

**Method.**

HCT-8 cells were cultured in 6-well plates until 80 % confluent and incubated with EGCG, H$_2$O$_2$ and Fe$^{3+}$ (alone and in combination), in 5 ml FBS-free media for 24 hours, under standard conditions of 37 °C and 5 % CO$_2$ in a humid environment. At various time points (0, 10, 20, 30 minutes, 1, 2, 4, and 24 hours), aliquots of incubation media (500 µl) were taken and assayed for H$_2$O$_2$ content. In addition, a standard curve was constructed using 0-2 mM H$_2$O$_2$. Assay tubes were prepared by the addition of 50 µl Triton X-100 and 1 ml imidazole (20 mM imidazole-HCl, pH 7, containing 0.1 % (w/v) BSA), followed by 500 µl of incubation media samples/H$_2$O$_2$ standards and finally 1.5 ml of 2.25 g/L Ti(SO$_4$)$_2$ (prepared in 1 M sulphuric acid). Assay tubes were then mixed by inversion and centrifuged at 1000 rpm for 5 minutes, before the supernatant was transferred to cuvettes, and absorbance read on an Ultrospec II 4050 spectrophotometer (Pharmacia LKB Biotechnology Inc) at 405 nm. Using the standard curve, H$_2$O$_2$ content for each sample was calculated, and plotted over time.

4.2.6 **Polymer treatment of HCT-8 cells, prior to attachment.**

**Materials.** (Detailed in 4.2.1).

**Method.**

During this experiment, HCT-8 cells were incubated either as normal, after attachment to the substratum, or while still in suspension, before attachment. Cells were, therefore, cultured (as detailed in 2.2.1) in 6-well plates, at a cell density of $0.04 \times 10^6$ cells/cm$^2$,
which preliminary studies had shown to yield \(0.125 \times 10^6\) cells/cm\(^2\) following a 24-hour growth period. The “substratum” cells were then washed in PBS before addition of fresh FBS-free media containing polymer (1-2 \(\mu\)M), the positive control staurosporine (0.5 \(\mu\)M) or the DMSO vehicle only. In addition, “suspension” cells were prepared, in 6-well plates, using FBS-free media, at a cell density of \(0.125 \times 10^6\) cells/cm\(^2\), containing the above chemicals. The cells were then incubated for 24 hours under standard conditions of 37 °C and 5 % CO\(_2\) in a humid environment. During this time, the cells incubated whilst still in suspension were expected to attach to the substratum, due to the fact that HCT-8 cells are adherent in nature. After the incubation period, both attached and detached cell fractions of “substratum” and “suspension” treatments were harvested and cells were assessed for cell proliferation and apoptosis using trypan blue exclusion and Hoescht 33342 staining, respectively (as detailed in 2.2.3 and 2.2.4).

4.2.7 Assessment of Fas expression.

Materials.

- Cycloheximide (CHX): Sigma-Aldrich Company Ltd, Poole, UK.
- Human anti-Fas antibody: Merck Biosciences Ltd, Nottingham, UK.

Method.

Previous studies, using a monoclonal antibody against the Fas antigen, have reported rapid (<3 hours) induction of apoptosis in a variety of different cell lines. Moreover, in the presence of the protein synthesis inhibitor, CHX, the susceptibility of cells to Fas-mediated apoptosis increased (Weis et al., 1995; Jones et al., 1998; Hamada et al., 1999). HCT-8 cells were cultured (as detailed in 2.2.1) in 6-well plates until 80 % confluent, and then washed in PBS before addition of fresh FBS-free media containing human anti-Fas (150 ng/ml) and CHX (1 \(\mu\)g/ml), either alone or in combination.
Negative control cells were incubated in FBS-free media and the vehicle only. Cells were then incubated, under standard conditions (37 °C and 5 % CO$_2$ in a humid environment), being assessed via light microscopy, every hour for 6 hours, for signs of apoptotic morphology. Following a 6-hour incubation, cells were harvested, fixed and assessed for apoptosis via fluorescence microscopy (as detailed in 2.2.4).

4.3 RESULTS.

4.3.1 Influence of antioxidants in polyphenol-induced apoptosis: glutathione and ascorbic acid.

To determine whether the presence of ascorbic acid prevents polyphenol degradation, potentially causing an increase in cellular apoptosis, the role of ascorbic acid upon EGCG-induced apoptosis in HCT-8 cells was investigated. In addition, the effect of GSH on the apoptotic inducibility of the “active” polyphenols EGCG, theaflavins, curcumin and the polymer were studied, to determine whether GSH exerted any influence on polyphenol-induced apoptosis. Cells were incubated with ascorbic acid using concentrations of 10 and 200 μM, reported to enhance the cellular activity of flavonoids (Kandaswami et al., 1993; Noroozi et al., 1998), or using 5 mM GSH, a typical intracellular concentration, which may potentially exhibit pro-oxidant properties (Chaudiere & Ferrari-Iliou, 1999; Halliwell & Gutteridge, 1999). The results obtained indicated that 10 and 200 μM ascorbic acid had no effect upon apoptosis when incubated alone, or in the presence of 25 μM EGCG (Figure 4.1). Similarly, 5 mM GSH had no effect upon apoptosis, either alone or in the presence of 25 μM EGCG (Figure 4.2), indicating that apoptosis may be initiated by EGCG. Nevertheless, GSH does appear to improve cell viability, particularly in the case of 50 μM EGCG (legend to Figure 4.2) and 15 μM curcumin (legend to Figure 4.3), wherein cell viability fell
from 95 %, to 85.5 and 54.5 % respectively, in the absence of GSH. The presence of GSH, however, decreased both the theaflavins- and curcumin-induced apoptosis (Figure 4.3). In contrast, the apoptotic effect of the polymer increased in the presence of GSH (Figure 4.2), suggesting that the structure of a particular polyphenolic may be an important determinant of whether it interacts with a reducing agent, such as ascorbic acid or GSH. On the other hand, the fate of the aforementioned reducing agents must also be considered, both of which are prone to oxidation, and thus levels may be depleted in the culture media.

Figure 4.1: The influence of ascorbic acid on EGCG-induced apoptosis. HCT-8 cells were treated for 5 minutes with 10 and 200 μM ascorbic acid (AA), and then incubated for a further 24 hours following the addition of 25 μM EGCG to the FBS-free media. Results for percentage apoptosis were obtained using the Hoechst 33342 stain and percentage viability of >95.5 % was obtained by trypan blue exclusion. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 %.
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Figure 4.2: The influence of GSH upon EGCG- and polymer-induced apoptosis. HCT-8 cells were treated for 5 minutes with 5 mM GSH, and then incubated for a further 24 hours following the addition of EGCG or polymer to the FBS-free media. Percentage viability was >93 %, except for 50 μM EGCG (without GSH) at 85.5 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10 %.

Figure 4.3: The influence of GSH upon theaflavins- and curcumin-induced apoptosis. HCT-8 cells were treated for 5 minutes with 5 mM GSH, and then incubated for a further 24 hours following the addition of theaflavins (TF) or curcumin to the FBS-free media. Percentage viability was >91.5 %, except for 15 μM curcumin (without GSH) at 54.5 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >14 %.
4.3.2 The role of hydrogen peroxide in polyphenol-induced apoptosis.

A study by Long et al. (2000) investigated the possibility that \( \text{H}_2\text{O}_2 \), produced by polyphenols, in commonly used culture media, is responsible for polyphenol-induced apoptosis. The same workers also found that, in the absence of cells, 3-98 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) was produced, following the addition of 100 \( \mu \text{M} \) of EGCG, EGC, (+)-catechin, gallic acid or quercetin to RPMI media (Long et al., 2000). To determine whether the demonstrated EGCG-, theaflavins-, curcumin- and polymer-induced apoptosis (section 2.3.2 and 2.3.3), was initiated by the polyphenols themselves or indirectly, by the generation of ROS, the role of \( \text{H}_2\text{O}_2 \) in polyphenol-induced apoptosis was investigated.

HCT-8 cells were incubated with EGCG and theaflavins in the presence and absence of 50 and 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \), to assess the influence of \( \text{H}_2\text{O}_2 \) upon apoptosis, at concentrations that have been reported to induce apoptosis in cancer cell lines (Yang et al., 1998; Matsura et al., 1999; Ishisaka et al., 2002). With \( \text{H}_2\text{O}_2 \) alone, at concentrations of 50 and 100 \( \mu \text{M} \), apoptosis was induced dose-dependently, but the extent was very modest, being 6 and 14 \%, respectively (Figure 4.4). Clearly, \( \text{H}_2\text{O}_2 \) does not influence theaflavins- or EGCG-induced apoptosis and alone only has a modest impact on apoptosis in HCT-cells, at the concentrations tested, indicating that a higher concentration of \( \text{H}_2\text{O}_2 \) is potentially required, to considerably induce apoptosis in the HCT-8 cell line.

In light of the previous experiment, HCT-8 cells were incubated with higher concentrations of \( \text{H}_2\text{O}_2 \) (200-1000 \( \mu \text{M} \)) to determine what concentration of \( \text{H}_2\text{O}_2 \) the HCT-8 cells would tolerate, without inducing cytotoxicity. Results showed a concentration-dependent increase in apoptosis by \( \text{H}_2\text{O}_2 \), as well as evidence of cytotoxicity at concentrations greater than 900 \( \mu \text{M} \) (Figure 4.5). However, 700 \( \mu \text{M} \) was
the highest concentration of H$_2$O$_2$ able to induce maximal apoptosis (21.75 %), without inducing cytotoxicity (retaining an optimal viability of 94.5 %). These results indicate that the HCT-8 cell line appears to be tolerant to H$_2$O$_2$, given that, in a number of other cancer cell lines, H$_2$O$_2$ has been shown to induce apoptosis at concentrations of 30-100 µM (Yang et al., 1998; Matsura et al., 1999; Ishisaka et al., 2002).

Bearing in mind that, 700 µM appears to be the "optimal" concentration of H$_2$O$_2$ to induce apoptosis, in the absence of cytotoxicity, the concentration of H$_2$O$_2$ generated by EGCG in culture media was investigated, to ascertain whether the H$_2$O$_2$ generated was responsible for EGCG-induced apoptosis. Moreover, elimination of H$_2$O$_2$ from the culture media, over a 24-hour incubation period, was concurrently examined, since the duration of cellular exposure to H$_2$O$_2$ may also influence apoptosis. HCT-8 cells were therefore incubated in the presence of 25 µM EGCG alone, 700 µM H$_2$O$_2$ and a mixture of 200 µM H$_2$O$_2$, 25 µM EGCG and 100 µM Fe$^{3+}$, and assayed for the presence of H$_2$O$_2$. A concentration of 800 µM H$_2$O$_2$ was initially observed, exceeding the 700 µM added to the media. This peaked after 10 minutes at a concentration of 860 µM, after which H$_2$O$_2$ levels rapidly dropped and were virtually eliminated from the media, after a 4-hour incubation (Figure 4.6). EGCG (25 µM) appears to induce H$_2$O$_2$ generation, with H$_2$O$_2$ levels slowly rising to a 255 µM peak after a 60-minutes, and then gradually decreasing to 10 µM after a 4-hour incubation. However, media assayed from cells incubated with 200 µM H$_2$O$_2$, 25 µM EGCG and 100 µM Fe$^{3+}$ exhibited a sharp increase in H$_2$O$_2$ concentration, peaking after 20 minutes at 615 µM, after which H$_2$O$_2$ levels fell and were eliminated following a 4-hour incubation (Figure 4.6). These observations indicate that, in the presence of EGCG, H$_2$O$_2$ appears to be generated in culture media at concentrations capable of inducing some apoptosis in HCT-8 cells.
However, in the presence of H$_2$O$_2$, Fe$^{3+}$ and EGCG, the concentration of H$_2$O$_2$ observed was considerably higher than that initially added to the media, implying that H$_2$O$_2$ seems to be formed in the culture medium, at least transiently. In addition, it may be inferred from these results that it is not necessarily the duration of treatment that is important, but the peak or threshold concentration of H$_2$O$_2$ to which the cells are exposed.

![Figure 4.4: The effect of H$_2$O$_2$ upon EGCG- and theaflavins-induced apoptosis.](image)

HCT-8 cells were incubated with hydrogen peroxide (H$_2$O$_2$) and EGCG or theaflavins (TF), added consecutively to FBS-free media and then incubated for 24 hours. Percentage viability was >91%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >12%. 

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Figure 4.5: Influence of H₂O₂ upon apoptosis and viability in HCT-8 cells. Cells were incubated for 24 hours with H₂O₂ in FBS-free media. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >7%.

Figure 4.6: H₂O₂ content following exposure of HCT-8 cells to H₂O₂, EGCG and Fe³⁺. Cells were incubated with H₂O₂, EGCG and Fe³⁺ for 4 hours in FBS-free media. Aliquots of the media were removed at 0, 10, 20, 30 minutes, 1, 2 & 4 hours, and assayed for H₂O₂ content. At time zero, 200 µM H₂O₂ & 100 µM Fe³⁺ yielded 265 & 10 µM H₂O₂ respectively. Results are expressed as the average of duplicate determinations of culture media aliquots, which did not differ between them by >14%.
To investigate the potential role of iron and the Fenton reaction in generating H\textsubscript{2}O\textsubscript{2} during polyphenol-induced apoptosis, HCT-8 cells were incubated with EGCG in the presence and absence of the enzymes SOD and catalase, and the Fe\textsuperscript{3+} chelator desferrioxamine, to ascertain whether a reduction in or removal of ROS would instigate a similar effect in polyphenol-induced apoptosis. Catalase (100 U/ml) reduced the apoptotic potential of EGCG from 22.75 to 9.75 %, and a similar effect was seen in the presence of SOD, where EGCG-induced apoptosis dropped to 10.25 %. EGCG-induced apoptosis also decreased in the presence of desferrioxamine (14.5 %), implying that endogenous levels of Fe\textsuperscript{3+} are present in the cell culture media (Figure 4.7). In addition, cells were incubated in the presence of H\textsubscript{2}O\subscript{2}, and EGCG and Fe\textsuperscript{3+}, to assess whether the Fenton reaction was involved in EGCG-induced apoptosis. Results showed that induction of apoptosis by 100 μM Fe\textsuperscript{3+}, or 200 μM H\textsubscript{2}O\textsubscript{2} alone was minimal (8.75 and 13 % respectively), and individually, had no major influence upon EGCG-induced apoptosis. However, in combination with EGCG, apoptosis rose to 32.5 %, although viability was slightly compromised at 87 % (Figure 4.8). These observations suggest that pro-oxidant properties may contribute to the ability of EGCG to initiate apoptosis.
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Figure 4.7: The effects of catalase, SOD and desferrioxamine upon EGCG-induced apoptosis in HCT-8 cells. Cells were pre-treated for 1 hour with catalase, SOD or desferrioxamine (desferri.), then incubated for a further 24 hours following the addition of EGCG to FBS-free media. Percentage viability was >90.5%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >10%.

Figure 4.8: The effect of H$_2$O$_2$, EGCG and Fe$^{3+}$ on apoptosis in HCT-8 cells. Cells were incubated with H$_2$O$_2$, EGCG and Fe$^{3+}$, added consecutively to FBS-free media for 24 hours. Percentage viability was >91%, except for EGCG + Fe$^{3+}$ + H$_2$O$_2$, when it decreased to 87%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9%.

It has already been demonstrated (section 3.3.2 and 3.3.3) that caspase-3 and caspase-9, which are indicative of an intrinsic apoptotic pathway, may be activated during EGCG-
induced apoptosis. To ascertain whether the pro-oxidant properties of EGCG potentially involve a caspase-9 dependent apoptotic pathway, the effects of catalase, SOD and desferrioxamine upon EGCG-induced caspase-9 activity during apoptosis were investigated. HCT-8 cells were incubated as described in Figure 4.7, and then assayed for caspase-9 activity. Higher caspase-9 activity was obtained in cells incubated with EGCG in the presence of catalase or desferrioxamine, than with EGCG alone, this indicates specific activity, due to inhibition of caspase-9 activity, particularly in the case of desferrioxamine, in which complete inhibition was observed. However, EGCG-induced caspase-9 activity in the presence of SOD was negligible, and the caspase-9 inhibitor had no effect (Figure 4.9), indicating that other pathways, not mediated by caspase-9 may also be involved in EGCG-induced apoptosis.

![Caspase-9 Activation and Inhibition](image)

Figure 4.9: Caspase-9 activation of cells incubated with EGCG in the presence and absence of catalase, SOD and desferrioxamine and subsequent inhibition with Z-LEHD-FMK. HCT-8 cell lysates were pre-treated with catalase (Cat), SOD or desferrioxamine (Des) for 1 hour, before addition of EGCG, then incubated along with control samples of staurosporine (Stauro) or DMSO for a further 24 hours in FBS-free media, before being assessed for caspase-9 activity by microfluorimetry. In addition, recombinant caspase-9 and HL-60 cells incubated with 0.5 μg/ml actinomycin D for 19 hours (HL-60 positive control) were assayed, and samples were incubated with the caspase-9 inhibitor Z-LEHD-FMK. Results are expressed as mean RFU (relative fluorescence units) ± SEM of triplicate determinations for caspase-9 activity of HCT-8 cells, and as single values for caspase-9 inhibition and caspase-9 activity of recombinant caspase-9 and HL-60 positive control.
4.3.3 The effect of bile acids on polyphenol-induced apoptosis in HCT-8 cells.

Bile acids, particularly highly hydrophobic secondary bile acids such as DCA, considered to act as tumour promoters in the gastrointestinal tract, can reportedly mediate oxidative stress, potentially inducing cell death machinery (Qiao et al., 2001a; Lechner et al., 2002). Hence, the primary bile acid, CA, and secondary bile acid, DCA, were investigated to ascertain whether bile acids could modulate polyphenol-induced apoptosis in HCT-8 cells. Initially, cells were incubated with either CA or DCA, using a range of concentrations (50-500 μM) relevant to those found in the colonic lumen, which reportedly cause DCA-induced apoptosis in colon cancer cell lines (van Munster et al., 1993; Schlottmann et al., 2000; Milovic et al., 2002). Results clearly showed CA had no significant effect upon apoptosis (<7.5 %). However, DCA induced apoptosis at 100 and 250 μM DCA, dose-dependently (22 and 50 % respectively), without compromising viability (Figure 4.10). At the highest concentration of 500 μM, DCA was highly cytotoxic, with <20 cells observed in the attached cell fraction (data not shown).

Figure 4.10: Impact of bile acids upon apoptosis in HCT-8 cells. Cells were incubated with CA or DCA for 24 hours in FBS-free media. Percentage viability was >95 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >7 %.
The aim of subsequent experiments was to ascertain the effect of DCA (50, 100 and 250 μM) upon EGCG-, theaflavins-, curcumin- and polymer-induced apoptosis. DCA appears to have no effect upon the level of apoptosis induced by the polymer (Figure 4.11), whereas, in the presence of 50 and 100 μM DCA, an apparent additive effect was observed upon EGCG-induced apoptosis (35.5 and 50 % respectively), although this was not evident at the higher 250 μM DCA concentration (Figure 4.12). In addition, at the concentration of 100 μM only, DCA had a possible additive effect upon apoptosis induced by theaflavins (25 μg/ml) (Figure 4.13). This was further investigated to establish whether this concentration of DCA was capable of inducing an additive apoptotic response in the presence of a range of theaflavins concentrations. An additive effect was observed at all the concentrations of theaflavins tested, but, at the concentration of 100 μg/ml, theaflavins were highly cytotoxic (viability <58.5 %) (Figure 4.14). These observations suggest that DCA may potentially additively modulate the apoptotic effect of theaflavins and EGCG in HCT-8 cells.

Figure 4.11: Effect of DCA upon polymer-induced apoptosis in HCT-8 cells. Cells were treated with DCA for 5 minutes before addition of 2 μM polymer and incubation for a further 24 hours in FBS-free media. Percentage viability was >95 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >11 %.
Figure 4.12: Influence of DCA upon EGCG-induced apoptosis in HCT-8 cells. Cells were treated with DCA for 5 minutes before addition of 25 μM EGCG and incubation for a further 24 hours in FBS-free media. Percentage viability was >92.5 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 %.

Figure 4.13: Influence of DCA upon theaflavins-induced apoptosis in HCT-8 cells. Cells were treated with DCA for 5 minutes before addition of 25 μg/ml theaflavins and incubation for a further 24 hours in FBS-free media. Percentage viability was >93 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 %.
DCA appeared to exhibit a dose-dependent, and possibly synergistic, response on curcumin-induced apoptosis (at all concentrations of DCA tested) (Figure 4.15). Curcumin-induced caspase-9 activity, as previously discussed (section 3.3.3), indicated that apoptosis is likely to occur via an intrinsic caspase pathway. Therefore, to investigate the possibility of synergism between curcumin and DCA in the induction of apoptosis, cells were incubated, as described in Figure 4.15, and assayed for caspase-9 activity. Caspase-9 activity for curcumin was considerably higher than that of the DMSO control (>68 RFU). Caspase-9 activity induced by 50 and 100 μM DCA alone was negligible, although activity appeared to be non-specific for 50 μM DCA, as in the presence of the caspase-9 inhibitor Z-LEHD-FMK, very little inhibition was observed. Conversely, 100 μM DCA, which effectively activates caspase-9, demonstrated almost complete inhibition of caspase-9 activity in the presence of Z-LEHD-FMK, implying specificity (Figure 4.16). Curcumin-induced caspase-9 activity, increased dose-
dependently in the presence of DCA at concentrations of 50 and 100 μM (>16.2 and >49.7 RFU of DMSO activity, respectively), and also appeared specific, displaying almost complete caspase-9 inhibition. However, caspase-9 activity was lower with 10 μM curcumin and DCA, than with curcumin alone, almost implying antagonism, rather than synergism. Nevertheless, there appears to be a surprising interaction between curcumin and high concentrations of DCA, seeing that, in the presence of 250 μM DCA, curcumin-induced caspase-9 activity dramatically falls, to <33.9 RFU of the DMSO control (Figure 4.16), causing a complete reduction in caspase activity.

Figure 4.15: Influence of DCA upon curcumin-induced apoptosis in HCT-8 cells. Cells were treated with DCA for 5 minutes before addition of 10 μM curcumin and incubation for a further 24 hours in FBS-free media. Percentage viability was >92 %, except for 10 μM curcumin & 250 μM DCA, when it decreased to 89 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >13 %.
Figure 4.16: Caspase-9 activation of cells incubated with different concentrations of DCA in the presence and absence of 10 μM curcumin and subsequent inhibition with Z-LEHD-FMK. HCT-8 cell lysates were pre-treated with DCA for 5 minutes before addition of curcumin (Cure), and incubated along with control samples of staurosporine (Stauro) or DMSO for a further 24 hours in FBS-free media, before being assessed for caspase-9 activity by microfluorimetry. In addition, recombinant caspase-9 and HL-60 cells incubated with 0.5 μg/ml actinomycin D for 19 hours (HL-60 positive control) were assayed, and samples were incubated with the caspase-9 inhibitor Z-LEHD-FMK. Results are expressed as mean RFU (relative fluorescence units) ± SEM of triplicate determinations for caspase-9 activity of HCT-8 cells, and as single values for caspase-9 inhibition and caspase-9 activity of recombinant caspase-9 and HL-60 positive control.

These observations imply that curcumin interacts with DCA to influence apoptosis, and, to some extent, this may be achieved via the caspase-9 pathway. Moreover, in the presence of high concentrations of DCA, apoptosis was clearly observed by morphological assessment (Figure 4.15), indicating that other mechanisms may be potentially involved.

4.3.4 Possible interaction between staurosporine and EGCG in apoptosis.

Staurosporine, a known inducer of apoptosis, is reported to trigger apoptosis via involvement in an intrinsic caspase pathway (Ahlemeyer et al., 2002; Caballero-Benitez & Moran, 2003; Gil et al., 2003), whereas the proposed mechanisms for EGCG-induced apoptosis appear to be cell type dependent, with conflicting reports for intrinsic and/or
extrinsic caspase pathway involvement (Hayakawa et al., 2001; Hastak et al., 2003; Roy et al., 2003). To establish whether potential interactions between EGCG and staurosporine may enhance cellular apoptosis, their combined effect on HCT-8 cell apoptosis was investigated. In addition, the possibility that, due to its potency, staurosporine may in fact mask the apoptotic activity of EGCG (see section 2.3.7) was examined. Cells were therefore incubated in the presence of both EGCG and staurosporine, at concentrations previously shown to induce apoptosis (sections 2.3.2 and 2.3.3). It was observed that in the presence of 5 and 25 μM EGCG, and 0.5 and 1 μM staurosporine alone, apoptosis increased in a dose-dependent manner (Figure 4.17). Moreover, an increase in apoptotic response was observed in cells incubated with both EGCG and staurosporine. However, the increase was a modest, non-additive apoptotic response, compared to cells treated with staurosporine alone. These observations imply that any interaction between EGCG and staurosporine is weak. In addition, it seems unlikely that staurosporine masks the apoptotic activity of EGCG, due to the presence of a moderate increase in apoptosis, in the presence of both compounds.

Moreover, when assessed for apoptotic morphology (data not shown), cells incubated with staurosporine alone were more likely to appear as apoptotic bodies, indicative of the final stage of apoptosis, whereas, cells incubated with EGCG alone were more likely to display condensation of chromatin on the nuclear membrane, indicative of an earlier event in apoptosis (Curtin & Cotter, 2003). However, nuclear fragmentation was also evident, in cells incubated with EGCG and staurosporine alone, and thus, neither type of morphology was sufficiently predominant to be able to identify, in the presence of both compounds, what proportion of cells was killed by EGCG or staurosporine, alone. These findings imply that EGCG and staurosporine may induce apoptosis over
different time frames, during a 24-hour incubation, which may help explain their differential apoptotic effect, but gives no indication of their potential pathways of apoptotic induction.

Figure 4.17: The effect of EGCG and staurosporine on HCT-8 cell apoptosis. Cells were treated with EGCG for 5 minutes before the addition of staurosporine (Stauro), then incubated for a further 24 hours in FBS-free media. Percentage viability was >95%. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >5%.

4.3.5 Effect of grape seed polymer upon apoptosis and cell proliferation.

Cells that were already attached to a substratum, as well as cells that were still in suspension, were incubated with the grape seed polymer, to investigate the potential of HCT-8 cells still in suspension, to present a greater surface area for polymer binding and, therefore, potentially a more pronounced apoptotic effect. Furthermore, to ascertain whether the addition of polymer to cells still in suspension would impair their ability to attach to the substratum, both attached and detached cell fractions were counted, following a 24-hour incubation, using trypan blue exclusion. The polymer dose-dependently induced apoptosis, whether polymer was added to cells that were still in suspension, or already attached to the substratum, but appeared more effective at inducing apoptosis in the latter situation. In addition, no difference in apoptosis was
observed between “substratum” and “suspension” control cell samples incubated with 0.5 μM staurosporine, or the solvent vehicle DMSO (Figure 4.18). In the detached cell fraction of cells incubated with the polymer (data not shown), apoptosis ranged 78.5-100 %, but, viability was low (50-83.5 %) and in most samples <100 cells were counted. In the attached cell fraction, a concentration-dependent inhibition of cell proliferation was observed in the presence of the polymer, which appears to be similar in both “substratum” and “suspension” cells, except in “suspension” cells, at the highest polymer concentration (2 μM), which exceeded cell growth of 1.5 μM polymer (Figure 4.19). After 24-hours >99.5 % of the “suspension” cells incubated in the presence of polymer had attached to the substratum, compared with 99.9 % in the DMSO control (data not shown), indicating that the presence of the polymer in the suspended cells does not impair subsequent attachment. In addition, an apparent increase in cell surface area, by using “suspension” cells, does not appear to correlate with an increase in polymer-induced apoptosis.

![Graph showing apoptosis comparison](image)

**Figure 4.18:** Comparison of apoptosis in HCT-8 cells incubated with polymer whilst attached or still in suspension. Cells already attached to the substratum and cells still in suspension, with the equivalent of $0.125 \times 10^6$ cells/cm$^2$ at time zero, were incubated with the polymer for 24 hours in FBS-free media. Percentage viability was >91 %. Results are expressed as the average of duplicate determinations of attached cell fractions, which did not differ between them by >9 %.
4.3.6 Expression of Fas in HCT-8 cells.

To ascertain whether the Fas-activated extracellular death receptor pathway was potentially involved in polyphenol-induced apoptosis (Hayakawa et al., 2001), it was important to evaluate the expression of Fas in HCT-8 cells. Cells were therefore incubated with the human anti-Fas antibody in the presence and absence of the protein synthesis inhibitor CHX, which has been reported to increase cell susceptibility to Fas-mediated apoptosis, using concentrations shown to rapidly (<3 hours) induce apoptosis (Weis et al., 1995; Jones et al., 1998; Hamada et al., 1999). Following a 6-hour incubation, apoptosis was not evident (Figure 4.20) suggesting that expression of Fas at the surface of HCT-8 cells was low. Therefore, it appears that Fas is unlikely to be responsible for a polyphenol-induced death receptor apoptotic pathway, although other death receptors, such as TNF, DR3 or TRAIL, may also be present at the cell surface, caspase-8 and -9 activity data (section 3.3.3) seem to indicate that polyphenols are likely to induce apoptosis via an intrinsic pathway.
4.4 DISCUSSION.

The polyphenolics EGCG, theaflavins, curcumin and the grape seed polymer, previously demonstrated to induce apoptosis, by Hoechst 33342 staining, in HCT-8 cells (section 2.3.3), were further investigated to ascertain whether pro- or antioxidant properties contributed to the mechanistics of apoptosis induction.

_Hydrogen peroxide generation: effect on polyphenol-induced apoptosis._

To determine whether polyphenol-induced apoptosis was initiated by the polyphenols themselves or mediated by \( \text{H}_2\text{O}_2 \) generation (Long _et al._, 2000), cells were incubated with \( \text{H}_2\text{O}_2 \), using concentrations known to induce apoptosis, in a variety of cell lines (Yang _et al._, 1998; Matsura _et al._, 1999; Ishisaka _et al._, 2002), in the presence of EGCG or theaflavins. However, \( \text{H}_2\text{O}_2 \) was unable to induce apoptosis in HCT-8 cells, at the concentrations tested, and had no effect upon EGCG- or theaflavins-induced apoptosis. These observations suggest that a higher concentration of \( \text{H}_2\text{O}_2 \) might be required to
induce apoptosis in HCT-8 cells. This is possibly due to H$_2$O$_2$ degradation, as demonstrated during the present study, by the rapid elimination of H$_2$O$_2$ from culture media, which may be as a result of powerful antioxidant defences present in the extracellular compartment, including traces of catalase, reported to sometimes leak from cells, and low GPx activity, both capable of breaking down H$_2$O$_2$ (Halliwell & Gutteridge, 1999). Incubation of HCT-8 cells, with higher concentrations of H$_2$O$_2$ (200 µM to 1 mM), established that 700 µM was the concentration of H$_2$O$_2$ able to induce maximal apoptosis, whilst maintaining optimal cell viability. It may be inferred from these studies that the HCT-8 cell line appears to be tolerant to high levels of H$_2$O$_2$. Similar observations have been made in studies investigating H$_2$O$_2$-induced apoptosis, wherein high concentrations of H$_2$O$_2$ reportedly stimulated apoptosis in a variety of cell lines (Hiraoka et al., 1997; Lee et al., 2000; Deshpande et al., 2002). Cellular tolerance to H$_2$O$_2$ may be explained by Bcl-2 overexpression, or the presence of low concentrations of H$_2$O$_2$, which are reported to induce cellular resistance, to subsequently added higher concentrations, possibly by elevating production of the antioxidants, GSH, catalase or GPx (Gardner et al., 1997; Wang et al., 1998; Amstad et al., 2001; Kim et al., 2001b; Kowaltowski et al., 2004). Although HCT-8 cells are clearly a good example of cells well adapted to cope with oxygen diversity, for that same reason, they are probably not the most “sensitive cell type” to study the pro-oxidant mechanisms of polyphenol–induced apoptosis.

Polyphenolic pro-oxidant activity was examined by investigating the potential role of iron and the Fenton reaction in generating H$_2$O$_2$ during polyphenol-induced apoptosis. Cells were, therefore, pre-incubated with the enzymes SOD and catalase, and the Fe$^{3+}$ chelator desferrioxamine, before incubation with EGCG, resulting in the inhibition of
EGCG-induced apoptosis, which appeared more pronounced in the presence of catalase. These observations concord with findings of studies undertaken, in a number of cell lines, which reported that apoptosis induced by galloatechins, such as EGC and EGCG, is inhibited by exogenously added catalase or SOD (Yang et al., 1998, 2000; Nakagawa et al., 2002; Nakayama et al., 2002). One possible explanation for the lower activity of SOD may be attributed to enzyme stability. Although SOD was added to the culture media, its stability and thus activity, during a 24-hour incubation, is uncertain. Furthermore, inhibition of EGCG-induced apoptosis by desferrioxamine, in the absence of added Fe³⁺, implies that endogenous Fe³⁺ is present in the culture media. However, polyphenols may also exert their pro-oxidant properties, such as ROS generation, by reacting with other transition metals including copper ions (Cu²⁺) (Azam et al., 2004; Yoshino et al., 2004). In addition, Furukawa et al. (2003) reported that desferrioxamine inhibited Fe³⁺-mediated, EGCG-induced damage of isolated and cellular DNA.

The potential involvement of the Fenton reaction in EGCG-induced apoptosis was further investigated. In cells that were incubated in the presence of EGCG, with the addition of either H₂O₂ or Fe³⁺, EGCG-induced apoptosis was unaffected. However, in the presence of all three compounds, HCT-8 cell apoptosis increased substantially (Figure 4.8). Thus, a requirement for H₂O₂ and Fe³⁺ to significantly increase EGCG-induced apoptosis may be explained by a proposed mechanism, where, in cell culture media, EGCG is able to promote the reduction of Fe³⁺ to Fe²⁺. In turn, Fe²⁺ interacts with H₂O₂ via the Fenton reaction, producing ROS, capable of instigating direct DNA breakage culminating in apoptosis (Nakagawa et al., 2002). In addition, a Fenton type reaction has been reported to occur in the presence of copper ions, whereby polyphenols such as EGCG, curcumin and quercetin, promote the reduction of Cu²⁺ to Cu⁺,
mediating a copper redox cycle, which appears to play a principal role in ROS generation, and potentially site specific DNA oxidative damage (Yoshino et al., 1999; 2004; Malik et al., 2003; Azam et al., 2004).

To ascertain whether EGCG pro-oxidant activity induces apoptosis by H$_2$O$_2$ generation, and to what extent the Fenton reaction may be involved, HCT-8 cells were incubated with EGCG alone, H$_2$O$_2$ alone, or a mixture of H$_2$O$_2$, EGCG and Fe$^{3+}$, and then H$_2$O$_2$ content was determined. Elimination of H$_2$O$_2$ from the culture media, over a 24-hour incubation period, was concurrently examined, to determine whether duration of exposure to cellular H$_2$O$_2$ influences apoptosis. Considerable increases in H$_2$O$_2$ concentration were observed in the culture media, rapidly exceeding any exogenous concentrations of H$_2$O$_2$ initially added to the media. Moreover, after a period of about 120 minutes, the H$_2$O$_2$ added to the media was almost completely eliminated. Alone, EGCG appeared to generate H$_2$O$_2$ (255 μM), but, in the presence of exogenously added H$_2$O$_2$ and Fe$^{3+}$, H$_2$O$_2$ production was exacerbated (415 μM). H$_2$O$_2$ also appeared to be generated in the absence of EGCG, but at somewhat lower concentrations (160 μM). These findings suggest that H$_2$O$_2$ may be formed by the cells or in the culture medium, at least transiently, and potentially that the cells' "H$_2$O$_2$ threshold concentration" may be considered more important than the duration of treatment. Similar observations have been made in studies investigating the effect of H$_2$O$_2$ upon DNA damage and apoptosis in JURKAT cells, wherein, after the addition of 150 μM H$_2$O$_2$, to a cell suspension, the concentration of H$_2$O$_2$ continuously decreased and was no longer apparent after about 60 minutes (Barbouti et al., 2002). In contrast to the above observations, Long et al. (2000), reported that, in RPMI media, following a 60-minute incubation, 100 μM EGCG generated only 70 μM of H$_2$O$_2$, which continued to increase over time.
However, these incubations were carried out in the absence of cells (Long et al., 2000), which may explain the discrepancy with the current findings, and indicates that cells may also influence \( \text{H}_2\text{O}_2 \) production and elimination in culture media. It is clearly apparent that extracellular \( \text{H}_2\text{O}_2 \) is being produced in the culture media, and that this process is associated with induction of apoptosis in HCT-8 cells, which concords with previous studies (Suhr & Kim, 1999; Ogawa et al., 2003). Moreover, exogenous hydrogen peroxide can reportedly increase intracellular \( \text{H}_2\text{O}_2 \) accumulation (Yang et al., 1998; Halliwell & Gutteridge, 1999; Ogawa et al., 2003), inducing apoptosis via the formation of MPTP (Dumont et al., 1999; Ishisaka et al., 2002; Ogawa et al., 2003). Taken together, these studies indicate that exogenous \( \text{H}_2\text{O}_2 \) appears to activate apoptosis via an intrinsic caspase cascade.

**Antioxidants: influence upon polyphenol-induced apoptosis.**

The antioxidants, ascorbic acid and GSH, were added to the extracellular culture medium, to determine whether they were able to prevent polyphenol oxidation and degradation, and thus influence the apoptotic outcome of polyphenols in cell culture. The antioxidants, GSH and ascorbic acid, were unable to induce apoptosis in HCT-8 cells, or have any influence upon EGCG-induced apoptosis. A small inhibition in theaflavins- and curcumin-induced apoptosis was observed in the presence of GSH. A similar study by Chen et al. (2003b), demonstrated that pre-incubation with the same concentration of GSH (5 mM) dramatically inhibited the apoptotic effect of EGCG (at the higher concentration of 100 \( \mu\text{M} \)) in human colon adenocarcinoma HT-29 cells. In addition, a number of studies show that polyphenol-induced apoptosis might be a consequence of rapid \( \text{H}_2\text{O}_2 \) generation in culture media (Long et al., 2000; Sakagami et al., 2001; Nakayama et al., 2002; Roques et al., 2002). Collectively, these findings
suggest that polyphenols may potentially be involved in the production of $\text{H}_2\text{O}_2$, while GSH, acting as an antioxidant, may scavenge ROS, thus impairing apoptosis. However, these findings are inconsistent with those observed during the current study, using HCT-8 cells. Additionally, GSH and ascorbic acid are themselves prone to oxidation, under conditions of oxidative stress (Halliwell & Gutteridge, 1999; Syng-ai et al., 2004; Nakazato et al., 2005), as a result active concentrations may have been depleted in the culture media, thereby impeding their antioxidant activities. Conversely, an increase in polymer-induced apoptosis was observed in the presence of GSH. Ascorbic acid, in addition to other antioxidants, such as GSH, has been reported to also exhibit pro-oxidant effects (Sakagami & Satoh, 1997; Halliwell & Gutteridge, 1999). Therefore, the possibility of a pro-oxidant response from GSH may explain the increase in polymer-induced apoptosis seen with GSH. However, the influence of antioxidants upon polyphenol-induced apoptosis remains unclear, and may be related to polyphenol structure.

Effect of bile acids on polyphenol-induced apoptosis.

CA and DCA were employed to investigate the apoptotic effect of bile acids. In accordance with a number of studies investigating the apoptotic effects of bile acids at physiological concentrations (Schlottmann et al., 2000; Qiao et al., 2001b; Glinghammar et al., 2002; Milovic et al., 2002), HCT-8 cells incubated with CA alone, displayed near basal levels of apoptosis, whilst cells incubated with highly hydrophobic DCA exhibited apoptosis that increased in a dose-dependent manner. Bile acids, including DCA and CDCA, which are considered as potent tumour promoters, are increasingly being implicated in colon tumour development via induction of apoptosis. However counterintuitive this may seem, at least two hypotheses are consistent with
this notion. One proposes that DCA can cause extensive elimination of cells in the colonic epithelium, which leads to an increase in cell proliferation, and, subsequently, a cell population more prone to DNA damage, mutations and neoplastic transformation. Alternatively, increasing concentrations of faecal DCA may lead to increased apoptosis, and potentially apoptosis-resistant cells (Martinez et al., 1998; Qiao et al., 2001a).

To determine whether the apoptotic potential of DCA could influence polyphenol-induced apoptosis, HCT-8 cells were incubated with the polyphenols EGCG, theaflavins, curcumin and the polymer, in the presence of DCA. Even though DCA appeared to have a negligible effect upon polymer-induced apoptosis, in the presence of DCA (100 μM), an additive effect was observed upon both EGCG- and theaflavins-induced apoptosis. These findings suggest that DCA has the potential to modulate the apoptotic response induced by the monomeric polyphenols. It is possible that as a result of its natural detergent activity DCA may increase cell permeability (Vyvoda et al., 1977; Gordon et al., 1985; Nielsen & Rassing, 1999), thus facilitating polyphenol cellular uptake, leading to increased apoptotic potency. The concentration at which this was observed, for example 100 μM, is in the physiological range for DCA in faecal water, which is more likely to be in direct contact with the colonic epithelium than components that are bound to food residues within the faeces (van Munster et al., 1993; Milovic et al., 2002). DCA appeared to synergistically enhance curcumin-induced apoptosis, in HCT-8 cells. Curcumin is reported to induce oxidative stress, leading to activation of an intrinsic caspase cascade (Bhaumik et al., 1999; Kim et al., 2001c; Woo et al., 2003). It is therefore possible that in the presence of curcumin and DCA, also reported to induce oxidative stress (Lechner et al., 2002), consequent ROS production is likely. Moreover, depletion of intracellular GSH may render cells more susceptible to
apoptotic induction by subsequent stimuli, such as curcumin and DCA, due to potential increases in oxidative stress (Halliwell & Gutteridge, 1999; Chandra et al., 2000; Carmody & Cotter, 2001; Townsend et al., 2003) and, thus, a synergistic apoptotic response may well be achieved.

To investigate the potential synergism of DCA upon curcumin-induced apoptosis, and in light of the fact that curcumin induces caspase-9 activity in HCT-8 cells (section 3.3.3), cells were incubated with DCA and curcumin, and then assayed for caspase-9 activity. DCA, alone, effectively induced caspase-9 activity in a concentration-dependent manner, however curcumin-induced caspase-9 activity was dramatically reduced, in the presence of DCA, indicating the absence of even an additive response. These observations indicate that an interaction between curcumin and DCA, which considerably suppresses caspase-9 activity, should also initiate a reduction in apoptosis, but this is inconsistent with the apoptosis morphology data. It is therefore possible that, at lower concentrations, DCA-induced apoptosis may occur via a pathway involving caspase-8 activation (Schlottmann et al., 2000; Qiao et al., 2001b). Moreover, high concentrations of DCA are reported to induce high levels of caspase-3 activity (in colonic cancer cells), which fell in the presence of curcumin (Glinghammar et al., 2002). Furthermore, DCA has been reported to induce oxidative stress, intracellularly, in colon cancer cells (Lechner et al., 2002). Taken together, these findings imply that high concentrations of DCA may potentially provoke ROS production, which curcumin attenuated through its antioxidant actions.
Evidence for an extrinsic pathway.

The possibility that cells in suspension may present a greater surface area for the binding of polyphenols, and thus elevate apoptosis, was investigated by incubating the grape seed polymer with HCT-8 cells that were still in suspension, prior to attachment or cells already attached to a substratum. Furthermore, the possibility that, once bound, the polymer might impair the ability of “suspension” cells to attach to the substratum was investigated. The polymer induced apoptosis in a concentration-dependent manner, with the degree of apoptosis being more pronounced in “substratum” cells. Similarly, the polymer induced a dose-dependent inhibition of HCT-8 cell proliferation, which appears comparable between the “substratum” and “suspension” cells. However, in “substratum” cells, incubated with the polymer, at a concentration of 2 μM, the number of cells was much lower than in their “suspension” counterparts. In addition, the percentage of “suspension” cells recovered in the attached cell fraction was comparable to that of the DMSO vehicle control, indicating that the presence of the polymer in culture media does not impair cell attachment. Taken together these observations imply that a decrease in polymer-induced apoptosis might be a consequence of cells remaining in suspension, compared to those fully attached to a substratum. However, these findings give no indication of whether the polymer, with its relatively large molecular weight, induces apoptosis via a different pathway from that of smaller monomeric polyphenols. Although, results from previous caspase assays (section 3.3.3) appear to indicate that the polymer may induce apoptosis via an intrinsic pathway. Studies undertaken by Deprez et al. (2000) and Scalbert et al. (2000) indicate that polymeric proanthocyanidins, such as the polymer, may be degraded into smaller monomeric components, by colonic microflora, before traversing the cellular membrane. However, it is unclear whether degradation occurs in the absence of colonic
microflora, and thus, the aforementioned degradation of polymer may be unlikely to arise under "cell culture" conditions.

Polymer-induced apoptosis may also be influenced by cell adhesion receptors. In normal cells, loss of adhesion from the extracellular matrix (ECM), and consequent increases in p53 or Bax, are sufficient to trigger apoptosis. However, in transformed cells, detachment from the ECM is not sufficient to trigger apoptosis, and ligandation of integrins, receptors that mediate attachment and transduce signals that regulate cell growth, survival and gene expression, are not required for cell survival. Moreover, in a variety of cancerous cells, induction of apoptosis, mediated by integrin ligandation, predominantly occurred in adherent cells (Bachelder et al., 1999; McArthur Lewis et al., 2002). Consequently, in the presence of the grape seed polymer, integrin-mediated cell adhesion may have the potential to positively regulate DNA damage, and this may explain why HCT-8 cells maintained in suspension appear to display an attenuated response to polymer-induced apoptosis, compared with adherent cells.

Fas is constitutively expressed in normal epithelium, but expression appears considerably diminished in most colorectal carcinomas (Koshiji et al., 1998; Naujokat et al., 1999; Ogawa et al., 2004). To ascertain whether the Fas-activated extracellular death receptor pathway was involved in polyphenol-induced apoptosis (Hayakawa et al., 2001), it was important to firstly evaluate the expression of Fas in HCT-8 cells. In the current studies, it was observed that, even after a 6-hour incubation with anti-Fas, apoptosis was not evident in HCT-8 cells, indicating that Fas expression at the cell surface was low, and, therefore, unlikely to be directly responsible for polyphenol-induced apoptosis. Although other death receptors, such as TNF, DR3 or TRAIL, may
potentially be present at the cell surface, results from caspase-8 and -9 activity assays (section 3.3.3) indicate that, in HCT-8 cells, polyphenols are likely to induce apoptosis via an intrinsic pathway. During these preliminary studies, Fas was incubated in the presence of CHX, a protein synthesis inhibitor reported to increase cells susceptibility to Fas-mediated apoptosis (Weis et al., 1995; Jones et al., 1998; Hamada et al., 1999), but with no effect. However, studies investigating Fas expression have reported that pre-incubation of colorectal cells with the cytokine interferon-gamma (IFN-γ), recognised for its anti-viral and anti-neoplastic activity, actually increased Fas expression in cells which previously had low or no Fas expression (Koshiji et al., 1998; Abreu-Martin et al., 1999; Naujokat et al., 1999). It would, therefore, have been interesting to determine whether IFN-γ has a similar effect upon Fas expression in HCT-8 cells. Recent studies have reported that stimulation of the Fas receptor by a ligand resulted in the rapid generation of \( \text{H}_2\text{O}_2 \), which in turn may trigger the intracellular mitochondrial apoptotic pathway (Suzuki et al., 1998; Chun et al., 2003; Devadas et al., 2003). However, this appears not to be the case in HCT-8 cells, since no apoptotic response was observed. Therefore, to assess whether polyphenols induced apoptosis via a Fas-activated pathway, another cell line, which expresses Fas, should be utilised.

Conclusions.

Studies to elucidate potential mechanisms involved in polyphenol-induced apoptosis appear to indicate that the "active" polyphenols EGCG, theaflavins, curcumin and grape seed polymer induce apoptosis in HCT-8 cells via pro-oxidant activity. In the presence of DCA, additive or synergetic increases in polyphenol-induced apoptosis were apparent that, to some extent, may involve caspase-9 activation. This would concur
with preliminary findings indicating that HCT-8 cells express low levels of Fas. Moreover, H₂O₂ generation was clearly evident in the HCT-8 cell culture media, which had a substantial influence upon EGCG-induced apoptosis, as, in the presence of the antioxidant enzymes, catalase and SOD, apoptosis activity was appreciably reduced. In conclusion, it seems likely that polyphenols induce apoptosis in HCT-8 cells predominantly via a pro-oxidant mechanism, principally involving H₂O₂ generation and caspase-9 activation.
CHAPTER 5.

Interactions of Polyphenols with the Aryl Hydrocarbon Receptor.
Chapter 5. Interactions of Polyphenols with the Aryl Hydrocarbon Receptor.

5.1 INTRODUCTION.

The cytosolic Ah receptor, a ligand-dependent transcription factor, is ubiquitously expressed in mammalian tissues and plays a key role in the metabolism of a variety of carcinogenic compounds, including PAH, aromatic amines and HA. Although the Ah receptor is an orphan receptor with no endogenous ligand being identified, so far, the highly toxic, halogenated aromatic hydrocarbon, TCDD, is the highest known affinity ligand for the receptor (Hankinson, 1995). The liganded Ah receptor complex (as described in 1.6) mediates a number of genes, including some CYP450 and phase II xenobiotic-metabolising enzymes, such as CYP1A1 and glutathione-S-transferase. The pleiotropic biological effects of TCDD, mediated by the Ah receptor, give rise to the hepatotoxic consequences of porphyria, immunotoxicity, and developmental and reproductive toxicity. It can also modulate the expression of a number of genes and gene products involved in cell proliferation and differentiation, including plasminogen activator inhibitor-2 (PAI-2), interleukin-1β (IL-1β), and the oncogenes c-fos and c-jun (Hankinson, 1995; Sogawa & Fujii-Kuriyama, 1997). In consequence, chemicals that act antagonistically with the Ah receptor may inhibit chemical carcinogen bioactivation and suppress cell proliferation, thus providing protection against their carcinogenic effects.

A variety of naturally-occurring polyphenols, including green tea extracts, were shown, at dietary levels, by Northern blotting, electrophoretic mobility shift assay (EMSA) and luciferase gene reporter assay (using 101L cells), to antagonistically suppress transformation of the Ah receptor, induced by TCDD, and hence CYP1A1 expression, demonstrating suppression of dioxin toxicity (Ashida et al., 2000; Allen et al., 2001). The Ah receptor has been found to favour compounds that are hydrophobic, with van
der Waals dimensions of $14 \times 12 \times 5 \, \text{Å}$, chemical characteristics common to flavonoids, however, the flavonoid glycosides, containing a large sugar moiety, consequently have a lower affinity for the Ah receptor than their corresponding aglycone (Ashida et al., 2000). In a more detailed study, Williams et al. (2000) employed human hepatoma primary cultures and HepG2 cells to investigate the antagonistic effects of green tea extracts upon the Ah receptor, using the luciferase gene reporter assay, EMSA, Western and Northern blotting. In addition to green tea extracts-treated guinea pig liver cytosol, that were analysed for cytosolic Ah receptor transformation, and DNA binding, in vitro, (GRAB) (Williams et al., 2000), it was concluded that green tea extracts blocked TCDD-induced binding of the Ah receptor to DNA in HepG2 cells and isolated hepatic cytosol, thus inhibiting CYP1A transcription. Subsequently, four major green tea catechins were tested, establishing that only EGCG was able to inhibit TCDD-induced binding of the Ah receptor and successive CYP1A1 transcription, but was less effective than green tea extracts. However, in the same study the agonistic activity of green tea extracts and catechins was investigated, concluding that green tea extracts can exert weak agonist activity by elevating CYP1A1 mRNA and protein levels, while EGCG functions solely as an Ah receptor antagonist (Williams et al., 2000). Moreover, after cytosolic receptor binding, Northern blotting, luciferase gene reporter assay (using Hepa-2Dlux.3A4 cells), EMSA, immunoblotting and immunocytofluorescence experiments, demonstrated significant inhibition of TCDD-induced Ah receptor transformation by substituted methoxyflavones. This implies that potent antagonists, such as substituted methoxyflavones, bind with high affinity to the Ah receptor, but are unable to initiate Ah receptor transformation and nuclear localisation (Lu et al., 1996; Henry et al., 1999). The flavonoid 3',4'-DMF was reported as a pure and highly potent Ah receptor antagonist in human breast cancer cells, transfected with the Ah-responsive
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construct pRNH11c, by ethoxyresorufin-O-deethylase (EROD) analysis and the chloramphenicol acetyltransferase (CAT) reporter gene assay, thus inhibiting Ah receptor-dependent CYP1A1 induction (Lee & Safe, 2000).

In the present study, the binding of various polyphenols to the Ah receptor was investigated by using the chemically activated luciferase gene expression (CALUX) assay. This assay employs the recombinant mouse hepatoma cell line (H1L1.1c2) expressing a firefly luciferase reporter gene plasmid, under control of the MMTV LTR viral promoter (Garrison et al., 1996), to measure Ah receptor-dependent gene expression in a time-, dose-, chemical-, and Ah receptor-dependent manner (Seidel et al., 2000). Initially, the CALUX assay was validated using the established AhR ligands, TCDD, 2,3,7,8-tetrachlorodibenzo-p-furan (TCDF), B[α]P and β-naphthoflavone. Subsequently, an array of naturally-occurring and synthetic polyphenols were investigated, for their ability to bind to the Ah receptor as agonists/antagonists.

5.2 MATERIALS AND METHODS.

5.2.1 Cell Culture of H1L1.1c2 cells.

Materials.

- PBS: Oxoid Ltd, Basingstoke, UK.
- Minimum Essential Medium Alpha with L-glutamine, without ribonucleosides and deoxyribonucleosides (α-MEM), FBS, trypsin-EDTA 1X and Geneticin selective antibiotic (G418): Invitrogen Ltd, Paisley, UK.
- 50 units penicillin- 50 μg/ml streptomycin- 100 μg/ml neomycin antibiotic solution: Sigma-Aldrich Company Ltd, Poole, UK.
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Method.

The recombinant mouse hepatoma cell line H1L1.1c2, transfected by Dr. Michael Denison, University of California, Davis, USA (Garrison et al., 1996) and donated by Prof. Aldo Roda, University of Bologna, Italy, were cultured in α-MEM, supplemented with 10 % FBS and 400 µg/ml G418. Cells were maintained under standard conditions of 37 °C and 5 % CO₂ in a humid environment. During experimental work and prior to re-isolation, α-MEM media containing 10 % FBS was either antibiotic-free or supplemented with penicillin-streptomycin-neomycin antibiotic solution.

5.2.2 Re-isolation of H1L1.1c2 cells:

Materials. (Detailed in 5.2.1)

Method.

H1L1.1c2 cells underwent re-isolation, since initial experiments had revealed a reduction in luciferase activity with each cell passage. Cells of a low passage number were revived from liquid nitrogen, and grown in 25 cm² flasks until 80 % confluent in α-MEM media, supplemented with 10 % FBS and penicillin-streptomycin-neomycin antibiotic solution. Cells were washed in PBS, and cell growth continued in α-MEM, supplemented with 10 % FBS and 600 µg/ml G418 for two days, before G418 concentration was increased to 700 µg/ml. At this point, massive cell death occurred of cells containing the firefly luciferase gene, but without the pGudLuc1.1 vector, wherein nearly all the cells were washed away from the substratum. However, following regular changes of media, colonies of stable cells (containing pGudLuc1.1) were clearly visible after two weeks.

Stable transfected cells in 25 cm² flasks were washed in PBS and, following the addition of 5 ml of PBS containing 5 % Trypsin-EDTA, the isolated colonies were
transferred to 24-well plates containing α-MEM with 700 μg/ml G418. Cells were then cultured until 80% confluent before being transferred to 6-well plates and, subsequently, 25 cm$^2$ flasks. The constitutive luciferase activity of the re-isolated H1L1.1c2 cells was determined by incubation with $10^{-10}$ M TCDD, using the CALUX assay (as detailed in 5.2.3). Isolates with the highest ratio of inducible to constitutive activity were stored in liquid nitrogen for later use. The best of which were cultured for subsequent experiments in α-MEM supplemented with 10% FBS and 400 μg/ml G418.

5.2.3 CALUX Assay.

Materials and instrumentation.

- Chrysin, (Cl 1); 8-C-(3,3-dimethylallyl)-chrysin, (Cl 2); 8-C-(1,1-dimethylallyl)-chrysin, (Cl 3); 5-7-dihydroxy-3,2',4',5'-tetramethoxyflavone, (Cl 6); 5-7-dihydroxy-3,2',3',4'-tetramethoxyflavone, (Cl 7); 5-7-dihydroxy-3,3',4',5'-tetramethoxyflavone, (Cl 8) (Figure 5.1): Donated by Prof. Denis Barron, University of Lyon, France.
- Cell culture lysis reagent (5X) and luciferase assay reagent: Promega UK Ltd, Southampton, UK.
- TCDD and TCDF: LGC Promochem Ltd, Hatfield, UK.
- B[a]P and β-naphthoflavone: Sigma-Aldrich Company Ltd, Poole, UK.
- 3',4'-DMF: Lancaster Synthesis Ltd, Morecambe, UK.
- EGCG (Figure 1.1): Donated by Dr. Conrad Astill, Unilever UK, Colworth, UK.
- Proanthocyanidin B1 (Figure 2.1): Donated by Prof. Celestino Santos Buelga, University of Salamanca, Spain.
• Packard Lumicount microplate luminometer, with PlateReader software: Packard Instrument Company, Meriden, USA.

![Chemical structures of polyphenols](image)

Figure 5.1: Chemical structures of polyphenols used in the CALUX assay, donated by Prof. Denis Barron, that are not commercially available.

**Method.**

The CALUX assay was used to measure the ability of a chemical to activate Ah receptor-dependent gene expression in cultured cells, thus enabling the detection and relative quantitation of Ah receptor agonists and antagonists. Stably integrated H1L1.1c2 cells were cultured in 24-well plates using α-MEM media, supplemented with 10 % FBS and penicillin-streptomycin-neomycin antibiotic solution, at a seeding density of $7 \times 10^4$ cells/ml, then cultured for 24 hours until 50-70 % confluent. Solutions of the test compounds investigated were initially prepared in DMSO and subsequently diluted in fresh antibiotic-free α-MEM (with 10 % FBS). The H1L1.1c2 cells were gently washed in PBS prior to addition of the compounds in media (1-2 %...
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(\%\textsubscript{v/v}) DMSO final concentrations) and incubated under standard conditions of 37 °C and 5 % CO\textsubscript{2} in a humid environment, for either a 4- or 24-hour period. Cells were then washed in PBS and lysed by incubation with 100 μl of cell culture lysis reagent (1X) for 15 minutes at room temperature. Cell lysates were collected by scraping, transferred to eppendorf tubes and placed on ice. Subsequently, they were vortexed for 15 seconds, prior to centrifugation at 12,000 rpm for two minutes at 4°C, to pellet out any remaining cell debris.

Luciferase activity of cleared cell lysates was determined using the Promega stabilised luciferase assay system, as described by the manufacturer. Briefly, 20 μl of lysates was added to a white 96-well microtitre plate (using every third well to avoid cross-talk), followed by 100 μl of luciferase assay reagent. Subsequently, the microtitre plate was shaken for 90 seconds in a Packard Lumicount microplate luminometer with PlateReader software (Packard Instrument Company), and readings were taken precisely two minutes after the addition of the luciferase assay reagent. Light was measured as relative light units (RLU) (example shown in Table 5.1), corrected for gain and normalised for cell number, thus luciferase activity is expressed as 10\textsuperscript{3} RLU/10\textsuperscript{6} cells, with DMSO control values being deducted from all sample values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RLU (1\textsuperscript{st} reading)</th>
<th>RLU (2\textsuperscript{nd} reading)</th>
<th>RLU (3\textsuperscript{rd} reading)</th>
<th>Gain level</th>
</tr>
</thead>
<tbody>
<tr>
<td>10\textsuperscript{9} M TCDD</td>
<td>315,560</td>
<td>327,931</td>
<td>321,631</td>
<td>4.4</td>
</tr>
<tr>
<td>10\textsuperscript{9} M 3',4'-DMF</td>
<td>172,001</td>
<td>166,228</td>
<td>113,025</td>
<td>6.7</td>
</tr>
<tr>
<td>10\textsuperscript{8} M EGCG</td>
<td>139,533</td>
<td>169,179</td>
<td>249,594</td>
<td>6.8</td>
</tr>
<tr>
<td>DMSO control</td>
<td>17,977</td>
<td>49,268</td>
<td>13,806</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Table 5.1: Example of luminometry readings (RLU and gain level) obtained for cell lysates treated with 10\textsuperscript{9} M TCDD, 10\textsuperscript{9} M 3',4'-DMF, 10\textsuperscript{8} M EGCG and DMSO. Data was subsequently corrected for gain level and normalised for cell number. Luciferase activity was finally expressed as 10\textsuperscript{3} RLU/10\textsuperscript{6} cells, after DMSO control values were deducted from all sample values.
5.2.4 Measurement of H1L1.1c2 cell viability.

Materials.

- Trypan Blue: Sigma-Aldrich Company Ltd, Poole, UK.

Method.

Cell viability of H1L1.1c2 cells incubated with high concentrations of compounds was determined via the trypan blue exclusion method, to ascertain if cell death could be responsible for low luciferase readings. Cells were grown in 24-well plates and treated with the compounds under investigation, as described in section 5.2.3, then harvested by trypsinisation and resuspended in media, to a total volume of 500 µl. Cell numbers and viability were counted in 10 µl aliquots of cell suspension mixed with trypan blue (4 %) at a ratio of 1:1 (v/v) using a Modified Fuch’s Rosenthal counting chamber, under phase contrast light microscopy. Non-coloured cells were scored as live/viable and blue cells were scored as dead/non-viable. Trypan blue exclusion was also used during cell passage to obtain healthy cell numbers whilst preparing a seeding density of $7 \times 10^4$ H1L1.1c2 cells/ml (as detailed in 5.2.3).

5.3 RESULTS.

5.3.1 Interaction of known ligands with the Ah receptor via the CALUX assay.

Known ligands of the Ah receptor were used to validate the CALUX assay and investigate the effect of incubation time upon Ah receptor binding. Using TCDD as the model ligand, a higher level of interaction was observed following a short 4-hour incubation period compared to that of a longer 24-hour period (Figure 5.2). However, the luciferase activity obtained for TCCD was appreciably lower than expected from previous reported studies (Garrison et al., 1996; Machala et al., 2001). After consultation with Dr. Denison, it was decided to re-isolate the H1L1.1c2 cells, as
detailed in 5.2.2. Isolate "f" gave the highest constitutive luciferase activity and was selected for use in all subsequent assays (Figure 5.3). The synthetic flavonoid β-naphthoflavone also bound to the receptor, as expected (Figure 5.4), although maximum binding data was unobtainable, because at a concentration of $10^{-3}$ M, solubility problems were encountered.

Figure 5.2: Effects of incubation time on the binding of TCDD to the Ah receptor. H11L1.1c2 cells were incubated with TCDD for 4 or 24 hours. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.
Figure 5.3: Constitutive luciferase activity of HIIL1.lc2 isolates. Cells were incubated with TCDD (0.1 nM), dissolved in DMSO. Twenty-nine cultures of isolated cells (a-C) were investigated along with cells grown in non-G418 media (N). Cells were incubated for 4 hours and luciferase activity determined via the CALUX assay. Results are expressed as the mean RLU (relative light units) ± SEM of triplicate determinations for TCDD and as single values for DMSO.

Figure 5.4: Interaction of β-naphthoflavone with the Ah receptor using the CALUX assay. HIIL1.lc2 cells were incubated for 4 hours with β-naphthoflavone (β-NF). Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.
Initially, the BioRad assay (section 3.2.3) was being utilised for protein determination of cell lysates intended for data normalisation, but in the presence of $>1\%$ ($\gamma_\circ$) of the cell lysis reagent, corresponding to the sample dilutions, a standard curve was unachievable. BSA standard curves containing 0, 2 and 10 % ($\gamma_\circ$) cell lysis reagent were compared using the BioRad and Lowry assays (data not shown), where an increase in cell lysis reagent alters the protein standard curve in both methods, and, on further investigation it became apparent that the cell lysis reagent was incompatible with both assays. Following consultation with Dr Denison, it was considered acceptable to present data as normalised by "a very defined number of cells", hence running every assay with the same cell density.

After H11.1.1c2 cell re-isolation and use of cell number for data normalisation, TCDD was again employed as an Ah receptor ligand (Figure 5.5), at the short incubation time of four hours, yielding a high level of interaction at a concentration of $10^{-9}$ M. The polycyclic aromatic hydrocarbon, B[a]P induced a higher level of binding after a 4-hour incubation, compared with a 24-hour incubation period (Figure 5.6). Moreover, as seen with $\beta$-naphthoflavone, B[a]P did not bind as avidly as TCDD to the receptor. In addition, concentrations higher than $10^{-3}$ M could not be investigated, due to insolubility, as observed by a cloudy appearance in the media. Another established high affinity ligand, TCDF also proved to have a high level of interaction with the Ah receptor (Figure 5.7), again demonstrating higher binding after a four-hour incubation, compared with a 24-hour incubation. TCDF appears to have higher affinity for the receptor than $\beta$-naphthoflavone and B[a]P, but was not as avid as TCDD. At a concentration of $10^{-5}$ M, binding of TCDF to the Ah receptor dropped markedly, although still remained above DMSO values.
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**Figure 5.5: Interaction of TCDD with the Ah receptor.** H1L1.1c2 cells ($7 \times 10^4$ cells/ml) were incubated with TCDD for 4 hours. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

**Figure 5.6: Effect of incubation time on the interaction of B[a]P with the Ah receptor.** H1L1.1c2 cells ($7 \times 10^4$ cells/ml) were incubated with B[a]P for 4 or 24 hours. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.
Figure 5.7: Effect of incubation time on the interaction of TCDF with the Ah receptor. H1L1.1c2 cells (7 × 10⁴ cells/ml) were incubated with TCDF for 4 or 24 hours. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

5.3.2 Interactions of a variety of polyphenols including 3',4'-dimethoxyflavone with the Ah receptor.

In contrast to the above observations of the interaction of known ligands with the Ah receptor, when 3',4'-DMF served as a ligand, no reproducible interaction spectra were obtained. Figure 5.8 illustrates observations made via the CALUX assay, when this polyphenol was investigated on three separate occasions. Furthermore, at concentrations of 10⁻⁷ M to 10⁻⁵ M luminometry readings for 3',4'-DMF fell below that of the DMSO control. Suspecting compromised cell viability to be the cause, this was further investigated in 3',4'-DMF-treated cells via trypan blue exclusion and established as being over 97% (Figure 5.9). Only two of the polyphenolic compounds examined for cell viability displayed cytotoxicity. At a concentration of 10⁻⁴ M, cell viability of 86.4 and 76.5% was observed for chrysin (CI 1) and 8-C-(1,1-dimethylallyl)-chrysin (CI 3), respectively, with all other compounds exhibiting viability >92.4%, indicating that cell viability is not responsible for assay reproducibility.
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Figure 5.8: Interaction of 3',4'-DMF with the Ah receptor. Three separate experiments are shown. H1L1.1c2 cells were incubated with 3',4'-DMF for 4 hours at a seeding density of $7 \times 10^4$ cells/ml. Cell viability was >97%. Luciferase activity for $10^{-9}$ M TCDD was $57430 \pm 885$ 10^3RLU/10^6 cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

Figure 5.9: Cell viability of H1L1.1c2 cells via trypan blue exclusion following polyphenol treatment. H1L1.1c2 cells were incubated with 3',4'-DMF, chrysin (Cl 1), 8-C-(3,3-dimethylallyl)-chrysin (Cl 2), 8-C-(1,1-dimethylallyl)-chrysin (Cl 3), 5,7-dihydroxy-3,2',4',5'-tetramethoxyflavone (Cl 6) and solvent control (DMSO) for 4 hours at a seeding density of $7 \times 10^4$ cells/ml. Results are expressed as the mean ± SEM of triplicate determinations.
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Figure 5.10: Interaction of synthetic dihydroxy-tetramethoxyflavones with the Ah receptor. H1L1.1c2 cells were incubated for 4 hours at a seeding density of $7 \times 10^4$ cells/ml. Luciferase activity for $10^{-6}$ M TCDD was $57430 \pm 885$ RLU/10^6 cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

Cl 6: 5-7-dihydroxy-3,2',4',4'-tetramethoxyflavone. Cell viability >96.5%.
Cl 7: 5-7-dihydroxy-3,2',3',4'-tetramethoxyflavone.
Cl 8: 5-7-dihydroxy-3,3',4',5'-tetramethoxyflavone.

Similarly, unexpected binding spectra were observed for 5-7-dihydroxy-3,2',4',4'-tetramethoxyflavone (Cl 6), 5-7-dihydroxy-3,2',3',4'-tetramethoxyflavone (Cl 7) and 5-7-dihydroxy-3,3',4',5'-tetramethoxyflavone (Cl 8), as illustrated in Figure 5.10. Luciferase activity levels of Cl 8 dropped below that of the DMSO control at concentrations of $10^{-6}$ to $10^{-4}$ M, although cell viability remained above 96.5 % (Figure 5.10). Equally, there was no indication of any binding with the synthetic polyphenols of Cl 1, 8-C-(3,3-dimethylallyl)-chrysine (Cl 2) and Cl 3 (Figure 5.11) or for EGCG and the proanthocyanidin B1 dimer (Figure 5.12).
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Figure 5.11: Interaction of synthetic polyphenols with the Ah receptor. H1L1.1c2 cells were incubated for 4 hours at a seeding density of $7 \times 10^4$ cells/ml. Luciferase activity of $10^{-9}$ M TCDD was $37608 \pm 2104 \times 10^3$ RLU/10$^6$ cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

CI 1: Chrysin. Cell viability >86.4%.
CI 2: 8-C-(3,3-dimethylallyl)-chrysin. Cell viability >92.4%.
CI 3: 8-C-(1,1-dimethylallyl)-chrysin. Cell viability >76.5%.

Figure 5.12: Interaction of EGCG and proanthocyanidin B1 with the Ah receptor. H1L1.1c2 cells were incubated with EGCG or B1 for 4 hours at a seeding density of $7 \times 10^4$ cells/ml. Luciferase activity for $10^{-9}$ M TCDD was $52889 \pm 6021 \times 10^3$ RLU/10$^6$ cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.
Moreover, in previous experiments at the higher concentration of $10^{-4}$ M, luciferase activity of both CI 1 and CI 3, fell below the control (data not shown). Cell viability was investigated for the synthetic polyphenols using trypan blue exclusion, displaying $>86.4\%$ and $>76.5\%$ viability for CI 1 and CI 3, respectively. However, low cell viability does not appear to explain the unexpected binding spectra exhibited by the polyphenols, as cell viability remained above $92.4\%$ for the other polyphenolics tested (Figure 5.9).

5.3.3 **Confirmation that polyphenols do not interfere with the CALUX assay.**

Further studies were carried out to provide a rationale for the unexpected results generated by the polyphenols. Initially, it was considered that the polyphenols might be interfering with the reagents used in the CALUX assay and, therefore, following a 4-hour incubation with TCDD, the effect of a number of polyphenols, in H1L1.1c2 cell lysates, was compared with that of the solvent vehicle DMSO. As illustrated by 3',4'-DMF (Figure 5.13), and also observed in proanthocyanidin B1, EGCG and CI 8 (5–7-dihydroxy–3,3',4',5'-tetramethoxyflavone) (data not shown) treated cells, these polyphenolic compounds did not interfere with the CALUX assay. The unexpected findings obtained with polyphenols were further investigated, by examining their response after an extended incubation period of 24 hours, compared with 4 hours, to investigate whether polyphenols had a better chance of binding to the Ah receptor. EGCG and 3',4'-DMF were initially considered (data not shown), both exhibiting non-sigmoidal spectra, indicating that longer incubation time had no effect upon polyphenol Ah receptor binding.
5.3.4 Possible role of reactive oxygen species on the lack of interaction between polyphenols and the Ah receptor.

It was possible that polyphenols displayed pro-oxidant activity, and production of ROS may be detrimental to the cells, preventing interaction with the Ah receptor and subsequent translocation to the nucleus. Using the CALUX assay, 3',4'-DMF was incubated in the presence of 850 U/ml catalase (Figure 5.14), 50 U/ml SOD (Figure 5.15), or 16 µM of the Fe³⁺ chelator desferrioxamine (Figure 5.16). No true differentiation was detectable in luciferase activity spectra, indicating that ROS are unlikely to be responsible for the irreproducible data obtained using polyphenolic compounds.
Figure 5.14: The effect of catalase on the interaction of 3',4'-DMF to the Ah receptor. H1L1.1c2 cells were incubated for 4 and 24 hours at a seeding density of $7 \times 10^4$ cells/ml. Concentration of catalase (Cat) used was 850 U/ml. Luciferase activity for $10^{-5}$ M TCDD at 4 & 24 hours was $58949 \pm 991$ and $18419 \pm 924$ $10^3$ RLU/10^6 cells, respectively. Results are expressed as the mean $\pm$ SEM of triplicate determinations, with solvent values being deducted from all sample values.

Figure 5.15: The effect of SOD on the interaction of 3',4'-DMF to the Ah receptor. H1L1.1c2 cells were incubated for 4 and 24 hours at a seeding density of $7 \times 10^4$ cells/ml. Concentration of SOD used was 50 U/ml. Luciferase activity for $10^{-5}$ M TCDD at 4 & 24 hours was $55693 \pm 5583$ and $29961 \pm 6048$ $10^3$ RLU/10^6 cells, respectively. Results are expressed as the mean $\pm$ SEM of triplicate determinations, with solvent values being deducted from all sample values.
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Figure 5.16: The effect of desferrioxamine on the interaction of 3',4'-DMF to the Ah receptor. H1L1.1c2 cells incubated for 4 and 24 hours at a seeding density of 7 x 10^4 cells/ml. Concentration of desferrioxamine (Des) used was 16 pM. Luciferase activity for 10^9 M TCDD at 4 & 24 hours was 77675 ± 6791 and 46575 ± 1075 10^3RLU/10^6 cells, respectively. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

5.3.5 Influence of 3',4'-dimethoxyflavone on the binding of TCDD to the Ah receptor.

Studies were undertaken to evaluate whether the binding of TCDD to the Ah receptor was modulated in the presence of 3',4'-DMF, to ascertain whether this flavonoid displayed any antagonistic activity. At a concentration of 1 μM, the flavonoid had no effect on the binding of TCDD to the Ah receptor (Figure 5.17). Conversely, at the higher concentration of 100 μM (Figure 5.18), 3',4'-DMF appeared to increase the binding of TCDD at lower concentrations (10^-14 to 10^-10 M) while reducing TCDD binding at the higher concentrations (10^-10 to 10^-5 M). In consequence, 3',4'-DMF, at a concentration of 100 μM, appears to function either as a weak Ah receptor agonist or an antagonist, depending on the concentration of TCDD.
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Figure 5.17: The effect of 1 pM 3',4'-DMF on the binding of TCDD to the Ah receptor. H1L1.1c2 cells incubated for 4 hours at a seeding density of 7 x 10^4 cells/ml. Luciferase activity for 1 pM 3',4'-DMF was 1332 ± 330 10^3 RLU/10^6 cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.

Figure 5.18: The effect of 100 µM 3',4'-DMF on the binding of TCDD to the Ah receptor. H1L1.1c2 cells incubated for 4 hours at a seeding density of 7 x 10^4 cells/ml. Luciferase activity for 100 µM 3',4'-DMF was 8915 ± 747 10^3 RLU/10^6 cells. Results are expressed as the mean ± SEM of triplicate determinations, with solvent values being deducted from all sample values.
5.4 DISCUSSION.

The CALUX assay was validated using the established Ah receptor ligands TCDD, TCDF, B[a]P and β-naphthoflavone. The binding characteristics were similar to what was previously reported by other workers, using the same method (Garrison et al., 1996; Machala et al., 2001). Work undertaken during the present study looked into the binding of known ligands at two exposure times, concluding that the shorter 4-hour incubation time yielded higher luciferase activity. Similar conclusions were reached by Garrison et al. (1996) and Zhang et al. (2002), who demonstrated that during time course studies, TCDD induction of luciferase activity peaked after 4 hours, and then declined, reaching stability at 12-14 hours, probably due to a time-dependent stimulation of luciferase degradation by cellular proteins (Garrison et al., 1996). Consequently, the CALUX assay can be used as a simple, sensitive and rapid detection system of ligand binding to the Ah receptor.

Polyphenolics and the CALUX assay.

In contrast to the above observations for TCDD, which clearly demonstrate Ah receptor binding, the polyphenols investigated during the current study, including the reportedly highly-bound 3',4'-DMF (Lee & Safe, 2000), yielded irreproducible data. Studies were carried out to provide a rationale for the unexpected behaviour exhibited by the polyphenols. After ensuring that the polyphenols under investigation did not interfere with the CALUX assay procedure, the possibility was next considered that the polyphenols might require a longer-incubation time for binding and, therefore, 24 hours incubation was compared to the 4-hour incubation time. A slight increase was seen in 3',4'-DMF luciferase activity at 24 hours, and no significant variation was noted for EGCG, however, both compounds failed to produce a sigmoidal dose response curve.
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The possibility that the pro-oxidant activity of polyphenols, leading to the production of ROS, was harmful to the cells, thereby potentially impairing interactions with the Ah receptor and subsequent translocation to the nucleus, was also examined. Results indicated ROS were not responsible for the unusual behaviour exhibited by the polyphenols, as, even in the presence of catalase, SOD and desferrioxamine, 3',4'-DMF failed to produce a sigmoidal dose response curve. However, there is evidence to suggest that it is not only the polyphenols that may influence the oxidative state at the Ah receptor. Dalton et al. (2002) proposed that Ah receptor activation, by any class of ligand, can lead to an increase in $O_2^{\cdot-}$ production via an escalation in xanthine oxidase/dehydrogenase and monooxygenase expression, ultimately leading to a rise in $H_2O_2$ production, and a decreased capacity to detoxify it. Furthermore, Palermo et al. (2003), using the potent free radical scavenger, Trolox, investigated whether the antioxidant effects of green tea catechins inhibited TCDD-induced luciferase reporter gene activity, in Hepa-2D1uc cells, establishing that Trolox treatment was unable to inhibit TCDD luciferase reporter activity. Collectively, this indicates that a pro- or antioxidant response, by the polyphenols, is unlikely to be responsible for the irreproducible CALUX results obtained in this study.

One possible explanation for the irreproducible CALUX data obtained during the present study, is that the polyphenols were unable to gain entry to the cells across the plasma membrane due to their low bioavailability, which is generally determined by chemical structure and physiochemical properties, for example, as exhibited by the partition coefficient ($logP$), which indicates hydrophilicity (Scalbert & Williamson, 2000). Similar observations, in the accessibility of chemicals to the Ah receptor, have already been reported for a range imidazoline receptor ligands, including adrenaline and
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Phentolamine, and β-carboline compounds, such as norharman and harman (Seidel et al., 2000). Bearing in mind that a number of polyphenols are found in aqueous tea brew, partition coefficient values indicate that polyphenols are generally hydrophilic. In comparison, PAH and HAH, are highly hydrophobic, and can thus easily traverse the cell membrane. Moreover, the membrane export pump MRP2, which has been reportedly implicated in the efflux of isoflavone, genistein and chrysin from Caco2 cells (Walle et al., 1999; O’Leary et al., 2003), may be involved in the efflux of other polyphenols from hepatic cell lines, thus reducing polyphenolic concentration at the Ah receptor binding site.

Polyphenols may also readily undergo metabolism, intracellularly, by enzymes such as COMT, SULT and UGT, to various conjugated and/or methylated metabolites (Higdon & Frei, 2003; Lambert & Yang, 2003; Kroon et al., 2004), again reducing effective concentrations at the Ah receptor site. Following oral administration, catechins undergo extensive glucuronidation in the intestinal mucosa, sulphation in the liver and methylation in both the liver and kidney (Kohri et al., 2001; Rechner et al., 2002; Higdon & Frei, 2003). Even though the CALUX assay is rapid (4 hours), this is still sufficient time for many polyphenols to undergo extensive metabolic degradation, and may explain the lack of reproducible binding of the polyphenols to the Ah receptor. Conversely, HAH are resistant to cellular metabolism, thus contributing to their potency as Ah receptor ligands (Seidel et al., 2000).

Furthermore, polyphenols have high binding affinity for proteins, particularly for large hydrophobic proline-rich proteins with flexible structures (Hagerman & Butler, 1981; Luck et al., 1994). Similarly, when EGCG-induced apoptosis in HCT-8 cells, incubated
in 10% or FBS-free media, was investigated earlier in the current study, to determine what extent FBS masks the apoptotic activity of EGCG (section 2.3.2), EGCG was markedly more active in the absence of FBS, at the concentrations studied. Seidel et al. (2000) reported that polyphenolic compounds have to avoid sequestration by serum proteins, as this can also lead to a reduction in their concentration at the Ah receptor-binding site.

**Polyphenols: potential Ah receptor antagonists.**

Studies using ligand binding and luciferase gene reporter assays, and utilising the subclone Hepa-2Dluc.3A4 cell line, demonstrated that the flavone derivatives, 3'-methoxy-4'-iodoflavone, 3'-methoxy-4'-azidoflavone and 3'-methoxy-4'-nitroflavone bound to the Ah receptor (Henry et al., 1999), as well as, exhibiting antagonistic activity against TCDD. It was stated that maximal induction in the luciferase assay was achieved by the flavone derivatives tested at 0.5nM, a concentration in the range used in the present study, although interestingly no data was shown for the remaining range of concentrations (1-1000 nM), apparently investigated. In addition, the compounds investigated encountered flavone metabolism, eliciting a substantial effect upon compound efficacy. Nevertheless, in general, a good correlation was obtained between results from the two methods employed (Henry et al., 1999). The same researchers also reported that polyphenolic 3'-methoxyflavones compounds containing a 4' substituent are highly effective as Ah receptor antagonists, in both rat hepatic cytosol and recombinant murine cells (Henry et al., 1999). In a similar study, using Hepa-2Dluc cells, green tea polyphenols were found to have high antagonistic activity at the Ah receptor (Palermo et al., 2003), particularly the catechins EGCG and ECG, at
concentrations of 1-200 μM. However, concentrations higher than 25-50 μM cannot be achieved by normal dietary intake.

In the present study, investigations were undertaken to evaluate whether 3’,4’-DMF antagonised the binding of TCDD to the Ah receptor. In these studies, TCDD binding was determined in the presence of 3’,4’-DMF. Initially, 1 μM of 3’,4’-DMF was used, having no effect on the TCDD dose response curve, but when the concentration was increased to 100 μM 3’,4’-DMF, an increase in binding was observed at the lower TCDD concentrations, which subsequently decreased, as TCDD concentrations rose, implicating 3’,4’-DMF as an Ah receptor antagonist. It is therefore possible, that below concentrations of 0.1 nM TCDD, 3’,4’-DMF could have weak agonist activity, given that TCDD levels are too low to occupy the binding sites at the Ah receptor. However, when TCDD exceeds 0.1 nM, avidly binding to the Ah receptor, the antagonist response of 3’,4’-DMF becomes manifested, though it is unlikely that these high antagonistic polyphenol concentrations can be achieved naturally from dietary source. Similar observations have been made in studies investigating the ligand responses of polyphenols at plasma concentrations. Flavonoids (1-20 μM) incubated in 101L cells for 18 hours failed to bind to the Ah receptor, but on further investigation, some of the compounds were able to decrease TCDD-mediated induction of luciferase reporter gene activity (Allen et al., 2001), with naringenin being the most effective. Moreover, pre-treatment of 101L cells with green tea extracts (25-100 μg/ml) blocked TCDD-induced Ah receptor binding, determined via the luciferase assay (Williams et al., 2000), demonstrating an antagonistic response by the green tea extracts.
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Receptor binding and gene expression.

It is clear that data obtained from studies carried out via methodologies, which employ rat liver, or cell cytosol, may differ to those using cell lines (Henry et al., 1999). When utilising intact cells, as in the current study, the compound under investigation must be sufficiently lipophilic to allow it to cross the cell membrane and reach the Ah receptor-binding site, while lipid solubility is of little consequence in the homogenised cytosol. Compartmentalisation is also a factor in intact cells, with regards to Ah receptor nuclear translocation and transformation, since ARNT is localised in the nucleus. Hence, in the intact cell, compartmentalisation is likely to amplify any differences, however minor, relating to the ability of a test compound to traverse biological membranes, bind to the Ah receptor, and to elicit Ah receptor nuclear translocation and transformation (Henry et al., 1999; Seidel et al., 2000). Moreover, in the majority of studies where dietary plasma levels of polyphenols are employed, only a limited number of concentrations are tested, covering a narrow concentration range, (such as 1-100 μM), which is in stark comparison to the 11 concentrations and much wider concentration range (10 fM to 100 μM), employed during the present study (Ciolino et al., 1999; Ashida, 2000; Ashida et al., 2000; Allen et al., 2001).

It has recently been reported, from studies using mouse hepatoma Hepalclc7 cells, that TCDD can enhance the expression of both stably transfected luciferase reporter and endogenous Ah receptor genes (Zhou & Gasiewicz, 2003), whereas polyphenols, such as 3'-methoxy-4'-nitroflavone (3'M-4'NF) acted as an agonist for the CYP1A1 gene, but as an apparent antagonist for the luciferase reporter gene. Although, reportedly a low efficacy ligand, the ability of 3'M-4'NF to act as an Ah receptor agonist or antagonist appears dependent upon its concentration, and the promoter of the particular gene in
question. The same researchers also examined the polyphenols 3'M-4'NF and 3',4'-DMF by Western blot analysis, reporting both enhanced and weak CYP1A1 expression, respectively. However, both polyphenolic compounds failed to induce luciferase expression (Zhou & Gasiewicz, 2003), which may explain the lack of reproducible interaction spectra for 3',4'-DMF, in the present study.

Conclusions.

In conclusion, after validation of the CALUX assay via established Ah receptor ligands, both naturally-occurring and synthetic polyphenols failed to provide reproducible binding spectra to the Ah receptor. Following studies to rationalise this data, it is conceivable that polyphenols are unable to transverse the cellular membrane, or hinder the process, by slow passive cellular uptake and/or poor substrate specificity for membrane transporters, in addition, metabolism that occurs during assay incubation, may reduce the effective polyphenol concentration present at the Ah receptor-binding site.
CHAPTER 6.

General Discussion.
6.1 ARE POLYPHENOLS POTENTIAL CHEMOPREVENTORS?

Bearing in mind that a number of studies employing animal models (as described in 1.3) and human epidemiology (Franceschi et al., 1997; Ji et al., 1997; Zhong et al., 2001; Sun et al., 2002; Il'Yasova et al., 2003) demonstrate anticarcinogenic effects by naturally-occurring polyphenols in various cancer types, in the present study, a variety of naturally-occurring and novel polyphenolic compounds were investigated, to determine whether apoptosis is a potential mechanism through which they exert their chemopreventive activity. In addition, the ability of polyphenols to bind to the Ah receptor, agonistically or antagonistically, was assessed, to evaluate whether polyphenols can prevent, or reduce, the binding of carcinogens to the Ah receptor, thus potentially eliciting chemopreventive activity.

6.1.1 Polyphenols: structure-activity relationships and relevance to in vivo studies.

Since HT-29 and Caco-2 cells, both commonly used cell lines of human intestinal origin, were being employed elsewhere on the POLYBIND project, the HCT-8 cell line was chosen to complement findings from within the project. Derived from a non-metastatic human ileocecal adenocarcinoma, HCT-8 (human colon tumour-8) cells were selected for their ability to grow as discrete and tightly packed colonies of epithelial cells, each containing a large nucleus and scanty cytoplasm. Moreover, the presence of fairly uniform microvilli across the cell surface was reported to closely resemble the brush border characteristics of the intestinal epithelium (Tompkins et al., 1974). The HCT-8 cell line also exhibited well-defined apoptotic morphological features, including distinctly fragmented and condensed nuclei, membrane blebbing, chromatin condensation and apoptotic bodies, which were the principal methodological endpoint for assessment of apoptosis, during the present study.
Initially, to determine the apoptotic activity of selected polyphenols, HCT-8 cells were treated during the exponential growth phase, whereby compounds exhibiting >20% apoptosis were considered "active" and were used in subsequent investigations. The "active" polyphenols, that induced apoptosis at concentrations potentially achievable in plasma, by dietary intake (1-5 μM) (Kroon et al., 2004), during the present study, were all flavan-3-ols, with the exception of curcumin. But not all polyphenols within a specific group/class induced apoptosis, and thus the presence of particular structural moieties appears to be important in conferring potential apoptotic activity. This was clearly shown in the current study by EGCG, which was a potent inducer of apoptosis, however, other flavan-3-ols, including ECG, EGC, and dimers of (+)-catechin and (-)-epicatechin, elicited minimal or modest apoptotic activity, even at high concentrations (50 μM). This suggests that the presence of both a 3',4',5'-trihydroxy (gallo) B-ring and a 3-galloyl moiety are potentially important in conferring apoptotic activity to a compound, in accord with previous studies (Saeki et al., 1999b, 2000; Chung et al., 2001). Moreover, the grape seed polymer (composed of (+)-catechin, (-)-epicatechin and ECG monomers), with an average degree of polymerisation of 8.6 and 22% galloylated units, induced apoptosis in a concentration-dependent manner (1-2 μM), whereas, the grape seed oligomer (average degree of polymerisation of 3.3 and 11% galloylated units), produced little apoptosis (even at 5 μM). This indicates, in accordance with other studies (Matito et al., 2003), that an increase in the degree of polymerisation, and the percentage galloylation of proanthocyanidins, appears to be related to enhanced apoptotic activity.

However, it is noteworthy that the majority of in vitro studies employed polyphenolic concentrations that are considerably higher than those achievable in vivo (50-500 μM).
For example, in human plasma, the concentration of an individual polyphenol rarely exceeds 1 μM, after consumption of 10-100 mg of the corresponding compound, and the maximum plasma concentration attained after ingestion of a polyphenol-rich meal ranges from 0.1 to 10 μM. However, plasma concentrations may vary, depending upon the nature of the compound and food source, and the total plasma polyphenol concentration is likely to be higher due to the presence of polyphenol metabolites (Scalbert & Williamson, 2000; Kroon et al., 2004; Manach et al., 2004; Williamson & Manach, 2005).

Quercetin conjugates, particularly the glucuronides, are reported to be the major metabolites present in plasma after consumption of glycones, the most common dietary form (Day et al., 2000; Sesink et al., 2001; Johnson, 2004). In the current study, quercetin conjugates, including the 7-sulphate, displayed low apoptotic activity. Moreover, in anti-proliferation studies, undertaken as part of the POLYBIND project (as detailed in 1.7), quercetin glucuronides and sulphates, with the exception of the 7-sulphate, displayed no activity (Depeint et al., 2002). In addition, flavonoid glycosides were generally found to be ineffective in the present study, and this lack of activity is supported by published work (Depeint et al., 2002; Chen et al., 2003b; Shen et al., 2003), regardless of the nature or position of the sugar substitution. Together these findings suggest that potential apoptotic activity of polyphenols, in colonic cells, may primarily be due to luminal, rather than systemic, exposure, considering that flavonoid glycosides are not readily absorbed in their native form.
Possible implications of protein-binding of polyphenols to their apoptotic activity.

Since polyphenols have a high binding affinity for proteins (Haslam, 1974; Hagerman & Butler, 1981; Luck et al., 1994; Bennick, 2002), it is likely that polyphenol-protein interactions influence polyphenol-induced apoptosis, as observed during the current study, whereby removal of FBS during incubations, thus preventing binding to proteins, enabled the investigation of lower polyphenol concentrations, that are more physiologically relevant to humans (Day et al., 2000; Scalbert & Williamson, 2000; Kroon et al., 2004; Manach et al., 2004). It was considered more pertinent to assess polyphenol apoptotic activity at concentrations similar to those found in vivo, even using a "non-physiological" assay, than to employ extremely high concentrations, which are unattainable naturally by dietary intake. However, this approach may have implications for potential in vivo apoptotic activity, since it is possible that the low concentrations of polyphenols attainable in humans by dietary intake, may be decreased further, following polyphenol-protein interactions, potentially to albumin, which is present in plasma at a normal concentration range of 3.5-5 % (Bourdon et al., 1999; Petibois et al., 2001), thus impeding polyphenol tissue distribution.

Cancerous and non-cancerous cells: differing polyphenolic responses.

Results obtained during the present study, using HCT-8 cancerous and ICE-6 non-cancerous epithelial cells, which have similar morphological and growth characteristics (Tompkins et al., 1974; Quaroni et al., 1979), demonstrated that EGCG, curcumin, theaflavins and the grape seed polymer all induced apoptosis in HCT-8 cells, with little or no effect in ICE-6 cells. This is in concord with several studies, which have reported that polyphenols induce apoptosis in cancer cell lines, but not in their non-cancerous counterparts (Jiang et al., 1996; Ahmad et al., 2000; Huynh & Teel, 2000; Lu et al.,
2000; 2001). One possible mechanism for the differing apoptotic response between cancer and non-cancer cells may involve induction of the potent, p53 independent, cki p57. Hsu et al. (2002) reported that OSC2 squamous cell carcinoma subclones demonstrate a lack of p57 induction, leading to an increase in Apaf-1 expression and caspase-3 activation. In contrast, normal human keratinocytes, containing polyphenol-inducible p57, maintain constant Apaf-1 levels and basal caspase-3 activity, preventing apoptotic induction (Hsu et al., 2002). However, by preventing the biological activity of polyphenols in healthy cells and, potentially, cells in the early stages of carcinogenesis, this may inadvertently affect their ability as chemopreventive agents, implying that polyphenols should be also considered as chemotherapeutic agents.

Polyphenolic compounds have afforded protection against a variety of carcinogens, during the initiation and promotion stages of carcinogenesis, implying that polyphenol-induced chemoprevention may involve multiple mechanisms (as described in sections 1.2 and 1.3). Although some polyphenol aglycones are potent inducers of apoptosis in vitro, as demonstrated in the current study and concurred by previous studies (Chen et al., 2003b; Shen et al., 2003), polyphenol glycosides such as the glucuronides, which are predominantly present in the human circulation (O’Leary et al., 2001; Spencer, 2003), were not active as apoptotic inducers. However, animal studies which explore the potential of polyphenols to act as chemopreventive agents via apoptosis, have reported that significant increases in the apoptotic index of chemically-induced tumours was observed following polyphenolic treatment (Tanaka et al., 2000; Cademi et al., 2000; Li et al., 2002). Therefore, it is apparent that polyphenols are capable of inducing apoptosis in chemically-induced cancers, but the mechanisms through which this is achieved remains unclear. It may, therefore, be possible that deconjugation, for
example, of glycosides or glucuronides, reported to occur intracellularly, by CBG or β-glucuronidases, respectively, (O’Leary et al., 2001; 2003; Berrin et al., 2002; 2003; Spencer, 2003), may potentially lead to the intracellular release of the aglycone and thus induce apoptosis via an intrinsic pathway.

It should be pointed out that the studies herein were exclusively undertaken in cell culture, and, since it is unclear whether some of the phenomena observed in cell culture, also manifest themselves in vivo, the subsequent observations should be considered when extrapolating these results to in vivo situations. For instance, in culture, cells usually grow in monolayers, thus only exposing one side of the cell to the compound in question. Consequently, cellular uptake and accumulation may be underestimated (Hou et al., 2004). Moreover, the oxygen partial pressure in cell culture system is reported to be considerably higher (160 mmHg) than that found in plasma or tissues (<40 mmHg). Although flavonoids are reported to readily undergo oxidation in cell culture, implying that pro-oxidant properties may be responsible for promoting apoptosis, this may not reflect the situation in vivo, where, at low concentrations of polyphenols, oxidation is potentially limited owing to strict oxygen regulation, by low oxygen tension and efficient sequestration of transition metal ions, such as Fe$^{3+}$ and Cu$^{2+}$, which can participate in ROS production (Khokhar & Apenten, 2003; Hou et al., 2004; Spencer et al., 2004). Even though cell culture studies pose limitations, it should be borne in mind that such studies provide an important platform with which to develop relevant in vivo studies.
6.1.2 Polyphenol-induced apoptosis: potential mechanisms.

A series of caspase assays were employed to elucidate potential mechanism(s) of apoptotic induction for the “active” polyphenols. It appears that polyphenols induce apoptosis via activation of an intrinsic caspase cascade. However, this pathway remains to be clarified, since Bid cleavage and mitochondrial cytochrome c release were not specifically investigated in the present study. Moreover, several published studies concur, reporting that EGCG, curcumin and grape seed extract induced apoptosis via an intrinsic pathway, as exemplified by caspase-9 activation, cytochrome c release and suppression of Bcl-2 protein expression (Pal et al., 2001; Agarwal et al., 2002; Chen et al., 2003a; Yan et al., 2004). In addition, preliminary studies to assess Fas expression in HCT-8 cells suggested that low levels of Fas are expressed at the cell surface. Since Fas is an extrinsic trigger of apoptosis, its absence concurs with polyphenol-induced apoptosis via an intrinsic caspase pathway. Indeed, Fas expression is appreciably diminished, or completely lost, in a substantial fraction of colorectal carcinomas, but Fas expression has been reported to be increased, after pre-incubation with IFN-γ and TNF-α (Koshiji et al., 1998; Abreu-Martin et al., 1999; Naujokat et al., 1999; Ogawa et al., 2004). Furthermore, in recent studies, low concentrations of flavonoids have been shown to enhance the extrinsic apoptotic signal of TNF-α (Monasterio et al., 2004).

Induction of apoptosis by polymeric proanthocyanidins appears to be triggered via an intrinsic apoptotic pathway, involving caspase-9 activation, as demonstrated in the current study with the grape seed polymer, and in the work of Agarwal et al. (2002) using a grape seed extract. Nevertheless, this fails to explain how the grape seed polymer, with a considerably large molecular weight in the region of 2780, gained access to intracellular cytosolic compartment and, as observed in the current study,
induced caspase-9 activity. It may be inferred that polymeric compounds, such as the grape seed polymer, might potentially induce apoptosis via a novel pathway, involving some type of endocytosis (chapter 4). However, such a novel pathway has not yet been identified, and the mechanism of polymer-induced apoptosis remains unclear. Although studies investigating proanthocyanidin metabolism, in the presence of colonic microflora, have been reported to degrade polymers into smaller constituents, before undergoing cellular uptake (Deprez et al., 2000; Scalbert et al., 2000; Gonthier et al., 2003), the grape seed polymer is more likely to induce apoptosis by an extrinsic pathway, at least in vitro, due to the absence of human colonic microflora in cell culture.

It would, therefore, be pertinent to employ Fas and TNF-α binding studies (using a cell line with high Fas/TNF-α expression) to investigate whether the grape seed polymer can induce apoptosis via an extrinsic trigger.

Observations made during the current study imply that induction of apoptosis by polyphenols, via an intrinsic pathway, necessitates the presence of a transport system to ensure cellular uptake. Although aglycones, such as EGCG and genistein, are reported to traverse cell membranes by a passive diffusion process, albeit slowly, on the whole, polymeric compounds, with a degree of polymerisation greater than 7, are unable to do so (Scalbert & Williamson, 2000; Deprez et al., 2001; Hong et al., 2002; Liu & Hu, 2002). Alternatively, monomeric polyphenolic compounds that are unable to traverse the cell membrane passively, including glycones, may potentially access the cytosolic compartment actively, via a membrane transporter, such as SGLT1, or the more recently identified, monocarboxylate transporter, which has been implicated in ECG transport (Gee et al., 1998; Liu & Hu, 2002; Vaidyanathan & Walle, 2003). Moreover, studies indicate that (-)-epicatechin can significantly enhance the cellular uptake of [3H] EGCG,
thereby increasing its intracellular concentration (Suganuma et al., 1999). However, membrane transporters, such as MRP2 and MRP1, can also efflux polyphenols out of the cell, as reported for EGCG and ECG (Hong et al., 2002; Vaidyanathan & Walle, 2003; Walle, 2004), thus, potentially, reducing intracellular polyphenol concentration. Taken together, the aforesaid factors appear to contribute to the low bioavailability of polyphenols, and may inadvertently affect the potency of a particular compound to induce apoptosis. Then again, perhaps it possible that some polyphenols are unable to access the cell, but instead bind to a cell surface death receptor, and produce an intermediate messenger, which may induce apoptosis intracellularly via an intrinsic pathway, involving cytochrome c release. Ceramide could be one such messenger.

Possible polyphenol interactions: implications for apoptotic potency.

Since polyphenols occur naturally, they may potentially interact to induce apoptosis additively and/or synergistically. Such a response was clearly observed, during the current study, between individual polyphenols and whole green and black teas. For example, whole green tea, at a concentration the equivalent of 10 μM EGCG, induced apoptosis comparable to that of 25 μM EGCG, in HCT-8 cells. Hence, the concentration at which an individual polyphenol induces apoptosis may effectively decrease. This is supported by recent evidence, where the potential additive and/or synergistic interactions between the grape polyphenols, ellagic acid, resveratrol and quercetin, was investigated in human leukaemia cells (Mertens-Talcott & Percival, 2005). Wherein, most notably, the effective EC_{35} (in 35 % of treated cells) concentration of ellagic acid that induced caspase-3 activity fell by 4-fold in the presence of resveratrol (Mertens-Talcott & Percival, 2005). Moreover, it is possible that these interactions may be associated with augmented cellular uptake, as described
by Suganuma et al. (1999), in which co-treatment of (-)-epicatechin, with EGCG, ECG or EGC induced a synergistic increase in apoptosis. Moreover, the apoptotic effect of polyphenols “acting in concert” was observed, in the present study, in which incubation of EGCG or theaflavins with the bile acid, DCA, produced an additive effect upon apoptosis, whereas a synergistic interaction was apparent with curcumin and DCA. It is possible that owing to its natural detergent activity, DCA may perturb the cell membrane, potentially increasing cell permeability (Vyvoda et al., 1977; Gordon et al., 1985; Nielsen & Rassing, 1999), thus facilitating cellular uptake of the monomeric polyphenols into the intracellular compartment, which may have contributed to their apoptotic potency.

**Potential pro-oxidant properties of polyphenols.**

Catalase, exogenously added to cell culture media, has been reported to prevent EGCG-induced apoptosis (Yang et al., 1998; 2000; Nakagawa et al., 2002), an observation compatible with the findings in the present study, suggesting that H$_2$O$_2$ generation may play a role in apoptotic induction. Indeed, in the current study, incubation of HCT-8 cells with EGCG led to the production of H$_2$O$_2$, in accordance with previous studies (Yang et al., 1998). In spite of this, H$_2$O$_2$ generation was more pronounced in the presence of EGCG, Fe$^{3+}$, and H$_2$O$_2$, collectively, suggesting that the pro-oxidant properties of polyphenols may potentially be involved in apoptosis. For example, during the present study, H$_2$O$_2$ and Fe$^{3+}$ incubated in combination with EGCG, exhibited a more marked effect on EGCG-induced apoptosis, than when incubated individually, and this may involve a Fenton-type reaction. Moreover, polyphenols such as EGCG and curcumin have been reported to promote the reduction of Cu$^{2+}$ or Fe$^{3+}$, potentially producing ROS, capable of instigating site-specific DNA oxidative damage.
that may culminate in apoptosis (Nakagawa et al., 2002; 2004; Yoshino et al., 1999; 2004). Furthermore, a wide range of cell lines, including HCT-8 cells, appear remarkably tolerant to H$_2$O$_2$ (Hiraoka et al., 1997; Lee et al., 2000; Deshpande et al., 2002). In light of their H$_2$O$_2$ tolerance, HCT-8 cells are unlikely to be the most “sensitive cell type” to investigate the pro-oxidant mechanisms of polyphenol-induced apoptosis, and, as a result, mechanisms of apoptosis should be investigated in tumour cell lines that are not influenced by H$_2$O$_2$ tolerance.

6.1.3 Polyphenols and the Ah receptor.

Unfortunately, conclusive results regarding the binding of naturally-occurring and synthetic polyphenols to the Ah receptor were not obtained in the present study. This may be due to the fact that, in comparison with the highly hydrophobic PAH and HAH established Ah receptor ligands, the bioavailability of polyphenolics is low (Scalbert & Williamson, 2000; Seidel et al., 2000). Generally, this may be attributed to a combination of slow passive cellular uptake, poor substrate specificity for membrane transporters, intracellular metabolism, sequestration by intracellular proteins and/or efflux by MRP2 (Seidel et al., 2000; Kohri et al., 2001; Vaidyanathan & Walle, 2001; Liu & Hu, 2002; Kroon et al., 2004, Walle, 2004). For example, transport studies revealed that intracellular accumulation of chrysin was seriously limited by efficient conjugative metabolism, and subsequent efflux of the resulting glucuronide and sulphate metabolites (Walle et al., 1999). Moreover, in a study by Hong et al. (2002), comparable results were reported for EGCG, whereby, following passive uptake into the cell, resulting glucuronide and methyl conjugates were effluxed by MRP transporters. Potentially, the above factors may culminate in a decrease in polyphenol concentration at the Ah receptor-binding site, which may explain the lack of response observed in the
current studies using the CALUX assay. However, for the same reasons, the CALUX assay may be a more valid model for studying Ah receptor binding, as it reflects the *in vivo* situation, implying that the chemopreventive activity of polyphenols appears unlikely to occur via a mechanism involving the Ah receptor.

Since no reproducible binding spectra were obtained for polyphenols using the CALUX assay, and in light of the fact that Ah receptor binding leads to expression of CYP450 isoenzymes, such as CYP1A1 and CYP1A2, it would be pertinent to assess expression of these enzymes directly. Such studies would be carried out to confirm whether polyphenols can agonistically bind to the Ah receptor, establish their potential antagonistic influence against TCDD and other ligands, such as the PAH, and determine a definitive binding constant, relative to that of TCDD, with a view to highlight whether future studies, in which the Ah receptor is investigated as a potential mechanism for polyphenol chemopreventive activity, are justifiable. This could be achieved by employing truly "*in vitro*", but less appropriate methodologies, such as Northern and Western blotting, and classical EROD analysis (Williams *et al.*, 2000; 2003; Thapliyal & Maru, 2001).

### 6.2 FUTURE WORK.

The ability of polyphenols to associate with cells, either by membrane interactions or by uptake, may have potential implications for their apoptotic activity, and thus warrants further investigation. Studies to ascertain the ability of [*H*] polyphenols, to associate with a cell, either by binding with the cell membrane, or by cellular uptake, after addition to the culture media, are proposed. It would be of particular interest to investigate monomeric and polymeric polyphenols (theaflavins and the grape seed
polymer, respectively), to ascertain whether the mechanism(s) through which they induce apoptosis involve intrinsic or extrinsic pathways.

Polyphenols are readily metabolised *in vivo*, pre-systemically, either by mammalian intestinal enzymes or by colonic microflora (Rechner *et al.*, 2002; Olthof *et al.*, 2003). For instance, polyphenols, such as (+)-catechin and (-)-epicatechin, undergo extensive microbial metabolism, by colonic microflora to generate metabolites such as phenylvalerolactones (Catterall *et al.*, 2003; Spencer, 2003; Manach *et al.*, 2005). Therefore, considering the former information, studies are proposed to determine the extent to which colonic microflora metabolites of orally administered parent compounds, such as theaflavins and curcumin, can potentially protect against chemically-induced colon carcinogenesis, by a mechanism involving increased apoptosis in tumours. This will be achieved by employing gnotobiotic (germ-free) and conventional rats, some of which will be pre-treated with anti-microbial drugs, to suppress colonic microflora. The apoptotic index will be determined by morphological evaluation of haematoxylin and eosin paraffin embedded sections.

Although polyphenol metabolites, such as glucuronides and sulphates are prevalent in the blood stream (O’Leary *et al.*, 2001), quercetin glucuronides and sulphates were inactive at inducing apoptosis in the *in vitro* system. However, animal studies have reported the presence of apoptosis following oral administration of polyphenols (Tanaka *et al.*, 2000; Cademi *et al.*, 2000; Li *et al.*, 2002). Such research possibly merits further work, and, therefore, it may be pertinent to investigate potential mechanism(s) of polyphenol metabolites, in comparison to parent compounds, using relevant physiological concentrations, which may lead to apoptosis. This could be investigated
by employing a cell line, such as HepG2, which contains endogenous human β-glucuronidase, an intracellularly distributed enzyme, involved in the deconjugation reaction of flavonoid glucuronides (O’Leary et al., 2001; 2003). A cell line devoid of endogenous human β-glucuronidase activity should also be used, for comparison. Such a study would provide information as to whether deconjugation of polyphenol metabolites is potentially involved in induction of apoptosis via an intrinsic pathway.

Since it is possible that exogenous H$_2$O$_2$ may act as a trigger for apoptosis, it would be prudent to decipher the potential mechanism(s) of H$_2$O$_2$-induced apoptosis, as they may well be a factor in the pro-oxidant apoptotic mechanism(s) of polyphenol-induced apoptosis. This study would employ a cell line that is not tolerant to H$_2$O$_2$, and pay particular attention to Bid cleavage and cytochrome c release, to determine whether an extrinsic or intrinsic apoptotic pathway was involved.

6.3 CONCLUSIONS.

Naturally-occurring polyphenols are prevalent in nature, constituting a considerable proportion of plant diets, diets rich in fresh fruits and vegetables have been strongly associated with a reduced risk of cancer incidence, as well as reportedly being beneficial against other disease states, including cardiovascular and neurodegenerative diseases (Weisburger, 2000; Higdon & Frei, 2003; Mandel & Youdim, 2004; Weinreb et al., 2004; Oak et al., 2005). As a result, polyphenols are potential chemopreventive agents that may be responsible for the benefits conferred by plant diets.

The present studies show that some polyphenols are able to induce apoptosis, in vitro, whereby, apoptotic activity appears to be related to structure, and the presence of
particular moieties, such as a 3-galloyl moiety and gallo B-ring, rather than to a specific
group/class of compounds. Moreover, previous animal studies have reported that
polyphenols afford a protective effect against chemically-induced carcinogenesis
through a mechanism, which may involve increased apoptosis in solid tumours
(Kawamori et al., 1999; Tanaka et al., 2000; Caderni et al., 2000; Li et al., 2002).

Therefore, apoptosis should be considered as a potential mechanism through which
dietary polyphenols exert their anticarcinogenic activity. However, it is noteworthy that
polyphenol-protein interactions may have implications for in vivo polyphenol
concentrations, attainable naturally by dietary intake, which may consequently affect
their apoptotic activity. Therefore, it would also be pertinent to promote the
development of polyphenols as dietary supplements, with a view to increasing their in
vivo concentrations, and potentially enhancing their chemopreventive activity, as long
as this does not lead to toxicity.


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