Piperine Causes Cell Cycle Arrest and Apoptosis in Colorectal Cancer Cells Via the Generation of Reactive Oxygen Species

by

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Abstract

Piperine is a black and long pepper plant-derived alkaloid with antimutagenic and tumour growth-inhibitory activities; however, the molecular basis for the anticancer activity of piperine remains unknown. In this study, human colon (HT-29) and rectal (HRT-18) adenocarcinoma cells were used to investigate the mechanisms underlying tumour cell-killing by piperine. Experimental determinations of the dose-dependent effects of piperine on colorectal cancer cell proliferation, cell cycle progression, cell viability, apoptosis induction, reactive oxygen species production, and endoplasmic reticulum stress were performed. Piperine inhibited HT-29 and HRT-18 cell proliferation in Oregon Green 488 staining assays. Flow cytometry of propidium iodide-stained HT-29 and HRT-18 cells showed that piperine caused G_1 phase cell cycle arrest in HT-29 cells, which was confirmed by western blotting. Piperine-treated HRT-18 cells were arrested at both G_1 and S phases of the cell cycle. Piperine also caused reduced HT-29 and HRT-18 cell viability in MTT assays. Annexin-V-Fluos/propidium iodide staining showed dose-dependent apoptosis induction in piperine-treated HT-29 and HRT-18 cells, which was associated with PARP cleavage and survivin downregulation. Piperine-induced apoptosis was also associated with mitochondrial membrane destabilization, as indicated by the detection of cytosolic cytochrome c by western blotting. Pan-caspase inhibition and western blotting of caspase-3 and -9 determined that piperine-mediated apoptosis is caspase-independent. Piperine was determined to upregulate SAPK/JNK and p38 mitogen-activated protein kinases. Expression of several endoplasmic reticulum stress pathway proteins was also upregulated with piperine treatment. Flow cytometry of dihydroethidium-stained HT-29 and HRT-18 cells revealed that piperine did not induce superoxide anion production. Conversely, analysis of 2',7' dichloro-fluorescin diacetate-stained HT-29 and HRT-18 cells showed that piperine exposure induced hydroxyl radical production. Piperine-induced apoptosis was reduced in the presence of exogenous glutathione, indicating an important role for reactive oxygen species. Finally, piperine-induced cytotoxicity was TRPV1 receptor-independent since TRPV1 antagonists did not protect colorectal cancer cells from apoptosis. In conclusion, piperine exerts an anti-proliferative and apoptosis-inducing effect on colorectal cancer cells that may have utility in colorectal cancer treatment.

List of Abbreviations and Symbols Used

Abbreviations

A	Adenine
AKT	v-akt murine thymoma viral oncogene homolog
AIF	Apoptosis-inducing factor
Apaf-1	Apoptotic protease activating factor 1
APC	Adenomatosis polyposis coli gene
ASK1	Apoptosis signal-regulating kinase 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine Triphosphate
B16F-10	Melanoma cells
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma 2
Bcl-X_L	B-cell lymphoma-extra large
BH3	Bcl-2 homology domain 3
Bim	Bcl-2-like protein 11
Bip	Binding immunoglobulin protein
Boc-D-fmk	BOC-Asp(OMe)-FMK Caspase Inhibitor
BRAF	B-RAF proto-oncogene serine/threonine-protein kinase
BSA	Bovine serum albumin
bZIP	Basic leucine zipper domain
C	Cytosine
°C	Degrees Celsius
C57BL/6	Mouse strain
$CaCl_2$	Calcium chloride
CaCo-2	Human colonic adenocarcinoma cell line
CAM	Cell-cell adhesion molecule
Carboxy-DFFDA, SE	Carboxylic acid diacetate, succinimidyl ester
Caspase	Cysteine aspartate-specific protease
CD95 (APO-1/Fas)	Cluster of Differentiation 95
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinases
CFDA SE	carboxyfluorescein diacetate
CHOP	C/EBP Homologous Protein
CIMP	CpG Island Methylator Phenotype
Cip1	Cdk-interacting protein 1
CKI	Cyclin-dependent kinase inhibitor
CO_2	Carbon Dioxide
CpG	Cytosine-phosphate-guanine
Cyt c	Cytochrome c
DCF	Dichlorofluorescein
DCFH	Dichlorofluorescin

DCFH-DA	Dichlorofluorescin diacetate
DHE	Dihydroethidium
DIABLO	Direct IAP binding protein with low PI
DISC	Death-inducing signaling complex
DLA	Dalton's lymphoma ascites
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EAC	Ehrlich ascites carcinoma
EC	Ethyl carbamate
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGCG	(-)-Epigallocatechin-3-gallate
EGTA	Ethylene glycol tetraacetic acid
eIF2α	Eukaryotic translation initiation factor 2A
Endo G	Endonuclease G
ER	Endoplasmic Reticulum
ERAD	ER-associated Protein Degradation
ERK	Extracellular signal-regulated kinases
ERo1-Lα	ERO1-like protein alpha
E2F	Family of transcription factors in higher eukaryotes
FADD	Fas-associated death domain
FGF	Fibroblast Growth Factor
FITC	Fluorescein isothiocyanate
FL1	Fluorescence label 1
FL2	Fluorescence label 2
5-FU	5-Fluorouracil
g	Gravity
G	Guanine
G_0	Quiescent Phase
G_1	Gap Phase 1
G_2	Gap Phase 2
G382D	Same as MUTYH
GADD153/CHOP	CCAAT/enhancer binding protein (C/EBP), epsilon
GRP78	Glucose regulated protein 78
•GS	Thiyl radical
GSH	Glutathione (Reduced)
GSSG	Glutathione (Oxidized)
GSK-3β	Glycogen synthase kinase 3 beta
γ-GT	γ-glutamyl transpeptidase
GTPase	Guanosine triphosphatase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HCT-116	Human colonic adenocarcinoma cell line
HEPES	5 mM N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid

HGF	Hepatocyte Growth Factor
HI-FBS	Heat-inactivated fetal bovine serum
HMEC	Human Mammary Epithelial Cells
HNPCC	Hereditary non-polyposis colon cancer
$HO^{\bullet}$	Hydroxyl radical
hr	Hours
HRP	Horseradish Peroxidase
HRT-18	Human rectal adenocarcinoma cell line
HT-29	Human colonic adenocarcinoma cell line
hTERT	human telomerase reverse transcriptase
HtrA2	Omi/high-temperature requirement protein A2
IAPs	Inhibitor of Apoptosis Proteins
IgG	Immunoglobulin G
INK4	Inhibitor of Cdk4
IRE1α	Inositol-requiring 1 alpha
JNK	c-Jun-N-terminal kinase
Kb	Kilobases
kDa	Kilodalton
kg	kilogram
KIP	Kinase Inhibitor Protein
L929	Immortalized mouse fibroblasts
LD50	Lethal Dose 50
LNCaP	Prostate cancer cell line
M	Medium
M Phase	Mitotic Phase
MAPK	Mitogen-activated protein kinase pathway
MAX	myc-associated factor x
Mcl-1	Induced myeloid leukemia cell differentiation protein Mcl-1
MEBM	Mammary epithelium basal medium
MEK	Mitogen activated protein kinase kinase
mg	milligram
ml	millilitre
MLH1	MutL homolog 1
mM	millimolar
MMS	Methane methylsulfonate
mRNA	Messenger ribonucleic acid
MSH2	MutS homolog 2
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUTYH or MYH	Mut Y Homologue
Myc gene	Myelocytomatosis oncogene
2N	Diploid
4N	Tetraploid
NaCl	Sodium chloride

NAD+	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
N-CAM	Neural cell adhesion molecule
NaH_2PO_4	Sodium dihydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
nm	nanometre
N-terminal	Amino-terminal
$O_2^{\bullet-}$	Superoxide anion
$p15^{INK4B}$	15 kDa Cyclin-dependent kinase inhibitor
$p16^{INK4A}$	16 kDa Cyclin-dependent kinase inhibitor
$p18^{INK4C}$	18 kDa Cyclin-dependent kinase inhibitor
$p19^{INK4D}$	19 kDa Cyclin-dependent kinase inhibitor
$p21^{Cip1/Waf1}$	21 kDa Cyclin-dependent kinase inhibitor
$p27^{Kip1}$	27 kDa Cyclin-dependent kinase inhibitor
p38	38 kDa MAPK
p53 or TP53	53 kDa tumour suppressor protein
$p57^{Kip2}$	57 kDa Cyclin-dependent kinase inhibitor
P450	Cytochrome p450 isoenzymes
PAK4	Serine/threonine-protein kinase PAK4
PAGE	Polyacrylamide Gel Electrophoresis
PAR	Poly (ADP-Ribose)
PARP	Poly (ADP-Ribose) Polymerase
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PERK	Double-strand RNA-activated protein-kinase like ER-kinase
pH	Power of hydrogen (measure of acidity)
PI	Propidium iodide
PI-3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
pRb	Phosphorylated Retinoblastoma Protein
PTEN	Phosphatase and tensin homolog
R Point	Restriction Point
Ras	Ras sarcoma oncoproteins
Raf	RAF proto-oncogene serine/threonine-protein kinase
Rb	Retinoblastoma Protein
rcf	Relative centrifugal force
RIPA	Radioimmunoprecipitation Assay Buffer
RNase	Ribonuclease
ROS	Reactive Oxygen Species
26S	26 Svedberg sedimentation coefficient proteasome subcomponent
S Phase	Synthesis Phase
SAPK	Stress-activated protein kinase
SB366791	N-Arylcinnamides
SDS	Sodium dodecylsulphate

Smac	Second mitochondria-derived activator of caspases
SMAD4	Mothers against decapentaplegic homolog 4
T	Thymine
tBid	truncated BH3 interacting domain death agonist
T-cell	T-lymphocytes
TCF-LEF	T-cell factor-lymphocyte enhancer factor family
TGF-β	Transforming Growth Factor-Beta
TGFBR2	Transforming Growth Factor-Beta Receptor 2
Thr	Threonine
TNF-α	Tumour Necrosis Factor alpha
TRAF2	Tumour Necrosis Factor receptor-associated-Factor 2
TRAIL	TNF-related apoptosis inducing ligand
Tris-HCl	Tris-Hydrochloric acid
Triton-X 100	Octylphenolpoly(ethyleneglycolether)$_x$
TRPV1	Transient receptor potential vanilloid-1
TrypLE	Phenol red negative trypsin replacement
T-TBS	Tween Tris-buffered saline
Tyr	Tyrosine
U	Units
UCN-01	Non-specific protein kinase inhibitor
μg	Microgram
μL	Microlitre
μm	Micron
UPR	Unfolded Protein Response
V	Vehicle control for antagonist (DMSO)
Vc	Vehicle control (DMSO)
VEGF	Vascular Endothelial Growth Factor
v/v	Volume per volume
Waf1	Wild-type p53-associated fragment 1
wnt	Wingless signaling pathway
w/v	Weight per volume
x	Multiples
Y165C	Same as MUTYH

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Chapter 1.0 Introduction

1.1 Cancer

Cancer is a class of diseases arising from normal cells via a series of genetic changes that affect controlled cell growth. Hanahan and Weinberg (2000) have explained that tumourigenesis is a multistep process reflecting genetic changes that transform normal cells into malignant cells. Specifically, these authors outline six essential cell physiological alterations that determine tumour growth. These are independence from growth signals, resistance to growth-inhibitory signals, evasion of programmed cell death, unlimited replicative potential, continued angiogenesis, and invasion and metastasis. Each of these represents evasion of an anticancer defense mechanism. Furthermore, these physiologic alterations serve as targets for many anti-cancer therapeutics.

While tremendous advances have been made in the decade since publication of Hanahan's and Weinberg's (2000) review, the basic principles of tumour biology remain remarkably unchanged. The role of tumour cell interaction with the tumour microenvironment has been increasingly recognized as an integral part of the initiation, growth and progression of cancer (Nannuru & Singh 2010). Furthermore, several of the cancer hallmarks described by Hanahan and Weinberg are provided by stromal components that include endothelial cells, pericytes, fibroblasts, leukocytes, and extra-cellular matrix (Pietras & Ostman 2010).

Mitogenic growth signals are transmitted into the cell via transmembrane receptors that bind different classes of signaling molecules (Nicholson et al 2007). These stimulatory signals are essential to transition from a quiescent state to active proliferation.

Signalling molecules involved include growth factors, cell adhesion molecules, and extracellular matrix components. For example, cancer-associated fibroblasts may provide growth factors, hormones and cytokines that directly stimulate tumour cell proliferation (Gonda et al 2010). Furthermore, malignant progression may be accompanied by stromal expansion, which augments mitogenic growth signals.

Cellular resistance to growth-inhibitory signals may operate through a number of distinct pathways. Proliferating cells may enter a quiescent state (G_0), which may be ultimately reversed under the influence of extracellular signals, such as cytokines (Zimmermann 2002). However, the cells may alternatively acquire differentiation-associated traits and may permanently lose their proliferative ability. Response to anti-proliferative signals is based on the cell cycle and factors that govern transition from G_1 phase. Cancer cells must resist these signals in order to proliferate.

As tumour cell expansion is determined by a balance between cell proliferation and cell death, evasion of apoptosis, or programmed cell death is an essential mechanism that favours cancer development. Apoptosis, first described by Kerr and Wylie (1972), involves a series of organized steps including cell membrane and cytoskeletal disruption, cytosolic extrusion, chromosomal degradation, nuclear fragmentation and chromatin condensation. As a footnote in Kerr and Wylie's seminal paper, the term "apoptosis" is intended to describe the "dropping off" of petals of flowers, or leaves from trees. Evasion of apoptosis is partly derived from stromal cell-derived pro-survival signals. Such survival signals include cancer-associated fibroblast-produced insulin-like growth factor-1 and -2 (LeBedis et al 2002, Strnad et al 2010).

While growth independence from external signals and resistance to apoptosis should be sufficient to enable limitless proliferation, most cells are limited by finite replicative potential, or senescence, as reviewed extensively by Hayflick (1997). Senescent cells remain metabolically active but lack replicative capacity. Cell division is limited to approximately 50 population doublings, often referred to as the Hayflick limit (Grimes & Chandra 2009). These cells also demonstrate morphological changes including enlarged size, flatness, and lack of vacuoles (Itahana et al 2004, Mooi & Peeper 2006, Sandhu et al 1994). They have altered gene expression and transcription factors (Itahana et al 2004). Senescence is distinguished from quiescence, which is a reversible state that enables cells to re-enter the cell cycle. Senescence, in contrast, involves an absence of responsiveness to mitogenic stimuli (Narita 2007). Senescence may be initiated via altered physiological conditions and cellular stress, as seen with the aging process. These cells may contribute to a pro-oncogenic environment by secreting inflammatory cytokines, growth factors, and extracellular matrix components, which disrupt tissue integrity (Campisi 2005). The process of senescence is related to reduction of telomere length with replication (Lendvay et al 1996). Specifically, telomeres lose approximately 100 base pairs with each population doubling. Once telomeres reach a critical length of less than 5 kilobases, p53 and retinoblastoma (Rb) protein pathways become activated, triggering senescence (Gorbunova et al 2002). The enzyme telomerase maintains telomere length, especially in germ cells and stem cells. Immortalization requires active telomerase, which is accompanied by simultaneous loss of p16 and Rb, which regulate the cell cycle (Rheinwald et al 2002). Telomere loss or dysfunction may result in genomic instability and DNA damage. Mitochondrial dysfunction may lead to

premature senescence related to production of high levels of reactive oxygen species, resulting in oxidative damage to cellular components (Passos et al 2007). Furthermore, reduction in proteasome activity, involved in degradation of oxidized proteins results in accumulation of oxidative proteins, now unable to undergo efficient degradation (Grimes & Chandra 2009).

Most cells require vasculature for survival. Specifically, residing within 100 μm of a capillary blood vessel is necessary to obtain proper supply of oxygen and nutrients (Bouck et al 1996). Angiogenesis, the carefully regulated and transitory process of new blood vessel formation, enables organogenesis. Similarly, in order to progress beyond a critical size, tumours must develop angiogenic capacity (Hanahan & Folkman 1996). Pro- and anti-angiogenic signals determine angiogenic ability. Signals favouring angiogenesis include vascular endothelial growth factor (VEGF) and acidic and basic fibroblast growth factor (FGF 1/2). Anti-angiogenic factors include thrombospondin-1 (Hanahan & Weinberg 2000). Cancer-associated fibroblasts play a role in provision of pro-angiogenic factors including VEGF and FGF (Pietras & Ostman 2010). The therapeutic potential of angiogenic signals has been exploited with anti-VEGF therapy, such as the agent Bevacizumab, which has associated overall and/or progression-free survival in colorectal, breast, and lung cancer, as well as in glioblastoma multiforme (Grothey & Galanis 2009).

Lastly, tissue invasion and metastasis are responsible for approximately 90% of cancer deaths (Sporn 1996). Primary tumour masses generate cells that found new colonies. This invasive and metastatic capacity is a complex process, which relies on the five previously discussed hallmarks of cancer. A number of protein classes involved in

cell adhesion are altered in cells with these capabilities (Aplin et al 1998). Cell-cell adhesion molecules (CAM), such as E-cadherin, N (Neural)-CAM, and integrin are involved in this process. Extracellular proteases are also involved, in order to invade nearby stroma, blood vessel walls, and through epithelial cell layers, via matrix degradation (Hanahan & Weinberg 2000). Cancer-associated fibroblasts further this invasiveness and metastatic potential by induction of epithelial-to-mesenchymal transition of tumour cells by Transforming Growth Factor (TGF)-β and Hepatocyte Growth Factor (HGF) secretion. These fibroblasts are also a source of protease activity via matrix metalloproteinases, cathepsins and plasminogen activators (Pietras & Ostman 2010). Epithelial cells exposed to stroma may undergo permanent pro-invasive changes (Sung et al 2008). Pericytes, a form of connective tissue cell, may also provide a physical barrier to metastatic spread (Pietras & Ostman 2010).

1.2 Cell Cycle

Understanding the cell cycle and its regulation at the molecular level is essential to understanding the fundamental differences that exist between normal and transformed cells. The cell cycle is a tightly coordinated series of events associated with DNA synthesis, mitosis, and gap phases between. Most normal cells are in G_0, or a quiescent phase. Following a sustained mitogenic stimulus, cells in G_0 or G_1 progress to a restriction (R) point. Beyond this point, cells are committed to enter S, or Synthesis phase (Pardee 1989). This restriction point also defines growth factor independence. Among the mechanisms cells use to respond to DNA damage is halting cell cycle progression (Kastan & Bartek 2004). Cancer cells have deregulated control mechanisms,

and therefore, have variable autonomy from extracellular signals promoting or inhibiting growth (Sherr 2000). Thus, cancer cells more readily transition through the R point. Key cell cycle complexes regulate progression through the cell cycle via their synthesis, assembly, activation, inactivation, dissociation and degradation. Furthermore, there is transcriptional, translational, and ubiquitin-mediated proteolysis for post-translational control. Subcellular protein localization provides additional regulation (Sherr et al 1994).

Cyclin-dependent kinases (Cdk) are a family of serine-threonine protein kinases catalyzing different cell cycle transitions. Cdk molecules bind to activator molecules, known as cyclins, which activate the Cdk. Cdk regulation occurs via phosphorylation events at a conserved threonine residue, and through binding of Cdk inhibitors. Cell division cycle (Cdc) phosphatases further remove inhibitory phosphates from threonine and tyrosine sites on Cdks, which activates Cdks, in a rate-limiting step necessary for S-phase entry (Sherr 2000).

Cyclins A to H have a shared conserved sequence of approximately 100 amino acids, known as the cyclin box. Cdks bind to different cyclins, followed by activation of cyclin-Cdk complexes, which phosphorylate target proteins to facilitate progression through specific cell cycle phases. Cyclin levels change during cell cycle progression, due to ubiquitin-mediated proteasomal degradation, thus coordinating with Cdk activation (Dirks & Rutka 1997).

G_1-to-S phase progression is regulated by Cyclins D, E, and A, along with their respective kinases. A further substrate is the Rb protein, which is hypophosphorylated early in G_1 and bound to E2F. Cyclin D- and E-dependent kinase activation during G_1-to-S progression results in pRb accumulation, allowing dissociation of E2F and activation

of gene transcription for S phase entry, including cyclin E and A, and DNA synthesis enzymes. Late G_1 phase Cyclin A-Cdk2 activation following Cyclin E-Cdk2 activation is a pre-requisite for S phase initiation and progression, as well as onset of mitosis. This is required for centrosomal duplication, allowing mitotic chromosomal segregation (Meraldi et al 1999).

Regulation of entry into and exit from mitosis occurs via cyclin –Cdk1 association. Cyclin B1, which normally shuttles between the nucleus and cytoplasm, becomes phosphorylated at the G_2/M phase transition, which prevents its nuclear export. Similarly, ubiquitin-mediated proteasomal degradation in late G_2/M phase promotes mitotic exit (Yoneda 2000).

Cyclin-dependent kinase inhibitors (CKI) are proteins that negatively regulate Cdks, resulting in G_1 phase arrest. The two families are the Kinase Inhibitory Protein (KIP) family and the Inhibitor of Cdk4 (INK4) family (Sherr & Roberts 1999). KIP family members include $p21^{Cip1/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$, which are homologous at their N-terminal Cdk inhibitory domain, thus they may inhibit all cyclin-Cdk complexes (Slingerland & Pagano 2000). p21 protein binds to Cdk2 and its gene expression is transcriptionally upregulated by p53 in response to DNA damage or cellular stress. p27 accumulates in Cyclin E-Cdk2 complexes and induces and/or maintains a G_1 arrest in the presence of anti-proliferative signals (Coats et al 1996). p27 expression is translationally-regulated and subject to ubiquitin-mediated proteolysis. p57 is associated with several cancers including Wilms' tumour, in which there is loss of heterozygosity of the p57 gene (Sherr 2000).

KIP family members also play a role in assembly, activation, and nuclear localization of the D-type cyclin-Cdk complexes. This is essential to accumulation of Cyclin D1 in the nucleus (Cheng et al 1999).

A separate family, known as the INK4 family of Cdk inhibitors, inhibit Cdk 4 and 6 in G_1 phase. INK4 family members are structurally related and include $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$. These Cdk inhibitors destabilize D-type cyclin associations with Cdk 4 and 6, resulting in an INK4-bound Cdk lacking an active cyclin (Sherr & Roberts 1999).

Cell proliferation is regulated by the *myc* gene, along with Max, which dimerizes with Myc protein. *Myc* expression depends on mitogenic signals, while suppression occurs through growth inhibitory or differentiation signals. When *myc* is constitutively expressed, a cell may enter the cell cycle in the absence of growth factors, via transcriptional targets such as Cyclins D1 and D2, Cdk 4, E2F-2, Cdc25A and B, p15, p27, and hTERT (Luscher 2001) .

Similarly, Ras activity is required for cell cycle progression. Ras activity plays a role in all cell cycle transitions. Ras plays a role in phosphorylation of retinoblastoma protein; however, sustained Ras activation in normal cells may trigger senescence (Kerkhoff & Rapp 1998). Ras activation is stimulated by the presence of mitogens, which facilitate entry of quiescent cells from G_0 to S phase. Ras then triggers the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway, in addition to the Ras-phosphoinositol-3-kinase (PI-3K)-protein kinase B (PKB) pathway, both of which regulate the cell cycle (Muise-Helmericks et al 1998). These pathways result in Cyclin

D1 gene transcription and assembly and activation of cyclin D1-Cdk4 complexes (Cheng et al 1998).

There are several mechanisms of DNA damage that affect the cell cycle. Free oxygen radicals break phosphodiester bonds in the DNA helix. If two breaks occur on opposite strands in close proximity, a double strand break occurs. Alkylating agents modify purine bases and result in adduct formation, the nature of which determines the repair process employed (Kastan & Bartek 2004).

As transformed cells have a deregulated cell cycle, there are significant implications. Differentiation, cellular senescence, and sensitivity to growth inhibitory stimuli are lost. This may result from overexpression of positive regulators such as cyclins and Cdc25 phosphatases, or due to deletion, mutation, or reduction in the levels of Cdk inhibitors (Broggini et al 2000). In addition, transformed cells bypass the restriction point, resulting in accumulation of genetic alterations, thus favouring overgrowth of cells with proliferative advantages. Cyclin overexpression may shorten the G_1-to-S phase interval and reduce responsiveness to growth inhibitory stimuli (Keyomarsi et al 2002). In contrast, negative regulators of cell cycle are frequent inactivation targets in cancer (Viglietto et al 2002). p53 mutation disrupts the p53/p21 response to DNA damage in coordination with cell cycle arrest. This results in accumulation of genetically altered cells. Drug development targeting the cell cycle includes Cdk inhibitors, such as flavopiridol (Cdk 1,2,and 4) and UCN-01 (protein kinase C and Cdk activity) (Kummar et al 2010). Cdc25 inhibitors have also been designed. Other targets include proteasome inhibition to promote cell cycle arrest and apoptosis, and inhibitors of MAPK and PI-3-K signaling (Kummar et al 2010 , Rizzolio et al 2009).

1.3 Colorectal Cancer

In 2009, there were an estimated 171,000 new cases of non-melanoma skin cancer, with an estimated 75,300 cancer-related deaths. According to current incidence rates, 45% of Canadian men and 40% women are expected to develop cancer (Canadian Cancer Society 2009). Among new cases, colorectal cancer is currently the third most common and the second leading cause of cancer mortality in Canada. Colorectal cancer incidence increased from 1980-1985 and decreased until the mid-1990's, followed by an increase through 2000. Colorectal cancer incidence has been decreasing over the past decade and there has been a corresponding reduction in mortality now estimated at 1.3% and 1.7% per year in males and females, respectively. This likely reflects both therapeutic advances such as chemotherapy, and improved screening (Canadian Cancer Society 2009). Furthermore, cancers of the lung, colon, prostate and breast are significantly less prevalent in Eastern countries, while cancers of the head and neck, and cervix are more common in India (Aggarwal & Shishodia 2006). Gastric cancer is much more common in Japan. Similarly, migration from a lower incidence nation to one of increased incidence elevates individual risk to that of the adopted country (Kolonel et al 2004).

Colorectal cancer begins as a benign adenomatous polyp, which progresses to advanced adenoma with high-grade dysplasia and subsequently, invasive cancer according to the adenoma-carcinoma sequence (Vogelstein et al 1988). Colorectal cancer progresses from confinement to the bowel wall (tumour-node-metastasis stages I and II) to regional lymph node spread (stage III), to distant metastasis (stage IV). Both stage I and II are cured by surgery, whereas stage III is curable by combination surgery and

adjuvant chemotherapy in 73% of cases. While incurable, treatment of stage IV disease with newer chemotherapeutic agents has resulted in improved survival (Markowitz & Bertagnolli 2009).

Understanding the molecular basis of colorectal cancer remains a formidable challenge. However, significant advancements have been made in determining factors that initiate tumour development, further progression, and determine therapeutic response or resistance. These factors include genomic instability, tumour-suppressor gene mutational inactivation, and oncogene activation (Grady & Markowitz 2002, Lengauer et al 1997).

Genomic instability facilitates the acquisition of numerous tumour-associated mutations. This promotes colorectal cancer development. The most common form is chromosomal instability, which involves changes in chromosome copy number and structure. This provides a mechanism for loss of a wild-type copy of tumour suppressor genes including APC, p53, and SMAD4 (Markowitz & Bertagnolli 2009). Normally, these genes counter malignant phenotypes. Colorectal cancer does not typically involve gene copy number amplification or gene rearrangement (Leary et al 2008).

Defective DNA repair represents another promoter of genomic instability in colorectal cancer. Genes necessary for repair of DNA base pair mismatches are collectively referred to as mismatch-repair genes (Kolodner et al 1999). Inactivation of these genes can be transmissible as hereditary nonpolyposis colon cancer (HNPCC), or Lynch Syndrome. Methylation-associated gene silencing represents an acquired form of gene inactivation. HNPCC germ-line mutations include MLH1 and MSH2 and carry an 80% lifetime risk of colorectal cancer, at an average age of 45 years. There is also

somatic inactivation of the wild-type parental allele (Kastrinos & Syngal 2007). The deficient DNA-mismatch repair gene therefore results in accelerated cancer development. Yearly colonoscopic surveillance is recommended as cancers may arise within 36 months of a normal colonoscopy (Jarvinen et al 2000). Similarly, development of high-grade lesions is an indication for prophylactic colectomy. Despite the high prevalence of DNA-mismatch repair gene inactivation in HNPCC, only 15% of patients with non-familial colorectal cancer have this mechanism, which is due to inactivation by gene promoter methylation (Herman et al 1998). Furthermore, DNA-mismatch repair gene inactivation is associated with microsatellite instability, in which there is inability to repair strand slippage in repetitive DNA sequence elements (Thibodeau et al 1993). This results in alteration of the size of mono- or di-nucleotide repeats, known as microsatellites. Loss of one of the mismatch repair proteins can be detected using immunohistochemistry (Hampel et al 2005). Clinically, mismatch-repair deficiency is associated with proximal colonic cancers, while sporadic cases are typified by older age and female sex (Lynch et al 2008). A different mechanism of colorectal carcinogenesis involves germ-line inactivation of a base excision repair gene, known as mutY homologue (MUTYH or MYH) (Jones et al 2002). This protein normally excises the 8-oxoguanine product of oxidative damage to guanine. Clinically, this manifests as a polyposis phenotype associated with near-certain development of colorectal cancer by 60 years of age. Diagnosis requires testing for the Y165C and G382D genes, accounting for 85% of cases. There is no known somatically-inactivated equivalent (Kastrinos & Syngal 2007).

The last major form of genomic instability in colorectal cancer is due to aberrant DNA methylation. Carbon 5 methylation of cytosine due to DNA methylases that

modify cytosines within CpG dinucleotides, creates a 5th DNA base (Toyota et al 1999). Normally, methylation of cytosines is limited to regions of DNA sequence repetition, outside of exons. It is mostly excluded from the CpG-rich islands in promoter regions of genes. In colorectal cancer, there is depletion of general cytosine methylation, but aberrant methylation within promoter associated CpG islands, which may induce silencing of gene expression (Nosho et al 2008, Weisenberger et al 2006). This epigenetic silencing accounts for blockage of MLH1 expression in sporadic colorectal cancer (Issa 2004). There is an associated phenotype known as the CpG island methylator phenotype (CIMP), in which there is a subgroup of colorectal cancers that are aberrantly methylated. This phenomenon is present in approximately 15% of colorectal cancers, especially MLH1 variants (Nosho et al 2008).

Tumour-suppressor gene mutational inactivation is another factor in the molecular basis of colorectal cancer. One such change is activation of the wnt signaling pathway, regarded as the initiating event in colorectal cancer (Markowitz & Bertagnolli 2009). This pathway proceeds as the oncoprotein β-catenin localizes to the nucleus and binds to T-cell factor-lymphocyte enhancer factor family (TCF-LEF) transcriptionally regulating cellular activation. β-catenin levels are controlled by proteolysis through the β-catenin degradation complex (Goss & Groden 2000). One component of this complex is APC, which both degrades β-catenin and inhibits its nuclear localization. APC gene inactivation represents the most common colorectal cancer mutation and results in inappropriate and constitutive activation of wnt signaling due to loss of β-catenin negative regulation (Morin et al 1997). Germline APC mutations result in familial adenomatous polyposis, clinically manifested by development of over 100 adenomatous

colonic polyps. APC gene mutational carriers have near complete penetrance with development of colorectal cancer by age 40 years (Lynch et al 2008). Furthermore, most sporadic colorectal adenomas and cancers have biallelic inactivation of APC via somatic mutation and deletion. A subgroup of tumours exists, which has wild-type APC in the presence of β-catenin mutations rendering resistance to the β-catenin degradation complex (Korinek et al 1997).

The second key step in colorectal carcinogenesis is p53 pathway inactivation via mutation of TP53. This is biallelic in most tumours, via combination of missense mutational inactivation of p53 transcriptional activity and 17p chromosomal deletion, eliminating the second TP53 allele (Grady & Markowitz 2002). Wild-type p53 functions in cell-cycle arrest and as a cell-death checkpoint, following activation in the presence of cellular stress (Vazquez et al 2008). TP53 inactivation typically occurs in concert with transition from large adenomas to invasive carcinoma. In colorectal cancers with defective mismatch-repair, wild-type TP53 often remains, though mutations in the BAX inducer of apoptosis likely reduce p53 activity (Markowitz & Bertagnolli 2009).

TGF-β mutational inactivation is another step in colorectal carcinogenesis. Somatic mutations inactivate TGFBR2 in one third of these cancers. TGF-β regulates cell proliferation and differentiation, embryonic development, angiogenesis and wound healing (Massagué 1998). Several disease states are related to altered TGF-β production including atherosclerosis and cancer (Blobe et al 2000). In the context of cancer, TGF-β signaling is important in promoting cancer cell proliferation, suppressing growth inhibition, regulating apoptosis, inducing angiogenesis, and promoting tumour metastasis (Ikushima & Miyazono 2010). Mutations inactivating the TGF-β signaling pathway

correspond to transition from adenoma to high-grade dysplasia or carcinoma (Grady et al 1998).

Another factor in the molecular basis of colorectal cancer is oncogenic pathway activation. Oncogenes promote cancer development and oncogenic mutations in colorectal cancer include RAS (37%) and BRAF (13%), activators of the mitogen-activated protein kinase (MAPK) signaling pathway (Downward 1998). RAS mutations activate GTPase activity, signaling directly to RAF (Davies et al 2002). BRAF mutations, which are detectable in small polyps, hyperplastic polyps, serrated adenomas and proximal cancers signal BRAF serine-threonine kinase activity, driving the MAPK signaling cascade (Markowitz & Bertagnolli 2009).

Phosphatidylinositol-3-kinase (PI3K) somatic mutations are found in one third of colorectal cancers (Yin & Shen 2008). Loss of PTEN, an inhibitor of PI3K, activation of insulin receptor substrate 2 (IRS2), which functions as an upstream activator of PI3K, and coamplification of AKT and PAK4, which are downstream mediators of PI3K may substitute for PI3K mutations and provide similar effects (Markowitz & Bertagnolli 2009).

1.3.1 Colorectal Cancer Epidemiology

Genetic influences on disease susceptibility of populations are a further area of study. Heredity plays a major influence on disease states, including a 50% disease concordance in monozygotic twins and 10-15% dizygotic concordance in schizophrenia (Cardno & Gottesman 2000). The implications for the remaining discordance are accounted for by the influences of environment on phenotype, in addition to epigenetic

phenomena. Furthermore, the environmental influence on phenotypic variation is difficult to measure (Wong et al 2005). Epidemiological and twin studies have determined that 35-100% of colorectal cancers and adenomas develop in individuals with an inherited susceptibility (Lichtenstein et al 2000). Despite absence of evidence of mismatch-repair defects, some families appear to have an HNPCC-like syndrome. Linkage studies have found genomic loci harbouring genetic susceptibility with high penetrance (Wiesner et al 2003). Mutations underlying this susceptibility are unknown. Similarly, germline DNA variants with strong susceptibility to colorectal cancer development have been identified through association studies (Zanke et al 2007).

1.4 Phytochemicals and Cancer Therapy

Dietary influences on cancer development were published as early as the 1920's in the context of animal experiments documenting malignant epithelial transformation following vitamin A deprivation (Wolbach & Howe 1925). This concept was further advanced in 1953 with the proposal of the "field effect" to account for the presence of multiple cancers in damaged tissue, such as the oral cavity through tobacco exposure (Lippman & Hawk 2009, Slaughter et al 1953). Therefore, new cancers may develop in tissues from which previous tumours have been successfully excised, due to tissue damage, as opposed to recurrence of the original tumour. Nutrient chemoprevention trials have identified β-carotene as a promoter of cancer in smokers at risk for lung cancer (Omenn et al 1996). This same compound, in combination with selenium and α-tocopherol has conversely been shown to reduce the total and disease-specific mortality rate in high-risk Chinese patients with gastric cancer. Furthermore, in one trial, patients

treated with selenium-rich yeast for skin cancer prevention had decreased incidence of prostate cancer. Lippman and colleagues were not able to replicate this finding (Lippman et al 2009).

While cancer is generally believed to be etiologically-related to dietary factors, genetic predisposition, or environmental exposure, 35% of cancers are attributed to dietary factors. Colon cancer is believed to be disproportionally related to diet, accounting for approximately 80% of cases (Reddy et al 2003). Specifically, fruits and vegetables contain several compounds that affect cancer development, such as dietary fibre, vitamins (including selenium, folate, vitamins C and E, and carotenoids), and biologically-active phytochemicals (including flavonoids, indoles, and isothiocyanates). In a study by Mathew et al (2004), a positive association between red meat and fat intake and an inverse association between fruits, vegetables and dietary fibre with development of colorectal adenomas was identified (Mathew et al 2004).

Modulation of gene expression is likely involved, but the chemopreventive mechanisms of many of these factors remain largely unknown (de Kok et al 2009). The adverse effects and complications of conventional chemotherapeutic agents are especially problematic in certain populations such as elderly and immuno-compromised patients, and refractory or chemotherapy-resistant disease remains a clinical challenge. This underscores the need for less toxic and better tolerated therapeutic agents (Nakazato et al 2005).

A number of dietary phytochemicals have been shown to be protective against cancer. These include curcumin (turmeric), genistein (soybean), resveratrol (grapes), capsaicin (red chili), and flavonoids (tea plant). Proposed mechanisms include

suppression of the inflammatory processes resulting in transformation, overproliferation, and initiation of carcinogenesis, as well as inhibition of angiogenesis and metastasis (Dorai & Aggarwal 2004). These phytochemicals modulate a number of transcription factors, pro- and anti-apoptotic proteins, protein kinases, cell cycle proteins, cell adhesion molecules, and growth factor signaling pathways (Aggarwal & Shishodia 2006). Various dietary phytochemicals have been shown to prevent carcinogen uptake and inhibit enzymatic carcinogen activation via modulation of phase I and II detoxification or bioactivation pathways (Jacobs 1988, Lampe & Peterson 2002, Nijveldt et al 2001, Smith & Yang 2000, van Poppel et al 1999). Furthermore, dietary phytochemicals may scavenge free radicals and reactive oxygen species (Nijveldt et al 2001). Other phytochemicals inhibit DNA topoisomerase II, inhibit cell proliferation via the cell cycle or induce apoptosis (Bonnesen et al 2001, Cover et al 1998, Gamet-Payrastre et al 2000, Wenzel et al 2000).

1.5 Piperine

While certain phytochemicals modulate their effects via well-understood mechanisms, the anti-carcinogenic effects of other phytochemicals, such as piperine remain poorly understood. Piperine, an amide alkaloid extract of the fruits of black and long pepper plants (*Piper nigrum Linn and Piper longum Linn*), is an important medicinal plant commonly used in Asian, Pacific Island, and Indian traditional medicine (Sunila & Kuttan 2004). Piperine represents approximately 1.7-7.4% of black pepper (Kanaki et al 2008). Piperine has therapeutic application in a number of conditions including tuberculosis, sleeping problems, respiratory tract infections, menstrual pain, gonorrhea,

chronic gut-related pain, arthritis, and anxiety. Piperine has analgesic, diuretic and muscle relaxant properties and has been reported to have central nervous system depressant, antipyretic and anti-inflammatory effects (Stohr et al 2001). Piperine is known to inhibit first-pass metabolism by the liver via inhibition of the mixed function oxygenase system and P450 isoenzymes non-specifically. Lastly, piperine inhibits prostaglandin and leukotriene biosynthesis (Sunila & Kuttan 2004). The anti-tumour effects of piperine have been poorly studied.

Srinivasan reviewed the diverse physiological effects of piperine in 2007. Spices in general enhance gastric and salivary secretions in addition to providing flavour to food. Piperine has been reported to increase bile acid secretion up to 30% over control population values (Ganesh Bhat & Chandrasekhara 1987). Dietary intake of piperine increased both pancreatic lipase and amylase activity up to 30 and 87% of control values, respectively. Piperine also stimulates trypsin activity up to 150% (Platel & Srinivasan 1996, Platel & Srinivasan 2000).

1.5.1 Other Physiological Effects of Piperine

Piperine has found a therapeutic role due to its effects on drug metabolism and has been extensively studied in its role of enhancing drug bioavailability. A number of studies confirm this role attributable to nonspecific inhibition of different cytochrome p450 isoforms (Dalvi & Dalvi 1991). Similarly, piperine is commercially marketed based on its bioavailability-enhancing properties. While the precise basis for this property of piperine is poorly understood, it is believed to be due to increased gastrointestinal absorption and/or reduced first-pass metabolism by the liver (Atal et al

1981). Piperine has been shown to increase the bioavailability of (-)-Epigallocatechin-3-gallate (EGCG) from green tea by inhibition of glucuronidation and gastrointestinal transit (Lambert et al 2004). Piperine has also been shown to enhance bioavailability of propranolol and theophylline administered orally (Bano et al 1991). Piperine has been reported to alter the pharmacokinetics of a number of compounds including phenytoin (Hiwale et al 2002, Velpandian et al 2001). These studies concluded that piperine's bioavaility-enhancing properties were due to effects on microsomal metabolizing enzymes. In contrast, piperine's enhancement of plasma levels of coenzyme Q_{10} were determined to be non-specific (Badmaev et al 2000). Piperine was found to increase the bioavailability of curcumin in healthy human subjects by 2000% via oral administration (Shoba et al 1998).

Piperine has several effects on the gastrointestinal system. Piperine has been shown to cause gastric cell exfoliation and mucosal microhemorrhage. Piperine causes increased parietal cell and pepsin secretion in additional to potassium loss (Myers et al 1987). Conversely, other investigators showed an inhibitory effect of piperine on volume of gastric juice, gastric acidity, and pepsin activity (Bai & Xu 2000). Piperine has anti-diarrheal properties, which were experimentally challenged with castor oil, magnesium sulphate and arachidonic acid. The mechanism may involve capsaicin-sensitive neurons (Bajad et al 2001a, Capasso et al 2002).

In vitro experiments have identified a pro-absorptive function to piperine. Piperine appears to stimulate γ-glutamyl transpeptidase activity (γ-GT), enhance radiolabelled amino acid uptake, and increase lipid peroxidation in rat jejunal epithelial cells (Johri et al 1992). Therefore, the enhanced absorption due to piperine may be due to

altered lipid environment. Alternatively, piperine has a demonstrated association with increased microvilli length, in addition to a prominent increase in free ribosomes and ER-associated ribosomes in enterocytes. Thus, this effect may be secondary to altered cytoskeletal components or membrane proteins (Khajuria et al 2002).

Piperine has demonstrated effects on gastrointestinal motility, resulting in increased orocecal transit time in human subjects (Vazquez-Olivencia et al 1992). Furthermore, piperine has been shown to inhibit gastric emptying (Bajad et al 2001b). In contrast, other investigators have shown a significantly reduced intestinal transit time associated with piperine administration, likely due to the enhanced release of digestive enzymes and bile secretion seen with piperine (Srinivasan 2007).

Piperine, which remains one of the dominant spices in common use, has a well-known safety profile. LD50 values published from mouse experiments are 15.1, 43, 200, 330 and 400 mg/kg body weight for a single intravenous, intraperitoneal, subcutaneous, intragastric, and intramuscular administration, respectively (Piyachaturawat et al 1983). Most animals administered a lethal dose succumbed to respiratory paralysis within 3-17 minutes. Subacute toxicity studies in rats report death within 3-7 days of treatment. Post-mortem histopathologic analysis indicated multi-organ dysfunction as a cause of death and severe hemorrhagic necrosis and edema in the gastrointestinal tract, adrenal glands and urinary bladder. However, piperine has been shown to be safe at levels equivalent to typical human intake, or up to 250 times normal human intake, based on growth, organ weights and blood constituents (Piyachaturawat et al 1983).

Piperine has a number of deleterious effects on the reproductive system. In vitro experiments performed to explore the effect of piperine on the fertilizing ability of

hamster sperm were performed by Piyachaturawat and colleagues (1991). There was reduced percentage of eggs fertilized and degree of polyspermia in a dose-dependent manner between administered concentrations of 0.18-1.05 mM (Piyachaturawat et al 1991). Other studies have identified a significant reduction in both the weights of testis and accessory sexual organs, and severe damage to the seminiferous tubules and a decrease in seminiferous tubular and leydig cell nuclear diameter. Desquamation of spermatocytes and spermatids was also seen. Piperine caused increased serum gonadotropins and reduced intratesticular testosterone concentration (Malini et al 1999). Furthermore, piperine administration (10-20 mg/kg body weight) was performed in Swiss albino mice and piperine interfered with important reproductive events. Specifically, piperine increased the length of the diestrous phase, resulting in reduced mating performance and fertility. Litter growth and sperm structure were not affected at doses of piperine below 75 mg/kg body weight (Daware et al 2000).

Piperine metabolism has been extensively studied. Following oral administration of piperine in a rat model, urinary metabolites were identified as piperonylic acid, piperonyl alcohol, piperonal, and free form vanillic acid. Only piperic acid was detected in bile. Piperine is renally excreted, with no metabolite identified in feces. (Bhat & Chandrasekhara 1987). Khajuria et al determined that piperine is rapidly absorbed across the intestinal barrier. Furthermore, piperine may act as an apolar molecule forming a complex with drugs and solutes (Khajuria et al 1998).

1.5.2 Anticancer Properties of Piperine

Sunila and Kuttan performed a series of experiments to determine the anti-tumour activity of piperine. Dalton's lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) cells were treated with differential concentrations of piperine or *Piper longum* extract with viability determinations performed with the trypan blue exclusion method. Both piperine and *Piper longum* extracts had cytotoxic activity on these cells. L929 (immortalized mouse fibroblast) cells were treated with these compounds and found to be cytotoxic. DLA cells were injected subcutaneously into the right hind limbs of Swiss albino mice and *Piper longum* extract and piperine treatments were associated with tumour volume reduction. Lastly, these compounds were associated with increased lifespan of ascites tumour-bearing mice (Sunila & Kuttan 2004). In 1990, Unnikrishnan and Kuttan showed a 65% increased lifespan in mice transplanted with intraperitoneal Ehrlich ascites who were orally administered black pepper extract (Unnikrishnan & Kuttan 1990). Bezerra et al. (2008) studied the in vitro and in vivo antitumor effect of 5-FU combined with piperine and another black pepper extract, piplartine. Sarcoma 180 cells incubated with 5-FU and either piperine or piplartine experienced increased growth inhibition. These agents also improved immunocompetence reduced by 5-FU, by reversal of 5-FU-induced leucopenia (Bezerra et al 2008).

The antimetastatic potential of piperine has been explored by Pradeep and Kuttan (2008), who demonstrated an inhibitory effect of piperine on lung metastasis induced by B16F-10 melanoma cells in C57BL/6 mice (Pradeep & Kuttan 2002). This was associated with 95% reduction in tumour nodule formation, with histopathological correlation performed. Piperine-treated mice also survived the 90-day experiment.

Piperine has a cytoprotective effect on Benzo(α)pyrene-induced experimental lung cancer (Selvendiran et al 2003). This study determined that piperine may extend its chemopreventive effect by modulating lipid peroxidation and enhancing the antioxidant defense system. Specifically, activity of antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase were increased. Non-enzymatic antioxidants such as reduced glutathione, vitamin C, and vitamin E levels were also increased, relative to non-cancer affected animals.

The antimutagenic properties of black pepper were studied by El Hamss et al (El Hamss et al 2003). This study was based on the premise that the human diet is rich in natural carcinogens and mutagens in addition to antimutagenic and anticarcinogenic components. Using the Wing Somatic Mutation And Recombination Test in a *Drosophila melanogaster* model, these investigators tested black pepper in combination with the alkylating agent methyl methanesulfonate (MMS) and the promutagen ethyl carbamate (EC). Black pepper was found to be effective at reducing mutational events induced by EC alone. The authors proposed that black pepper exerts its antimutagenic action via suppression of metabolic activation or interaction with active groups of mutagens. However, piperine has been shown in several animal studies to promote differential tumour formation including liver, ileum, stomach, kidney and spleen in Egyptian toads (el-Mofty et al 1988), in addition to extensive demonstration of its anti-carcinogenic activity. Therefore, piperine's chemotherapeutic and chemopreventive effects remain poorly understood, which is the focus of the current research.

1.5.3 Piperine Receptor Activity

Notable for its "burning" aftertaste, piperine is detected based on its activity on heat and capsaicin receptor TRPV1. This receptor, also known as vanilloid receptor-1, was the first member of the vanilloid subgroup of transient receptor potential (TRP) channels to be identified (Caterina et al 1997). Agonists include vanilloid compounds such as resiniferitoxin and non-vanilloid compounds, such as piperine. TRPV1 expression is seen in human sensory neurons involved in pain pathways and gastrointestinal function. TRPV1 expression may be upregulated in neuropathic pain and inflammatory conditions. Furthermore, piperine has been shown to induce receptor desensitization (McNamara et al 2005). Piperine was determined to be a more potent agonist at the TRPV1 receptor than capsaicin. Furthermore, capsazepine has been determined to be a moderate TRPV1 receptor antagonist (Nocerino et al 2002). SB366791 is a more selective and more potent TRPV1 receptor antagonist, used in pain research (Niiyama et al 2009). TRPV1 receptor antagonism provides potential therapeutic benefit to limit the gustatory sensation of piperine, in addition to modulation of cancer pain symptoms.

1.6 Reactive Oxygen Species

Reactive oxygen species (ROS) are oxygen-containing reactive chemical species, including superoxide anions ($O_2^{\bullet -}$) and hydroxyl radicals ($HO^{\bullet}$), each containing an unpaired electron molecule. Hydrogen peroxide (H_2O_2) is a non-radical molecule ROS. Other ROS include singlet oxygen radicals and hypochlorous acid. ROS are generated constantly in biological systems via enzyme-catalyzed and non-enzymatic reactions

(Pelicano et al 2004). ROS are involved in microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and induction of damage to lipids, proteins and nucleic acids. ROS affect membrane fluidity due to peroxidation, altering the amounts of cell membrane unsaturated fatty acids and proteins (Pan et al 2009). Furthermore, ROS stress is intrinsic to cancer cells, due to enhanced ROS generation in cancer cells, increased accumulation of reaction products in these cells, and over-expression of antioxidant enzymes in response to oxidative stress. This ROS generation exceeds that seen in normal cells of similar type (Pelicano et al 2004). In contrast, free radical injury is a major etiological factor in the pathogenesis of atherosclerosis, aging, and cancer. Specifically, oxidative stress induces DNA damage resulting in genomic instability (Jackson & Loeb 2001). ROS also contributes to p53 mutations as a result of G-to-T transversions (Pan et al 2009). ROS generated from exposure to chemical carcinogens play an important role in initiation and progression of carcinogenesis. Antioxidants act as free radical scavengers to inhibit carcinogenesis. However, ROS have dual roles, also functioning in tumour suppression (Papaioannou et al 2010). These opposing roles may be related to the participation of ROS in MAPK signaling pathways that have opposing functions. In mammals, the MAPKs are divided into three subgroups: ERKs (extracellular signal-regulated kinases), JNKs (c-Jun-N-terminal kinases) and p38 MAPKs (Lewis et al 1998). The Ras-Raf-MEK1/2-ERK1/2 signaling cascade is involved in tumourigenesis, while the p38 MAPK pathway is involved in tumour suppression via cellular senescence, contact inhibition and DNA-damage responses (Pan et al 2009). Furthermore, apoptosis related to ROS is processed via the mitochondrial pathway, in a mechanism dependent on p38 activation. Apoptosis signal-regulating

kinase 1 (ASK1) is a MAP kinase that activates both JNK and p38 MAPK (Kuo et al 2007). These may also be triggered by ROS. ASK1-induced and ROS-dependent MAPK activation is critical for apoptosis. An ASK1-p38-TNF-α pathway may result in positive feedback, thus enhancing apoptosis. As well, p38 may stimulate ROS production via p53 (Bragado et al 2007). The relationship is complex, as p38-related apoptosis may be independent of ROS, in which JNK may perform an opposite function (Torres et al 2008).

Oxidative stress remains the backbone of ionizing radiation and cytotoxic therapy (Cadet et al 2003). These agents are effective via direct cellular injury in addition to inducing stress responses due to DNA or cytoskeletal damage. This may result in apoptotic cell death. Cells that have undergone oncogenic transformation activate a stress response as a protective measure, which further supports the function of oncogenes to induce senescence or cell death (Evan & Vousden 2001). Transformed cells therefore suppress stress signals, though incompletely, as they remain susceptible to anti-cancer agents (Benhar et al 2001). Thus, cells which have developed into tumours have been selected based on their survival abilities under environmental duress, including hypoxia and depleted survival or growth factors (Brown & Wouters 1999).

1.7 Antioxidants and Cancer

Antioxidants are important modulators of cellular defense against oxidative damage. Work with antioxidants has borne out the free radical theory of human diseases (Wu et al 2004). One such antioxidant, glutathione (GSH), in its reduced form is the most abundant non-protein thiol in mammalian cells. It is produced intracellularly in all

organs and types of cells, but is especially abundant in liver and lung tissues. Intracellular concentrations range from 1-10mM (Lu 2000). Glutathione may exist in one of two forms. Its reduced form, GSH accounts for over 99% of intracellular glutathione, in contrast to the oxidized form, GSSG (Jefferies et al 2003). GSSG efflux from cells contributes to net loss of intracellular GSH, in addition to reductions in cellular GSH concentration due to protein malnutrition, oxidative stress, and numerous pathological conditions (Griffith 1999). Most cellular glutathione is cytosolic (85-90%). The remainder is found in organelles, such as mitochondria, nuclear matrix, and peroxisomes (Wu et al 2004). Glutathione has increasingly been recognized for its role as a reducing agent, antioxidant, and mediator of diverse physiological reactions such as cellular signaling, metabolism, thiol disulfide exchange reactions, and as a cysteine reservoir (Chung et al 1990). Its depletion may be a causal factor or requirement for ROS production. Severe oxidative stress may impair glutathione reductase activity, resulting in accumulation of intracellular GSSG. Furthermore, the redox status of the cell is indicated by the GSH/GSSG balance (Schafer & Buettner 2001). Sufficient GSH concentrations are essential for cell proliferation, spermatogenesis, cytokine production and polymorphonuclear leukocyte activation (Wu et al 2004).

Glutathione is formed in two steps, the first of which involves formation of γ–glutamylcysteine from glutamate and cysteine, via γ–glutamylcysteine synthetase. The second enzymatic step involves formation of GSH via GSH synthetase using γ–glutamylcysteine and glycine as substrates (Njalsson et al 2001). Each step consumes one ATP molecule.

Glutathione has several roles in cellular reactions. GSH scavenges free radicals and other reactive oxygen species both directly through donation of electrons, and indirectly via enzymatic reactions. A thiyl radical (•GS) forms, which itself is pro-oxidative if not removed (Wlodek 2002). Two of these species then react to form GSSG, which is reduced by NADPH-dependent glutathione reductase to GSH. NADPH acts as an electron donor, maintained via the pentose phosphate shunt. Glutathione peroxidase catalyzes GSH-dependent reduction of hydrogen peroxide and other peroxides (Fang et al 2002). Glutathione reacts with a number of electrophiles, physiological metabolites, and xenobiotics to form mercapturates via glutathione-S-transferase. GSH also conjugates with nitric oxide to form an S-nitrosoglutathione adduct, which is then cleaved to release GSH and nitric oxide (Wu et al 2004).

GSH also acts as a substrate for formaldehyde dehydrogenase, converting GSH and formaldehyde to S-formyl-glutathione. GSH is needed for conversion of prostaglandin H_2 into D_2 and E_2, via endoperoxide isomerase. GSH is involved in conversion of methylglyoxal to D-lactate via the glyoxalase system, important in microorganisms. Lastly, protein glutathionylation is important in cellular physiology (Wu et al 2004).

Piperine is known to inhibit free radicals and ROS, thus protecting against oxidative damage. Specifically, piperine has been shown to scavenge hydroxyl radicals at low concentrations, in addition to generating hydroxyl radicals at higher concentrations, via the Fenton reaction (Mittal & Gupta 2000).

1.8 Apoptosis, Autophagy and Necrosis

In the event of failed stress defenses, cell death programs are initiated to remove these cells. These include apoptosis, necrosis, pyroptosis, or autophagy (Zakeri et al 1995). The mechanism of cell death depends on the conditions to which the cells are exposed. Apoptosis was first proposed to describe the morphological changes associated with cell death including shrinkage and blebbing of cells, nuclear rounding and fragmentation, chromatin condensation and phagocytosis of cell fragments (Kerr et al 1972). Apoptosis is a non-inflammatory means of cell death, in contrast to necrosis, which is typically associated with loss of control of ionic balance, water uptake, swelling and cell lysis. This process attracts immune cells and is pro-inflammatory (Fulda et al 2010).

Apoptosis is triggered by a variety of cellular stresses including radiation, oxidative stress, endoplasmic reticulum (ER) stress and chemotherapeutics (Szegezdi et al 2006). A series of cysteine proteases, known as caspases, serve as effectors in a common death pathway (Degterev et al 2003). Caspases are synthesized in an inactive form, which are activated via cleavage of substrates in the nucleus or cytoplasm. Caspase activation induces many of the changes associated with apoptotic death, including DNA fragmentation, loss of cell shape, and nuclear shrinking (Lockshin & Zakeri 2007).

Caspase activation occurs via different mechanisms. In the non-mitochondrial pathway, death receptors of the tumour necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas) or TNF-related apoptosis inducing ligand (TRAIL) receptors are stimulated by agonistic antibodies or ligands resulting in receptor aggregation and Fas-associated death domain (FADD) adaptor molecule and procaspase-8 recruitment,

forming the death-inducing signaling complex (DISC) (Kischkel et al 2000). Caspase-8 becomes activated, thus initiating apoptosis via direct cleavage of effector caspases (Ashkenazi 2008).

In contrast, the mitochondrial pathway begins through release of apoptogenic factors including cytochrome c, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low PI (DIABLO) or Omi/high-temperature requirement protein A2 (HtrA2) from the mitochondrial intermembrane space (Kroemer et al 2007). Cytosolic cytochrome c release activates caspase-3 via cytochrome c/Apaf-1/caspase-9-containing apoptosome complex formation (Zou et al 1999). Caspase activation is promoted by Omi/HtrA2 or Smac/DIABLO via neutralization of the inhibitory effects of Inhibitor of Apoptosis Proteins (IAPs) (LaCasse et al 2008). Caspase activation is tightly controlled to prevent damaging effects on cell survival in the event of inappropriate activation (LaCasse et al 2008).

Sensitivity to apoptosis is regulated by the ratio of anti-apoptotic (Bcl-2, Bcl-X_L, and Mcl-1) to pro-apoptotic (Bax, Bak, and BH3) Bcl-2 family proteins. There are two models of activation involved. In the direct activation model of Bcl-2 activation, BH3-only proteins functioning as direct activators (Bim, tBid), directly bind to Bax and Bak to stimulate their activation (Letai et al 2002). Binding to pro-survival Bcl-2 proteins by BH3-only proteins including Bad, promote apoptosis. In the indirect activation model, BH3-only proteins activate Bax and Bak indirectly through binding to multiple anti-apoptotic Bcl-2 proteins that inhibit Bax and Bak, thus releasing these proteins (Willis et al 2007).

A separate type of programmed cell death is autophagy, which is a multistep process, defined by sequestration and degradation of cytoplasmic proteins and organelles into double-membrane vesicles, termed autophagosomes. Fusion with lysosomes results in degradation of the autophagosome content by acid-dependent enzymes (Liang et al 1999). Autophagy is seen in cells under metabolic and therapeutic stresses, such as nutrient and growth factor deprivation, ischemia/reperfusion, inhibition of proteasomal degradation, ER stress, intracellular calcium accumulation, and inhibition of receptor tyrosine kinase/Akt/mammalian target of rapamycin (mTOR) signaling (Eskelinen 2008).

Reactive oxygen species have also been reported to lead to autophagy (Scherz-Shouval et al 2007). However, autophagy and cell death have a complex relationship in which autophagy may provide stress adaptation, preventing cell death, while alternatively it may result in cell death. Autophagy remains poorly understood and clinically it appears to have beneficial roles in protecting the heart against hemodynamic stress (Nakai et al 2007). Additionally, there appears to be a relationship between inhibition of apoptosis and induction of autophagy- associated or –dependent cell death (Shimizu et al 2004).

Another form of cell death is necrosis, which occurs in response to cellular stresses, such as ischemia or DNA damaging alkylating agents (Festjens et al 2007). Necrosis is associated with increased cell volume, organelle swelling, plasma membrane rupture, and loss of intracellular contents, due to this inflammatory process. Furthermore, necrosis involves numerous signal transduction cascades (Vanlangenakker et al 2008). Reactive oxygen species and calcium are both important mediators in propagation of necrosis, via organelle and macromolecule damage, resulting in loss of cell integrity.

Caspase inactivation by ROS and calcium-dependent pathways further inhibits the apoptotic machinery and drives necrosis (Samali et al 1999).

1.8.1 Caspase-Independent Cell Death

Evidence is accumulating from an increasing number of experimental models that apoptosis may occur independently of caspase activation. As it is widely regarded that caspase activation accounts for many of the cellular changes associated with traditional apoptosis, caspase-independent cell death entails partial chromatin condensation, in the absence of internucleosomal DNA fragmentation (Broker et al 2005). There are several known mitochondrial inducers of caspase-independent cell death, including flavoprotein apoptosis-inducing factor (AIF) and the endonuclease Endo G (van Loo et al 2002). Other investigators treated breast cancer cells and found a different PARP cleavage fragment at 60 kDa, instead of the typical 89 kDa fragment, which they attributed to activation of a calpain-like protease. Therefore, additional proteolytic pathways exist, about which little is known (Pink et al 2000). For example, Arimura et al (2003) reported in their study of evening primrose extract-induced apoptosis that caspase-3 activation was not observed, even though caspase-3 activation is one of the main consequences of cytochrome c release, which did occur. They stated that the precise mechanisms of this caspase-independent apoptosis are poorly understood (Arimura et al 2003). Therefore, the traditional understanding of apoptotic cell death is being challenged.

1.8.2 Cancer and Endoplasmic Reticulum Stress Pathways

The endoplasmic reticulum (ER) is an organelle responsible for several major functions in eukaryotic cells. These are protein folding and assembly, lipid and sterol biosynthesis, and free calcium storage (Hotamisligil 2010). Intracellular stress signaling pathways from the ER may result in altered transcription and translation in cells undergoing stress, referred to as Unfolded Protein Response (UPR) (Schroder & Kaufman 2005). These are crucial for survival versus death decision-making in these cells. Induction of ER stress and UPR in tumours and cancer cell survival has broadened the field of cancer research. These are believed to account for resistance to anti-cancer therapy as well (Healy et al 2009). ER stress results from a number of stimuli including ER calcium depletion, altered glycosylation, nutrient deprivation, oxidative stress, and DNA damage, which are handled by the ER through altered transcriptional and translational programs, initiated by the Unfolded Protein Response signaling pathways (Wang et al 2010). These include three main pathways in mammalian cells, operating through three protein stress sensors localized to the ER. These are IRE1α (Inositol-requiring 1 alpha), PERK (Double-strand RNA-activated protein-kinase like ER-kinase), and ATF6 (activating transcription factor 6) (Ron & Walter 2007). The UPR reduces ER stress by preventing translocation of proteins into the ER lumen, degrading ER-localized proteins and increasing their cytosolic translocation, and increasing the protein folding capacity of the ER. In the event of failure, the UPR induces cell death programs, thus removing stressed cells (Zhang & Kaufman 2008).

The UPR is an important mechanism for tumour cell survival during tumourigenesis and growth due to stress induced as a result of nutrient deprivation and

hypoxia. Furthermore, in tumourigenesis, the high proliferation rate demands increased ER protein folding, assembly and transport, all of which generate ER stress (Boelens et al 2007). Therefore, the UPR maintains malignancy and resistance to chemotherapy. This has generated interest in the UPR as a therapeutic target (Lee 2007).

In the event of ER stress, PERK homodimerizes and autophosphorylates, which phosphorylates transcription initiating factor eIF2α, which generally reduces protein translation (Manga et al 2010). This reduces ER workload. Under ER stress atf4 mRNA is selectively translated, activating expression of genes involved in cell metabolism, anti-oxidative response and apoptosis associated with ER stress (Harding et al 2003).

IRE1α and ATF6-mediated UPR pathways are also activated in response to ER stress. This increases protein folding capacity and protein degradation in the ER. Similar to PERK, IRE1α is activated via homodimerization and autophosphorylation, allowing it to function as an endoribonuclease (Schroder & Kaufman 2006). This ultimately aids in protein folding, maturation, secretion and degradation of misfolded protein. Additionally, phosphorylated IRE1α acts as a scaffold protein for recruitment of Tumour Necrosis Factor (TNF) receptor-associated-Factor 2 (TRAF2), which activates Jun N-terminal Kinase (JNK)-mediated signaling pathways (Urano et al 2000). Furthermore, IRE1α may interact with Bcl-2, providing a link between ER stress and apoptosis (Hetz et al 2006).

UPR activation also induces ATF6 release from the ER membrane and transfer to the golgi, where processing produces an activated bZIP transcription factor, activating UPR target gene expression. Protein secretion, degradation and folding in the ER also occur with ATF6 cleavage (Lee et al 2003). Failed UPR-mediated survival program

activation in the event of ER stress results in activation of the apoptotic pathway, mediated by a transcription factor, GADD153/CHOP, downstream of the PERK/eIF2α pathway (Marciniak et al 2004). As well, ER-associated Protein Degradation (ERAD) degrades aberrant or misfolded ER proteins. This entails translocation of targeted substrates to the cytoplasm, polyubiquitination, and 26S proteasomal degradation. This process maintains ER homeostasis, therefore, disruption of this process results in ER-stress induced apoptosis (Vembar & Brodsky 2008).

Cancer cells activate UPR-mediated survival signals to survive the stressful environment associated with rapid glucose metabolism and rapid growth, with its consequential poor vascularization, hypoxic environment, nutrient depletion, and altered pH (Dong et al 2008). Cancer cells also express mutant proteins that cannot undergo proper folding, have insufficient energy supply and altered redox environment, which generates ER stress and UPR activation. Cancer cells must adapt to ER stress through differential activation of UPR branches (Hersey & Zhang 2008).

An understanding of the molecular mechanisms of ER stress has provided therapeutic implications and has expanded the armamentarium in treating cancer (Healy et al 2009). GRP78, a molecular chaperone that exhibits a cytoprotective role in oncogenesis can be targeted for inhibition. Similarly, proteasome inhibitors, such as Bortezomib, result in growth inhibition and apoptosis and have a cytotoxic effect on a broad range of cancers. ERAD inhibitors, which result in accumulation of misfolded proteins, trigger ER stress-induced apoptosis (Wang et al 2010). A broader understanding of the ER stress pathways will enable development of improved targeted chemotherapeutic agents.

1.8.3 Poly ADP-Ribose Polymerase and Cancer

Poly (ADP-ribose) (PAR) polymers are involved in mitotic progression, DNA repair, transcriptional control, and caspase-independent cell death. The direct role played by PAR in cell death is complex (Sandhu et al 2010). PAR polymers are formed by an enzymatic reaction catalyzed by Poly (ADP-ribose) polymerase-1 (PARP) in response to genotoxic stress, using NAD+ as a substrate (Zaremba & Curtin 2007). Mild DNA damage leads to stimulation of PARP-1 activity, with nuclear accumulation of PAR. This subsequently allows DNA repair protein recruitment, which is protective against genotoxic stress. However, excessive PARP activation depletes cellular NAD+/ATP pools, favouring necrotic cell death (Virag 2005).

Therefore, PAR polymers exert a dual role in that they are needed for mitosis and stimulation of repair of DNA damage. Excessive PAR synthesis is key to caspase-independent cell death. In proliferating cells such as cancer cells, there is glycolytic generation of ATP, in contrast to oxidative phosphorylation, which occurs in non-proliferating cells (Gonin-Giraud et al 2002). Therefore, excessive PARP-1 activation in actively proliferating cells results in NAD+ depletion and thus ATP depletion. Non-replicating cells retain their mitochondrial ATP supply and can therefore undergo apoptosis (Zong et al 2004). Excessive DNA damage results in overactivation of PARP-1, producing large quantities of PAR polymers, which leads to AIF translocation. PARP-1 then dissociates from PAR and binds DNA strand breaks. This ultimately consumes greater quantities of NAD+ (Skaper 2003). This depletion may result in either caspase-independent apoptosis or necrosis (Yu et al 2006). Translocation of AIF from the

mitochondria to the nucleus was shown to induce chromatin condensation, DNA fragmentation, and cell death (Susin et al 1999). Caspase-dependent and-independent insults can then result in AIF release from the mitochondria, where it may then translocate to the nucleus, resulting in chromatin condensation. It also recruits nucleases including cyclophilin A and endonuclease G (Wang et al 2002). Activation of apoptosis is followed by PARP-1 cleavage by pro-apoptotic proteases or caspase-3 or -7, forming an 85 kDa catalytic fragment and a 25 kDa DNA-binding domain. The catalytic fragment continues to form PAR at a basal level, but is no longer stimulated in presence of DNA damage. Cleavage is believed to occur to prevent further PARP-1 activation, thus limiting the depletion of cellular ATP levels and the resulting necrosis (Kaufmann et al 1993).

1.9 Cancer and Colony Formation

Studying *in vitro* colony formation by cancer cells enables drug sensitivity testing in colonies of thirty or more cancer cells (Shoemaker et al 1985). The cancer stem cell hypothesis proposes that only a fraction of the cells within a tumour have unlimited proliferative capacity (Shipitsin & Polyak 2008). These colonies form after five or more rounds of cell division (Lee et al 1986). Furthermore, colony formation reflects tumour growth rate (Koppikar et al 2010). Therefore, studying colony formation provides another index of cancer cell viability. The colony-forming assay allows unbiased study of cell death as it detects cytotoxicity regardless of mechanism, based upon the cell's reproductive capacity to form progeny (Katz et al 2008). Nair et al (2004) showed that colony formation by prostate cancer cells was proportional to the aggressiveness of the

cancer cell line studied. They also found quercetin, a phytochemical found in citrus fruits, inhibited colony formation significantly in more aggressive cancer cells, while less aggressive cancer cells were unaffected (Nair et al 2004). Therefore, studying colony formation enables study of tumour biology and the cytotoxic effect of phytochemical treatment on cancer cells.

1.10 Research Rationale

Exploring the molecular targets of other dietary agents known to possess chemopreventive and chemotherapeutic effects is helpful in guiding a determination of piperine's mechanism of action. The molecular targets of well studied phytochemicals are detailed extensively in a review paper by Aggarwal and Shishodia (Aggarwal & Shishodia 2006). Well-studied naturally-derived compounds include curcumin (turmeric), resveratrol (red grapes, berries, and peanuts), genistein (soybean), diallyl sulfide (onion), allicin (garlic), lycopene (tomato), and capsaicin (red chili), among many others. Molecular targets of dietary agents already identified include protein kinases, anti-apoptotic proteins, pro-apoptotic proteins, transcription factors, cell cycle proteins, cell adhesion molecules, the growth factor pathway, metastasis, and others (Aggarwal & Shishodia 2006). These pathways have helped guide efforts to investigate the mechanisms of action of piperine on human cancer cell lines in the current research.

Chapter 2.0 Materials and Methods

2.1 Cell Lines

HT-29 and CaCo-2 colon adenocarcinoma, and HRT-18 rectal adenocarcinoma cell lines were a kind gift from Dr. J. Blay (Dalhousie University). Human mammary epithelial cells (HMEC) were purchased from Lonza Inc.,Walkersville, MD. McCoy's 5a Medium (Invitrogen Corp., Burlington, ON, Canada) was supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal bovine serum (HI-FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and L-glutamine (all Invitrogen, Corp., Burlington, ON, Canada) for HT-29 cells. HRT-18 and CaCo-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich Canada, Ltd., Oakville, ON, Canada) supplemented with 5 and 10% heat-inactivated fetal bovine serum (HI-FBS), respectively, 5 mM N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid (HEPES) buffer (pH 7.4), 100U/ml penicillin and 100 μg/ml streptomycin. All supplements were purchased from Invitrogen. HMECs were maintained in MEBM (mammary epithelium basal medium) containing recombinant human epidermal growth factor, recombinant human insulin, hydrocortisone, Gentamicin sulfate, amphotericin and bovine pituitary extract (Lonza Inc.)

2.2 Reagents

Piperine (97-98% pure; Sigma-Aldrich Canada) was dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich Canada) to 150 mM, then stored in aliquots at -20°C. Trypsin and TrypLE (phenol red negative trypsin replacement) were purchased from Invitrogen. The CellTrace™Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (Carboxy-DFFDA, SE) was purchased from Molecular Probes (Eugene, OR). Phosphate buffered saline (PBS), L-glutathione and N-acetylcysteine were purchased from Sigma-Aldrich Canada.

2.3 Proliferation Assay

The Oregon Green flow cytometric proliferation assay was performed to determine the anti-proliferative effect of piperine on HT-29 and HRT-18 cell lines. The cells were harvested from culture flasks using 3 ml of trypsin (HT-29 cells) or 5 ml TrypLE (HRT-18 cells). The cells were centrifuged at 500xg for 5 minutes and resuspended in 4 ml PBS and centrifuged for an additional 5 minutes at 500xg. The cells were resuspended in a solution of 4 ml PBS containing 1 µl Oregon Green and incubated in the dark on a shaker for 10 minutes at room temperature. Following incubation, 4 ml of HI-FBS were added to the Oregon Green-incubated cells. This solution was centrifuged for 5 minutes at 500xg. The cells were resuspended in 4 ml of TrypLE and incubated for 30 minutes at 37°C. Following incubation, 4 ml of McCoy's 5a medium (HT-29 cells) or 5% DMEM (HRT-18 cells) was added. The cells were then centrifuged at 500xg for 5 minutes and resuspended in 1 ml of the appropriate medium. The cells were then counted and plated at 50,000 cells per well on a 6-well plate. The plates were

incubated overnight at 37°C. After 24 hours, the non-proliferative control cell population was harvested by incubating the cells with 0.45 ml of TrypLE for 5 minutes at 37°C. Paraformaldehyde (4%) was added and the solution was stored in a 5ml tube wrapped in aluminum foil at 4°C. Piperine and control treatments were applied to the remaining plated cells. After 72 hours, control and treated cells were harvested using 0.5ml TrypLE (incubated at 37°C for 5 minutes). Flow cytometric analysis was performed on FL1 using a FACSCalibur flow cytometer. Oregon Green-488, or succinimidyl ester of carboxyfluorescein diacetate (CFDA SE) spontaneously and irreversibly couples to intracellular and cell-surface proteins via lysine side chain and other amine group reactions. Since there is an equal distribution of dye between daughter cells, an exponential reduction in fluorescence occurs with each succeeding generation of cells. The excitation/emission maxima are ~495/525 nm, which are readily detectable by flow cytometry. The staining protocol is based on information provided from Invitrogen and a modification of the protocol used by MacMillan et al (MacMillan et al 2009).

2.4 Cell Cycle Analysis

HT-29 and HRT-18 cells were cultured for 12 hours in serum-free medium and harvested at confluency from culture flasks using trypsin and TrypLE, respectively. Cells were plated at 100,000 cells per well for 48 hours in flat 6-well tissue culture plates. Following overnight incubation to allow adherence, the cells were treated with the desired concentrations of piperine and incubated for 48 hours at 37°C in a 5% (HT-29 cells) and 10% (HRT-18 cells) CO_2 humidified atmosphere. Following 48 hour incubation, the cells were harvested with TrypLE and centrifuged at 500xg and

resuspended in 0.5 ml PBS. 70% ethanol cooled to -20°C was added slowly to the cell solution during continous vortex to a final volume of 5 ml. The ethanol-suspended cells were stored for a minimum of 24 hours at -20°C. Following storage, the cells were centrifuged at 500xg and resuspended in 5 ml PBS. Cells were again centrifuged at 500xg and resuspended in propidium iodide (0.1% v/v Triton X-100 in PBS containing 0.2 mg/ml DNase-free RNase A and 0.02 mg/ml propidium iodide) and incubated for 30 minutes at room temperature. Analysis was performed on a FACSCalibur flow cytometer on FL2. Data was then analyzed using Mod-fit software. The protocol above is similar to that described in a recent paper (Cheng et al 2010).

2.5 Viability Assays

2.5.1 MTT Assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was used to determine cell line viability (Mosmann 1983). Yellow MTT (Sigma-Aldrich Canada) reagent is converted to purple formazan crystals in the presence of mitochondrial succinate dehydrogenase present in viable cells. All cell lines were harvested from culture flasks and control and piperine treatments were applied for 24-144 hours on 96-well plates. Two hours prior to the desired time point, 20 µl of MTT reagent was added to each well, followed by a 2-hour incubation at 37°C in 5 or 10% CO_2, depending on the culture medium. Following incubation, the 96-well plate was centrifuged at 1440xg for 5 minutes. The supernatants were then discarded and 0.1 ml DMSO was added to each well. A plate shaker was used to solubilize the formazan crystals in DMSO and an EL_X800 uv Universal Microplate Reader (BIO-TEK

instruments Inc., Winooski, VT) was used to read absorbance at 490 nm. Optical densities were plotted against treatment and control populations of cells and percent loss of viability was calculated.

2.5.2 Crystal Violet Assay

A crystal violet colorimetric assay was used according to the method provided by Dr. Volker Briese (Universitäts-Frauenklinik Rostock) to determine cell viability at 72 hours. Crystal violet binds to proteins and DNA within the cell. Cells were harvested and plated at a concentration of 5,000 cells per well on a 96-well plate. Piperine and control treatments were applied for 72 hours. The supernatants were discarded and 0.2 ml of PBS was added to each well. The PBS was then removed and 50 µl of 0.4% crystal violet (dissolved in methanol) was added to the wells for 10 minutes. Following a 10-minute incubation period, the plate was washed in 1-litre of water and this step was repeated. The plate was inverted and allowed to dry on a paper towel. After drying, 0.1 ml DMSO was added to each well to solubilize the crystal violet stain. A microplate reader was used to read absorbance at 570 nm. Optical densities were plotted against treatment and control populations and percentage loss of viability was calculated.

2.5.3 Trypan Blue Dye Exclusion Assay

A trypan blue dye exclusion assay was performed to determine cell viability (Joyner et al 2009). Trypan blue (Invitrogen) is a diazo dye vital stain used to selectively permeate dead or dying cells. Piperine-treated and control cell populations were grown at 100,000 cells per well for 24-72 hours, then harvested with TrypLE and centrifuged at 500xg.

The cells were resuspended in medium and 50 μl of cell solution was combined with 50 μl trypan blue for approximately 30 seconds. Cells with an intact membrane exclude the dye and are therefore viable, in contrast to dead or dying cells, which lack an intact membrane and take up the dye. Counting was performed in quadruplicate using a hemocytometer with cell solutions diluted to enable counting in the 75-150 cell range. Percentage of non-viable cells was determined in addition to total cell counts in the control and treatment populations.

2.6 TRPV1 Antagonists

Capsazepine is a competitive antagonist of moderate potency against the TRPV1 receptor (McNamara et al 2005). SB366791 is a selective antagonist of TRPV1 (Boudaka et al 2007). HT-29 and HRT-18 cell lines were harvested from culture flasks. Capsazepine pretreatments were applied for 30 minutes at concentrations of 0.5, 1, 5 and 10 mM. Similarly, SB366791 pretreatments were applied for 30 minutes at concentrations of 0.5, 1, and 2.5 mM. Control and piperine treatments were applied for 24-72 hours on 96-well plates. Two hours prior to the desired time point, 20 μl of MTT reagent was added to each well, followed by a 2-hour incubation at 37°C in a 5 or 10% CO_2, depending on the medium. Following incubation, the 96-well plate was centrifuged at 1440xg for 5 minutes. The supernatants were then discarded and 0.1 ml DMSO was added to each well. A plate shaker was used to solubilize the formazan crystals in DMSO and an EL_X800 uv Universal Microplate Reader (BIO-TEK instruments Inc., Winooski, VT) was used to read absorbance at 490 nm. Optical densities were plotted against treatment and control populations and percent loss of viability was calculated.

2.7 Reactive Oxygen Species Detection Assays

2.7.1 Dihydroethidium Reactive Oxygen Species Assay

Dihydroethidium (DHE; Invitrogen Corp.) is a non-polar, non-fluorescent compound that readily diffuses across plasma membranes and is converted to the fluorescent ethidium in the presence of superoxide anions. Confluent cells were harvested and plated at a concentration of 100,000 cells per well on a 6-well plate. 10 mM glutathione pre-treatments were applied for 30 minutes for comparison with control and piperine treatment groups. Piperine and control cells were harvested at 48 hours using TrypLE (incubated for 5 minutes at 37°C). The cells were then centrifuged at 500xg for 5 minutes, resuspended in 0.5 ml medium, and incubated for 15 minutes at 37°C in the presence of 20 μl of 0.01 μM DHE (Stock: 5mM in DMSO) diluted in medium. Flow cytometric analysis was performed on FL2 using the FACSCalibur flow cytometer.

2.7.2 2',7'-Dichlorofluorescin 3'6'-diacetate Reactive Oxygen Species Assay

2',7'-Dicholorofluorescin 3'6'-diacetate (DCFH-DA; Invitrogen Corp.) is a non-polar, non-fluorescent compound that readily diffuses across plasma membranes and is converted to the fluorescent dichlorofluorescein in the presence of hydroxyl radicals. Confluent cells were harvested and plated at a concentration of 100,000 cells per well on a 6-well plate. 10mM glutathione pre-treatments were applied for comparison with control and piperine treatment groups. Piperine and control cells were harvested at 48 hours using TrypLE (incubated for 5 minutes at 37°C). The cells were then centrifuged

at 500xg for 5 minutes and resuspended in 1 ml PBS. The cells were again centrifuged at 500xg for 5 minutes and resuspended in 0.5 ml medium containing 10 μM DCFH-DA (Stock: 2mM in DMSO). The cells were then incubated for 20 minutes at 37°C. Flow cytometric analysis was performed on FL1 using the FACSCalibur flow cytometer. A similar protocol was used in a recent paper (Olson et al 2008).

2.8 Annexin-V/Propidium Iodide Flow Cytometry Assay for Apoptosis and Necrosis

Annexin-V-Fluos/propidium iodide flow cytometric staining assays were performed to determine the presence of apoptosis and/or necrosis (Giladi et al 2010). HT-29 and HRT-18 cells were harvested from T75 cm^2 tissue culture flasks at confluency using trypsin and TrypLE, respectively. Cells were plated at a concentration of 50,000 per well on flat 6-well tissue culture plates and allowed to adhere overnight incubated at 37°C in a 5% or 10% CO_2 humidified atmosphere. Piperine or control treatments were applied and cells were incubated for 72 hours. Additionally, pre-treatments with 10mM glutathione and N-acetylcysteine were used for comparison with piperine-treated and control populations. Following incubation, cells were harvested with TrypLE and centrifuged at 500xg for 5 minutes. Cells were resuspended in 1 ml PBS and centrifuged at 500xg for 5 minutes. Cells were then labeled with Annexin-V-Fluos (Roche Diagnostics, Laval, QC) diluted according to manufacturer's protocol and propidium iodide (1 μg/ml) in detection buffer (10mM HEPES, 10mM NaCl, and 5mM $CaCl_2$). Dye incubation for 15 minutes at room temperature was followed by flow cytometric analysis using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada). Annexin-V is a calcium-dependent phospholipid-binding protein with high

affinity for phosphatidylserine residues, which are externalized to the outer leaflet of the lipid bilayer in apoptotic cells. Propidium iodide is a DNA-intercalating agent that readily diffuses across disrupted cell membranes, thus differentiating necrotic cells from apoptotic cells.

2.9 Colony-Forming Assays

Confluent HT-29 cells were harvested from culture flasks with TrypLE. Following centrifugation at 500xg for 5 minutes, cells were resuspended in McCoy's 5a medium and counted. Cells were plated at a concentration of 100,000 cells per well and incubated overnight at 37°C in a 5% humidified CO_2 atmosphere. Piperine-treated and control cells were harvested and centrifuged at 500xg for 5 minutes. Cells were plated at between 250-2000 cells on flat 6-well tissue culture plates. Medium was changed every 48 hours for a duration of 13 days. At the end of the 13-day period, the medium was removed and the wells were washed with 1 ml PBS. The PBS was removed and 0.5 ml of 0.4% crystal violet (in methanol) was added to each well. After 15 minutes, the crystal violet was removed and the wells were washed with de-ionized water until crystal violet staining was limited to colonies. The 6-well plates were then dried overnight and visible colonies were photographed and counted. The colony number was compared to the cell number that was originally plated.

2.10 Caspase Inhibition Assays

HT-29 colon cancer cells were harvested at confluency and plated at 5,000 cells per well on a 96-well flat plate. The cells were incubated at 37°C in a 5% CO_2 incubator for approximately 24 hours. The pan-caspase inhibitor, Boc-D-fmk (Calbiochem, Darmstadt, Germany) was applied at 40 μM for 30 minutes prior to control and piperine treatments. The plates were incubated at 37°C in a 5% CO_2 incubator for 72 hours. Two hours prior to the desired time point, 20 μl of MTT reagent was added to each well, followed by 2-hour incubation at 37°C in a 5% CO_2 incubator. Following incubation, the 96-well plate was centrifuged at 1440xg for 5 minutes. The supernatants were then discarded and 0.1 ml DMSO was added to each well. A plate shaker was used to solubilize the formazan crystals in DMSO and an EL_X800 uv Universal Microplate Reader (BIO-TEK instruments Inc., Winooski, VT) was used to read absorbance at 490 nm. Optical densities were plotted against treatment and control populations and percent loss of viability was calculated.

2.11 Western Blotting

HT-29 cells were harvested at confluency using trypsin. Following centrifugation at 500xg for 5 minutes, cells were resuspended in 1 ml McCoy's 5a medium and plated at 100,000 cells per well. Cells were incubated overnight at 37°C in a 5% humidified CO_2 atmosphere and subsequently piperine or control treatments were applied to the cells. Incubation was continued at 37°C in a 5% humidified CO_2 atmosphere for 48 or 72 hours. The cells were then harvested using TrypLE and centrifuged at 500xg for 5 minutes. The cells were resuspended in 1 ml PBS and transferred to 1.5 ml Eppendorf

tubes. Following centrifugation at 1000xg for 5 minutes, the supernatants were discarded and the cells were resuspended in 80 µl RIPA lysis buffer (0.1% NP-40, 0.25% sodium deoxycholate, 0.1% sodium dodecylsulphate (SDS), 50 mM Tris-HCl, 150 mM NaCl, 50 mM Na_2HPO_4, 5 mM EDTA, 5mM EGTA (pH 7.5), 5 µg/ml pepstatin, 10 µg/ml aprotinin, 5µg/ml leupeptin, 1mM phenylmethyl sulfonyl fluoride, 100 µM sodium orthovanadate, 1mM DTT, 10 mM sodium fluoride and 10 µM phenylarsine oxide). After incubation on ice for 30 minutes, cells were centrifuged at 14,000xg for 10 minutes at 4°C. The supernatants were transferred to new 1.5 ml Eppendorf tubes and protein concentration was determined using a Bradford Assay. A microplate reader was used to determine optical densities and a standard curve was plotted on Softmax Pro software. Equal amounts of protein were resolved on 15%, 12% or 7.5% polyacrylamide gels for 1 hour at 200 volts. After glycine SDS-PAGE, protein transfer to nitrocellulose membrane was performed using the iBlot transfer system according to manufacturer's protocol (Invitrogen Corp.). Blocking of nitrocellulose membranes was performed for 1 hour in Tris-buffered saline (200 mM Tris, 1.5 M NaCl (pH 7.6)) containing 0.05% Tween-20 (T-TBS) with 10% w/v skim milk powder). Western blots were then washed 3 times for 5 minutes each in T-TBS and incubated for 1 hour or overnight with the desired primary antibody in either 5% BSA in T-TBS or 5% w/v skim milk powder in T-TBS as per manufacturer specifications. Blots were then washed in T-TBS for 5 minutes, which was repeated 10 times. Blots were then incubated with the appropriate secondary antibody in either 5% BSA in T-TBS or 5% w/v skim milk powder in T-TBS as per manufacturer specifications. Following incubation, T-TBS washing was repeated as described above for primary antibody. Protein bands were detected upon film exposure using ECL

Western blotting detecting reagent (GE Healthcare). Even protein loading was confirmed by reprobing blots with goat anti-actin antibody (1:1000) dilution in 5% skim milk powder and T-TBS for 1 hour. Blots were then washed as described above for primary antibody. After washing, blots were incubated for 1 hour with HRP-conjugated bovine anti-goat IgG antibody. Washing of secondary antibody and protein band detection are as described above. Densitometry of all western blots was performed on the AlphaImager to quantify band density relative to actin band density. Cell cycle-based antibodies Cdk 4 mouse (#2906), Cdk 6 mouse (#3136), Cyclin D1 mouse (#2926), Cyclin D3 mouse (#2936), p15 INK4B rabbit (#4822), p21 Waf1/Cip1 mouse (#2946), p27 Kip1 rabbit(#2552), phospho-Rb (Ser795) rabbit (#9301), and phospho-Rb (Ser807/811) rabbit (#9308) were purchased from Cell Signaling Technology, Inc., Danvers, MA. Mitogen-activated protein kinase (MAPK) pathway antibodies total p38 MAPK rabbit (#9212), phospho-SAPK/JNK (Thr 183/Tyr 185) (#9255) and total JNK rabbit (#9252) were purchased from Cell Signaling Technology, Inc. The phospho-p38 MAPK rabbit (#368500) antibody was purchased from Biosource. Anti-survivin (6E4) mouse antibody (#2802), anti-phospho-AKT (Ser473) rabbit antibody (#9271), total AKT rabbit antibody (#9272), anti-rabbit IgG HRP-linked antibody (#7074), anti-mouse IgG HRP-linked antibody (#7076), anti-caspase-3 rabbit antibody (#9662), and anti-caspase-9 mouse antibody (#9502) were purchased from Cell Signaling Technology. Anti-PARP mouse antibody (#sc-8007) and anti-goat IgG HRP-linked antibody (#sc-2378) were purchased from Santa Cruz Biotechnology. ER stress protein antibodies Bip rabbit (#3177), calnexin (#2679) rabbit, CHOP (#2895) mouse, ERo1-Lα (#3264) rabbit, IRE1α (#3294) rabbit, and PDI (#3501) rabbit were purchased from Cell Signaling Technology. Anti-

cytochrome C mouse (#556433) antibody was purchased from BD Biosciences, Mississauga, ON.

2.11.1 Digitonin Lysis Assay

Digitonin lysis for mitochondrial isolation for cytochrome c protein determination was performed in a similar manner as described above. A digitonin lysis buffer was used in place of RIPA buffer (190 μg/ml digitonin, 75 mM NaCl, 1mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 5 μg/ml pepstatin, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethyl sulfonyl fluoride, 100 μM sodium orthovanadate, 1 mM DTT, 10 mM sodium fluoride and 10 μM phenylarsine oxide. De-ionized water was added to a final volume of 50 ml). Following centrifugation at 12,000xg for 15 minutes at 4°C the supernatant, representative of the cytoplasmic portion of the cell, was collected. Equal amounts of protein were resolved on a 15% polyacrylamide gel as described above, with the remaining steps equivalent to the procedures as performed above for standard western blotting.

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad In Stat version 3.00 (GraphPad Software, San Diego, CA). ANOVA with Bonferroni multiple comparisons test or the Student's t-test were used to analyze data.

Chapter 3.0 Results

3.1 Piperine Exerts an Antiproliferative Effect on Human Colon and Rectal Cancer Cells.

Proliferation assays were performed to understand piperine's effect on colon and rectal cancer cell proliferation. Piperine was determined to inhibit proliferation of HT-29 colon cancer cells at 150 μM in a statistically significant manner, via the Oregon Green-488 flow cytometric assay (Figure 1). In this assay, cells were incubated with the CellTrace™Oregon Green® 488 carboxylic acid diacetate, succinimidyl ester (Carboxy-DFFDA, SE). Some of the cells were harvested at 24 hours and fixed with 4% paraformaldehyde as a non-proliferative control. At the 72-hour time point, the remainder of the cells were harvested, fixed and analyzed by flow cytometry. The leftward shift of the medium and vehicle control cells represents approximately 3.5 rounds of proliferation, in contrast to the 150 μM piperine-treated cells, which have undergone approximately 2 rounds of proliferation. A similar effect was observed in HRT-18 rectal cancer cells (Figure 2).

3.2 Piperine Induces a G_1 Phase Cell Cycle Arrest in HT-29 Colon Cancer Cells

Cell cycle analysis was performed to determine the effect of piperine on cell cycle progression to account for its anti-proliferative effect. This was performed according to a similar method as described in a recent paper by Khan et al (Khan et al 2008). Piperine treatment at 100 μM was associated with a statistically significant increase in the percentage of HT-29 colon cancer cells in G_0/G_1 phase of the cell cycle, with a corresponding reduction in the percentage of cells in S and G_2/M phases (Figure 3). While there is a significant reduction in the percentage of HRT-18 cells in G_2/M phase of

the cell cycle following 100 μM piperine treatment, there was no statistically significant change in the percentage of cells in both G_0/G_1 and S phases (Figure 4).

The effects of piperine on cell cycle progression were also studied using western blotting of proteins associated with G_1 cell cycle arrest in HT-29 cells. Treatment of HT-29 cells with piperine for 48 hours was associated with statistically significant downregulation of cyclin D1 and D3 (Figures 5a, b, 6a, b), Cdk 4 and 6 (Figures 7a, b, 8a, b) and phospho-Rb (795, 807/811) (Figures 9a, b, 10a, b). Upregulation of the p21 Waf1/Cip1 (Figures 11a, b) and p27 Kip 1 (Figures 12a, b) was also observed. These changes in protein concentration are consistent with G_1 cell cycle arrest in HT-29 cells. Similarly, a reduction in phospho-AKT protein concentration is consistent with an anti-proliferative effect induced by piperine (Figures 13a-c). There were no significant changes in p15 INK4B protein concentration (Figures 14a, b).

3.3 Piperine is Cytotoxic to Human Colon and Rectal Cancer Cells

Several different cell viability assays were performed in order to determine the cytotoxic effect of piperine. The first type of viability assay used was the MTT assay. This assay, developed by Mosmann in 1983 uses 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow reagent, to measure cytotoxicity (Mosmann 1983). In the presence of viable mitochondria, which contain the enzyme succinate dehydrogenase, the yellow compound is converted to purple formazan crystals, which can be solubilized in dimethylsulfoxide and the absorbance measured. Greater intensity of purple colour determines increased viability. Failed conversion of the yellow reagent to purple formazan crystals implies loss of mitochondrial viability. MTT assays were performed on HT-29 and CaCo-2 colon cancer cells, HRT-18 rectal cancer cells, and

HMECs to determine the cytotoxic effect of piperine on both transformed cells and normal cells. Piperine was found to exert a cytotoxic effect in a dose and time-dependent manner on HT-29 cells at concentrations of 75 to 150 μM (Figure 15). This effect was seen consistently at 48 and 72 hours. The effect was not observed consistently at 144 hours with 150 μM piperine. In contrast, piperine was cytotoxic to HRT-18 rectal cancer cells at 150 μM at all time points while 100 μM piperine was cytotoxic at 72 and 144 hours, and 50 μM piperine was cytotoxic at 144 hours (Figure 16). Piperine was cytotoxic to CaCo-2 colon cancer cells at 25 μM to 100 μM at 144 hours (Figure 17). Cytotoxicity was also observed at 100 μM concentration of piperine at 48 hours. Note that 150 μM piperine was not tested in this cell line.

Colony-forming assays were also used to determine the effect of piperine on HT-29 colon cancer cell viability. 150 μM piperine was found to reduce the formation of colonies by 50% (Figure 18).

The cytotoxic effect of piperine on normal human cells was studied using HMECs. Through MTT assays, piperine was shown to exert an approximately 15% cytotoxic effect at 100 μM and 150 μM at 72 hours (Figure 19). There was negligible cytotoxicity to HMECs at 24 and 48 hours.

Viability was also studied using the trypan blue exclusion assay. This assay utilizes trypan blue, which is a toluidine-derived diazo vital stain that readily traverses non-viable cell membranes. Dye exclusion thus implies cell viability. This assay was performed at 24, 48 and 72 hours. The number of viable cells was significantly reduced by piperine concentrations of 50 μM (36%), 100 μM (75%) and 150 μM (85%) at 72 hours, as well as with 150 μM (75%) at 48 hours (Figure 20).

3.4 Piperine-Mediated Cytotoxicity is Due to Apoptosis

Since piperine was determined to exert a cytotoxic effect on HT-29 colon and HRT-18 rectal cancer cells, Annexin-V-FITC and propidium iodide staining of control and piperine-treated cells was performed to determine whether cells were dying by apoptosis or necrosis. Apoptosis is associated with plasma membrane structural changes that include expression of thrombospondin binding sites, loss of sialic acid residues and exposure of phosphatidylserine (Fadok et al 1992, Pytela et al 1985, Savill et al 1993). Phosphatidylserine is a negatively charged phospholipid, normally found on the inner leaflet of the plasma membrane bilayer (Op den Kamp 1979). Apoptosis results in loss of membrane phospholipid asymmetry, such that phosphatidylserines become exposed on the outer leaflet of the membrane.

In piperine-treated HT-29 colon cancer cells, there was an increase in early apoptotic cells, supporting an apoptotic mechanism of cell death induced by 150 μM piperine (Figure 21). HRT-18 rectal cancer cells treated with 150 μM piperine also underwent early apoptotic cell death (Figure 22).

3.5 Piperine-Mediated Apoptosis is Due to the Production of Reactive Oxygen Species

In order to determine the mechanism of apoptosis induction, reactive oxygen species production was studied. Reactive oxygen species are a family of highly reactive molecules formed from one-electron reductions of molecular oxygen in a stepwise, enzymatic manner. The result is generation of superoxide anions, hydroxyl radicals and other species. Superoxide anion production was detected using the DHE staining flow cytometric assay. DHE is a non-polar, non-fluorescent compound that readily traverses

plasma membranes, where it is oxidized to the fluorescent ethidium in the presence of superoxide anions. Ethidium, which is a DNA intercalating agent, binds to the DNA and fluoresces at an excitation wavelength of 500-530 nm, with an emission spectrum ranging from 590-620 nm (Peshavariya et al 2007).

HT-29 colon cancer cells treated with piperine exhibited a minimal rightward shift on flow cytometric analysis, indicating a lack of superoxide anion production (Figures 23 and 24). Additionally, exogenous pre-treatment of control and piperine-treated cells with glutathione, an antioxidant, did not alter the flow cytometry results. This suggests that superoxide anion production is not involved in piperine-induced apoptotic cell death in HT-29 cells. A similar result was seen in HRT-18 rectal cancer cells (Figures 25 and 26).

A second form of reactive oxygen species generation was also studied. Hydroxyl radical production can be detected using DCFH-DA, which is a stable, non-fluorescent compound that readily diffuses across plasma membranes. DCFH-DA becomes activated via alkaline hydrolysis to a second, non-fuorescent compound, 2',7'-dichlorofluorescin (DCFH), which remains stable for several hours. DCFH is then rapidly oxidized to 2',7'-dichlorofluorescein (DCF) due to the presence of hydrogen peroxide. The enzyme peroxidase enhances this reaction. DCF, due to its polarity, remains trapped within the cell. This trapped, fluorescent product can therefore be used to detect the production of hydroxyl radicals (Bass et al 1983, Brandt & Keston 1965, Keston & Brandt 1965).

HT-29 colon cancer cells treated with 100 μM piperine demonstrated a statistically significant rightward shift, indicating hydroxyl radical production (Figure 27). The addition of exogenous antioxidant glutathione reversed this rightward shift, thus protecting the cell from oxidative stress as induced by hydroxyl radical production

secondary to piperine treatment (Figure 28). A similar, but less statistically significant effect was observed in HRT-18 rectal cancer cells pre-treated with glutathione (Figures 29 and 30). I conclude from these experiments that piperine-mediated apoptosis is due to hydroxyl radical production, with absence of superoxide anion production.

To determine if HT-29 and HRT-18 apoptotic cell death was the result of reactive oxygen species production, specifically due to hydroxyl radical production, Annexin-V/ propidium iodide assays were performed with cells that were pre-treated with glutathione or N-acetylcysteine. HT-29 colon cancer cells pre-treated with glutathione followed by piperine treatment exhibited statistically significant reduction in early apoptotic cell death in comparison to piperine treatments alone, at both 100 μM and 150 μM (Figures 31 and 32). In contrast, HRT-18 rectal cancer cells pre-treated with glutathione did not show a reduced early apoptotic cell population at either concentration of piperine (Figure 33). Pre-treatment of HRT-18 cells with N-acetylcysteine was also associated with significant reduction of the early apoptotic cell population at both piperine concentrations (Figure 34). To better understand this phenomenon, crystal violet staining assays of viability were performed at 72 hours with glutathione and N-acetylcysteine pre-treated HT-29 colon cancer cells, and with N-acetylcysteine pre-treated HRT-18 rectal cancer cells.

Crystal violet is a 6-methyl dye compound that binds to cellular proteins and DNA. Increased violet colour indicates greater cell number and hence viability due to greater binding by the dye to DNA and proteins. Similar trends were seen in both MTT and crystal violet assays. With N-acetylcysteine and glutathione pre-treatments, there was a statistically significant reduction in the cytotoxic effect of piperine on HT-29 cells

at 100 and 150 μM (Figures 35 and 36). In contrast, the cytotoxic effect of piperine on HRT-18 rectal cancer cells at these concentrations was not reduced with N-acetylcysteine pre-treatments (Figure 37). This data further supports a mechanistic role of hydroxyl radical production in the effect of piperine on HT-29 colon cancer cells, whereas it appears that hydroxyl radical production is not responsible for the cytotoxic effect of piperine on HRT-18 rectal cancer cells.

3.6 Piperine-Mediated Apoptosis Involves Mitochondrial Membrane Destabilization

The role of mitochondria in apoptosis has historically involved the study of cytochrome c. Pro-apoptotic stimuli result in sustained elevations in calcium levels, which stimulates cytochrome c release from the mitochondria. Cytochrome c release subsequently activates caspase 9, followed by activation of caspases 3 and 7, responsible for cellular destruction (Suen et al 2008). Digitonin lysis was performed to isolate the cytosolic fraction of cells in order to determine cytochrome c levels of both control and piperine-treated cells at 48 and 72 hours. The digitonin lysis assay for cytochrome c showed significant cytochrome c release at 48 hours in both 75 and 100 μM treated cells (Figures 38a, b). In contrast, cytochrome c release was only significant in 100 μM piperine-treated cells at 72 hours (Figures 38c, d).

3.7 Piperine-Mediated Apoptosis is Caspase-Independent

Boc-D-fmk is a pan-caspase inhibitor that irreversibly binds to the catalytic site of caspases and, therefore, prevents activation of caspases by preventing cleavage of pro-caspase forms (Thornberry & Lazebnik 1998). Boc-D-fmk, which is typically used at

concentrations of 20-50 μM was used at a concentration of 40 μM in my studies (Gupta et al 2010, Uchide et al 2009). MTT viability assays were performed to determine if pan-caspase inhibition protected HT-29 colon cancer cells from piperine-induced cytotoxicity. There was no significant difference observed between cells pre-treated with Boc-D-fmk 30 minutes prior to piperine treatment and those treated with piperine alone (Figure 39). Therefore, piperine-mediated cytotoxicity is caspase-independent.

To further corroborate a caspase-independent mechanism of piperine effect, western blotting was performed to determine changes in caspase-3 and -9 protein expression following piperine treatment. There were no significant differences in caspase protein expression following treatment with 75 or 100 μM piperine (Figures 40a, b, and 41a, b). Further, no caspase cleavage products were identified. Together with the Boc-D-fmk data, this confirms that piperine's cytotoxic effect is caspase-independent.

Survivin protein expression was also studied as a link between cell cycle arrest and apoptosis. Survivin is a 16 kDa protein member of the Inhibitor of Apoptosis Proteins (IAP) family. Survivin is known to block apoptosis when overexpressed, in addition to its function as a G_2/M phase cell-cycle checkpoint regulator (Reed & Reed 1999). Western blotting for survivin protein expression determined that this protein is downregulated in cells treated with piperine at 100 μM (Figures 42a, b).

PARP protein expression was also studied since PARP is important in DNA damage repair, PARP cleavage depletes cellular ATP supplies, which may result in cell death. Production of PAR may stimulate mitochondrial release of apoptosis-inducing factor (AIF), which mediates caspase-independent apoptosis. Western blotting of PARP

determined the presence of a cleavage product at 85 kDa, supporting a role of piperine in inducing apoptosis (Figures 43a, b).

Western blotting of SAPK/JNK and p38 MAPK was performed due to the activation of these MAPKs by stressful stimuli such as pro-inflammatory cytokines and cellular stresses, such as genotoxic, osmotic, hypoxic, and oxidative stress (Kim & Choi 2010). Both phosphorylated JNK and p38 were upregulated in piperine-treated cells, supporting a possible mechanistic role of oxidative stress in piperine-induced cytotoxicity (Figures 44a-e, 45a-c).

3.8 Piperine-Mediated Apoptosis Involves Significant ER Stress

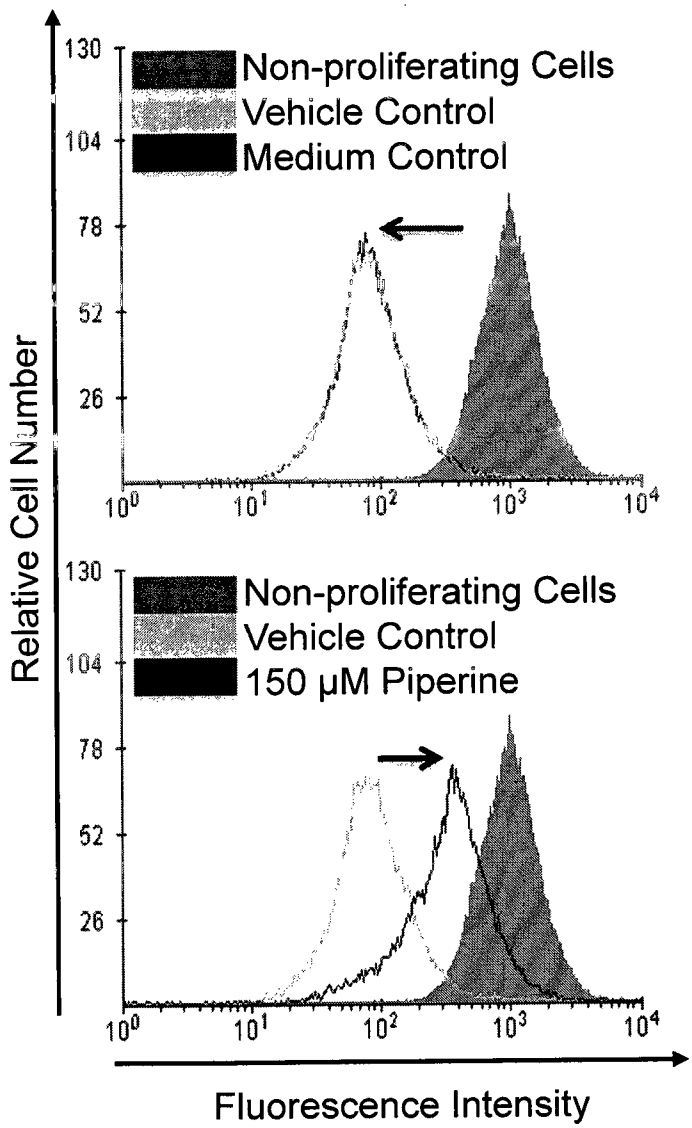
The effect of piperine on ER stress protein expression was determined through western blotting. IRE1-α, CHOP and Bip expression was upregulated following treatment with 100 μM piperine (Figures 46-48). Expression of calnexin (Figures 49a, b), ERo1-Lα (Figures 50a, b), and PDI (Figures 51 a, b) were unchanged following piperine treatment. Taken together, these data suggest that ER stress does play a role in piperine-induced cytotoxicity.

3.9 Piperine-Mediated Effects on Human Colon and Rectal Cancer Cells are Independent of TRPV1 Receptor Binding

Piperine is known to mediate some effects by binding to the TRPV1 receptor (Okumura et al 2010). To determine the effects of TRPV1 receptor blockade on HT-29 colon cancer and HRT-18 rectal cancer cell viability following piperine treatment, two different antagonists of the TRPV1 receptor were used to pretreat cancer cells prior to

piperine treatment. An MTT viability assay was then performed. Blockade with capsazepine, a moderate TRPV1 antagonist demonstrated no change in viability in comparison with HT-29 colon cancer cells treated with piperine alone (Figure 52). Similar effects were seen with capsazepine pre-treated HRT-18 rectal cancer cells (Figure 53). Pre-treatment with SB366791, a more potent and selective antagonist of the TRPV1 receptor also did not alter the cytotoxic effect of piperine on either HT-29 colon cancer cells (Figure 54) or HRT-18 rectal cancer cells (Figure 55).

Figure 1. Piperine Inhibits the Proliferation of HT-29 Colon Cancer Cells. Medium alone, the DMSO vehicle, or piperine was added to Oregon Green 488-labeled HT-29 colon cancer cells. After 72 hours of culture, cells were harvested and analyzed by flow cytometry. A non-proliferative control was harvested at 24 hours of culture and fixed with 4% paraformaldehyde to a final concentration of 1%. Data shown are representative of 3 independent experiments. Statistical significance in comparison to the vehicle control was determined by the Student's t-test; p=0.0013.


Relative Cell Number
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104
78
52
26
Non-proliferating Cells
Vehicle Control
Medium Control
10^0
10^1
10^2
10^3
10^4
130
104
78
52
26
Non-proliferating Cells
Vehicle Control
150 µM Piperine
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity

Figure 1.

Figure 2. Piperine Inhibits the Proliferation of HRT-18 Rectal Cancer Cells. Medium alone, the DMSO vehicle, or piperine was added to Oregon Green 488-labeled HRT-18 rectal cancer cells. After 72 hours of culture, cells were harvested and analyzed by flow cytometry. A non-proliferative control was harvested at 24 hours of culture and fixed with 4% paraformaldehyde to a final concentration of 1%. Data shown are representative of 4 independent experiments. Statistical significance in comparison to the vehicle control was determined by the Student's t-test; $p = 0.0015$.

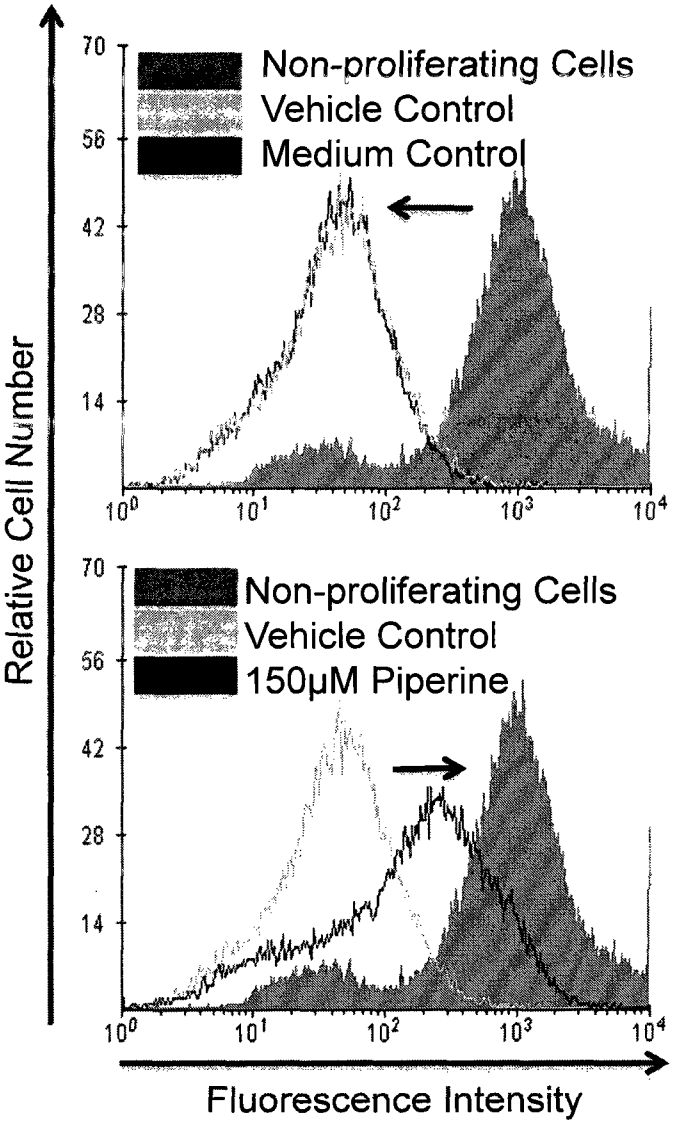

Relative Cell Number
70
56
42
28
14
Non-proliferating Cells
Vehicle Control
Medium Control
10^0
10^1
10^2
10^3
10^4
Non-proliferating Cells
Vehicle Control
150μM Piperine
Fluorescence Intensity

Figure 2.

Figure 3. Piperine Arrests HT-29 Colon Cancer Cells in the G_1 Phase of the Cell Cycle. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were cultured for 48 hours. Propidium-iodide staining of DNA was used to determine cell cycle distribution. Cellular DNA was analyzed by flow cytometry and ranges for G_0/G_1, S, and G_2/M are established based upon the corresponding DNA content of histograms. Data represent the mean of 4 separate experiments. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$, *** $p < 0.001$.

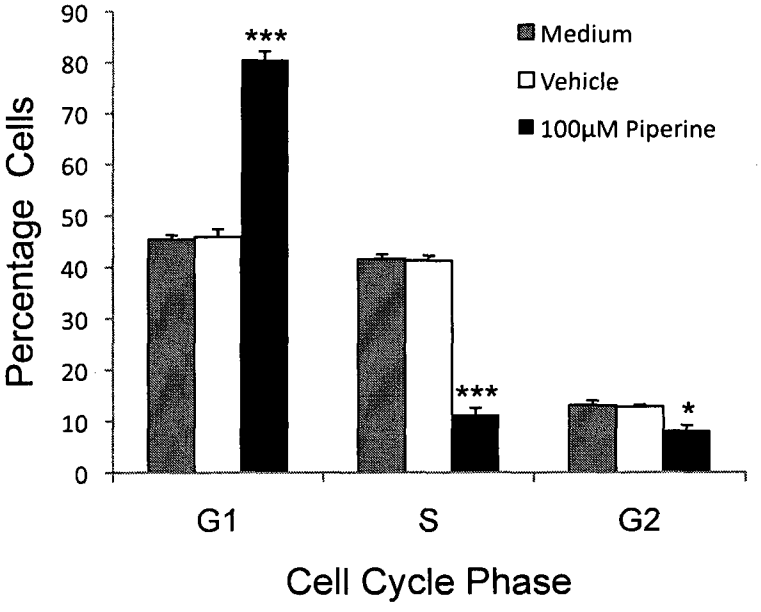

90
80
70
60
50
40
30
20
10
0
Percentage Cells
***
***
*
Medium
Vehicle
100µM Piperine
G1
S
G2
Cell Cycle Phase

Figure 3.

Figure 4. Piperine Arrests HRT-18 Rectal Cancer Cells in the G_1 Phase of the Cell Cycle. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were cultured for 48 hours. Propidium-iodide staining of DNA was used to determine cell cycle distribution. Cellular DNA was analyzed by flow cytometry and ranges for G_0/G_1, S, and G_2/M are established based upon the corresponding DNA content of histograms. Data represent the mean of 6 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; $***p < 0.001$.

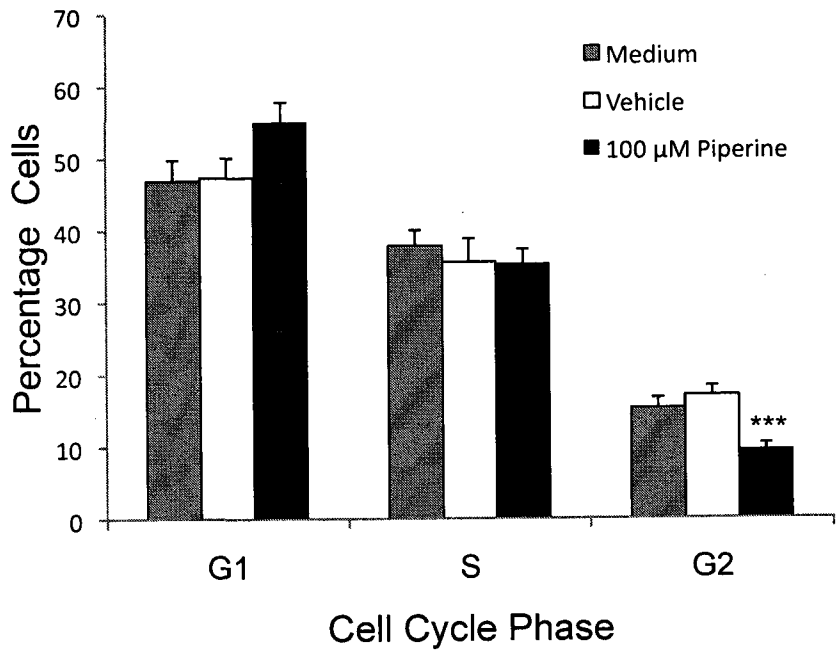

70
60
50
40
30
20
10
0
Percentage Cells
Medium
Vehicle
100 µM Piperine
***
G1
S
G2
Cell Cycle Phase

Figure 4.

Figure 5. Piperine Downregulates Cyclin D1 Protein Expression. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine on Cyclin D1 protein expression. Data are representative of 3 independent experiments. (b) Cyclin D1 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

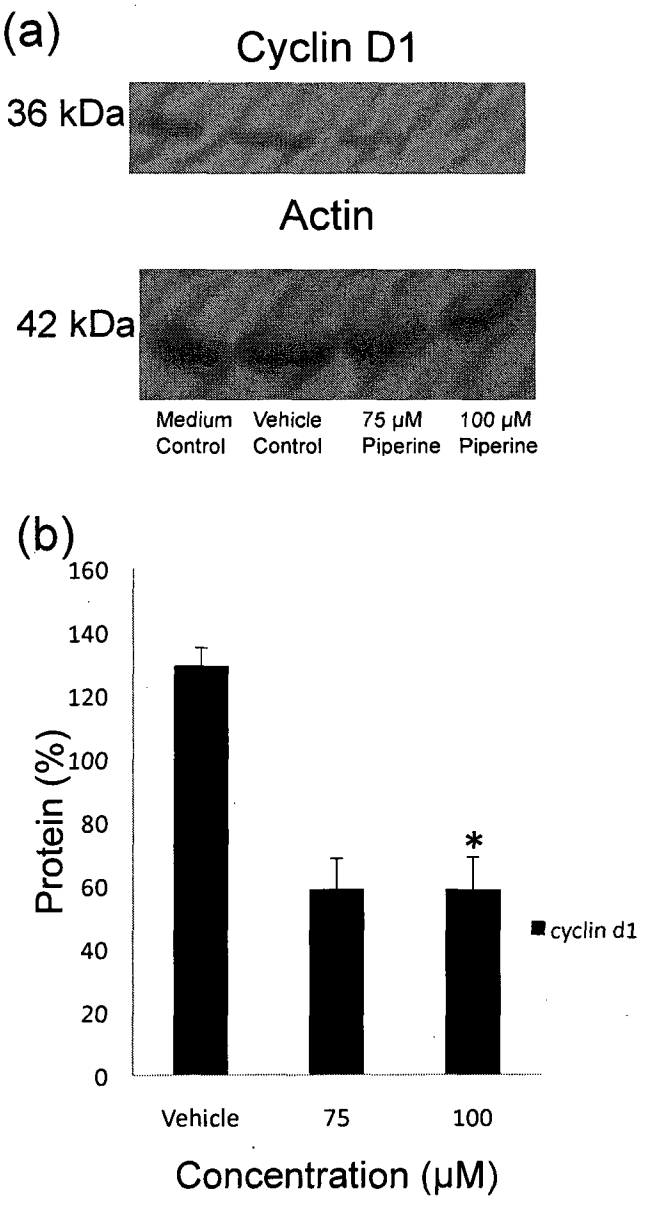

(a)
Cyclin D1
36 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
160
140
120
100
80
60
40
20
0
*
cyclin d1
Vehicle
75
100
Concentration (µM)

Figure 5.

Figure 6. Piperine Downregulates Cyclin D3 Protein Expression. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on Cyclin D3 protein expression. Data are representative of 3 independent experiments. (b) Cyclin D3 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

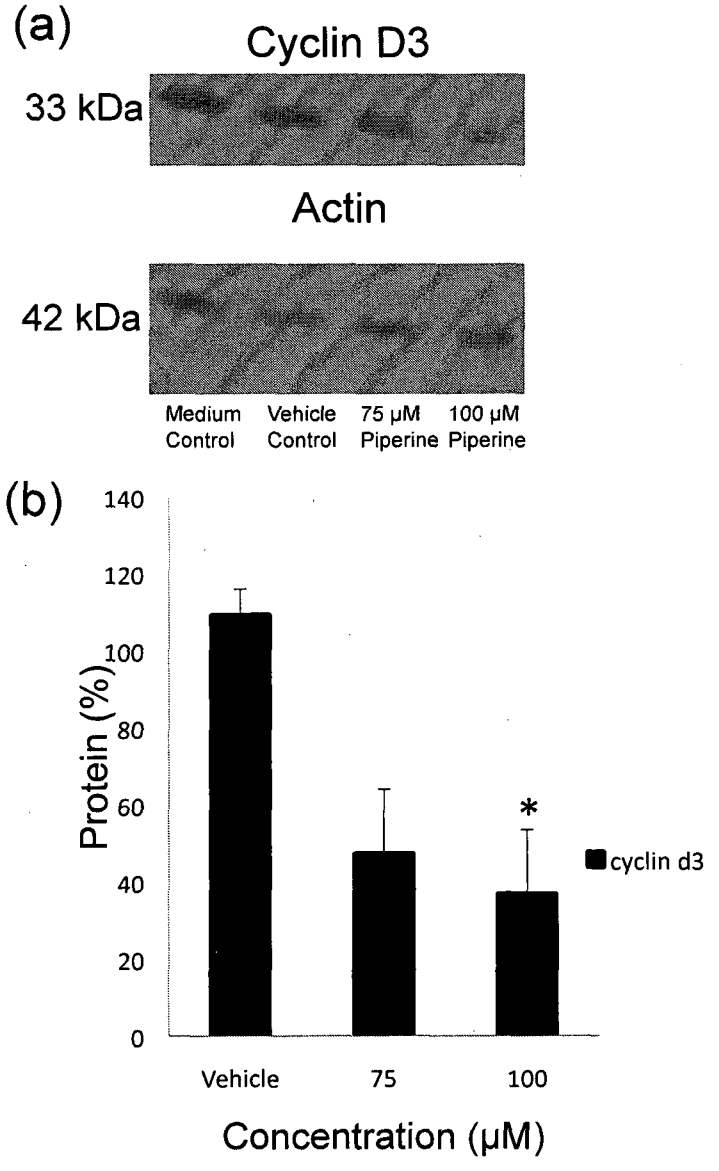

(a)
Cyclin D3
33 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
140
120
100
80
60
40
20
0
*
cyclin d3
Vehicle
75
100
Concentration (μM)

Figure 6.

Figure 7. Piperine Downregulates Cdk 4 Protein Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates determine the effect of piperine on Cdk 4 protein expression. Data are representative of 3 independent experiments. (b) Cdk4 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by ANOVA by the Bonferroni multiple comparisons test; * $p < 0.05$.

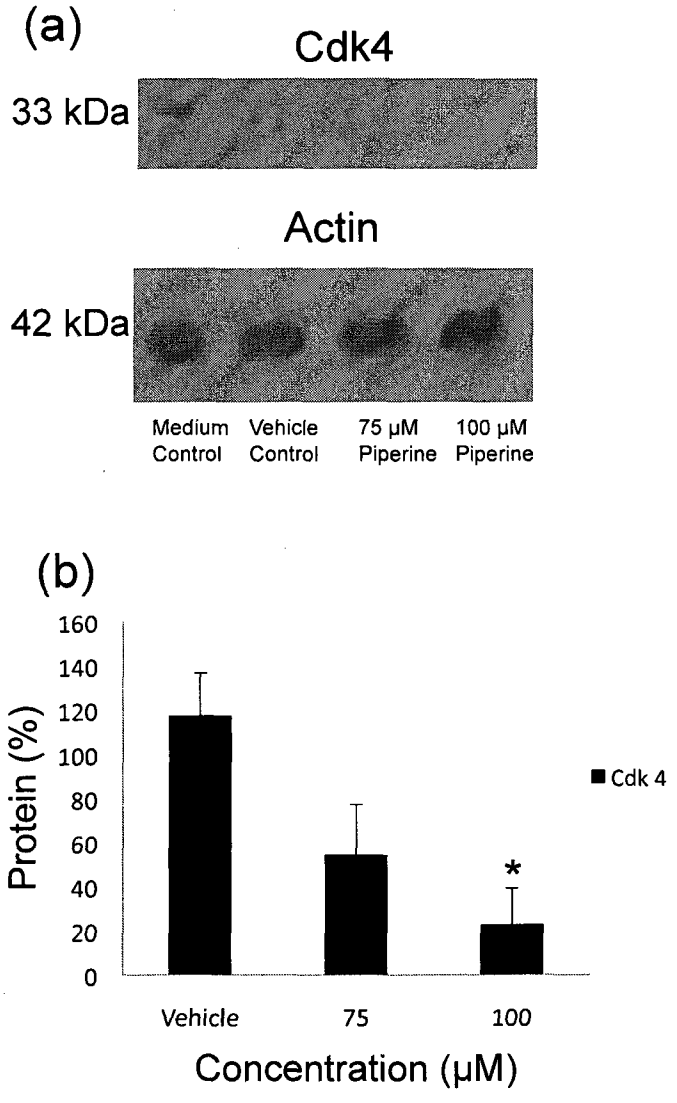
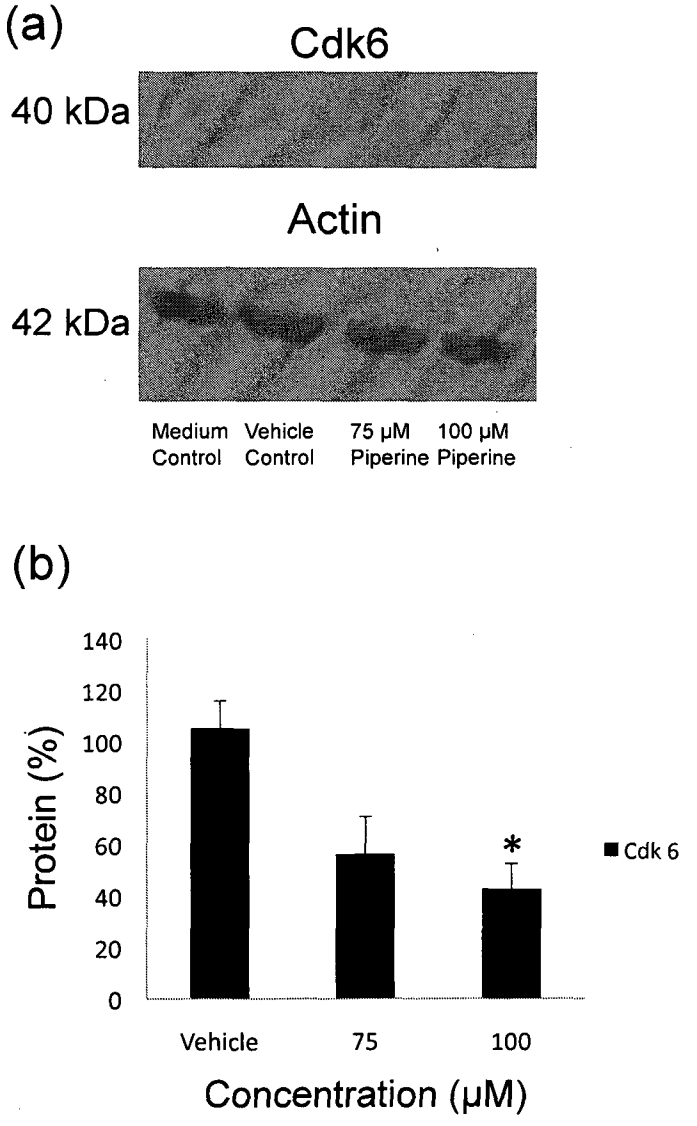

(a)
Cdk4
33 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
160
140
120
100
80
60
40
20
0
Cdk 4
*
Vehicle
75
100
Concentration (µM)

Figure 7.

Figure 8. Piperine Downregulates Cdk 6 Protein Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on Cdk 6 protein expression. Data are representative of 3 independent experiments. (b) Cdk 6 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.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40 kDa
Actin
42 kDa
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75 µM Piperine
100 µM Piperine
(b)
Protein (%)
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120
100
80
60
40
20
0
*
Cdk 6
Vehicle
75
100
Concentration (µM)

Figure 8.

Figure 9. Piperine Reduces Retinoblastoma Protein (795) Phosphorylation in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates determine the effect of piperine treatment on phospho-retinoblastoma protein (p-Rb 795) expression. (b) p-Rb 795 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; *** $p < 0.001$.

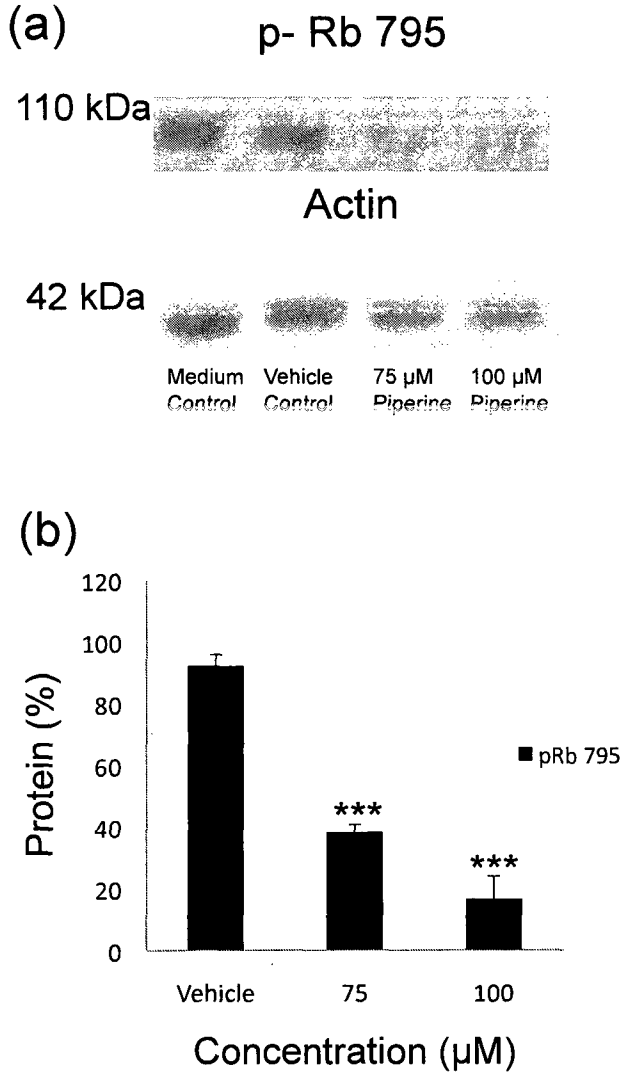
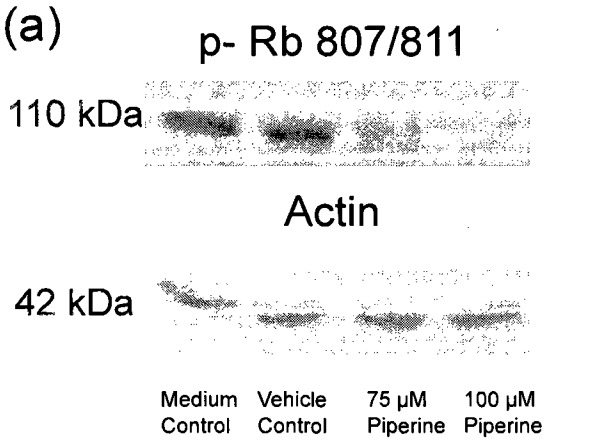

(a)
p- Rb 795
110 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
120
100
80
60
40
20
0
pRb 795
***
***
Vehicle
75
100
Concentration (µM)

Figure 9.

Figure 10. Piperine Reduces Retinoblastoma Protein (807/811) Phosphorylation in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates determine the effect of piperine treatment on phospho-retinoblastoma protein (p-Rb 807,811) expression. (b) p-Rb 807,811 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; ** $p < 0.01$.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110 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine

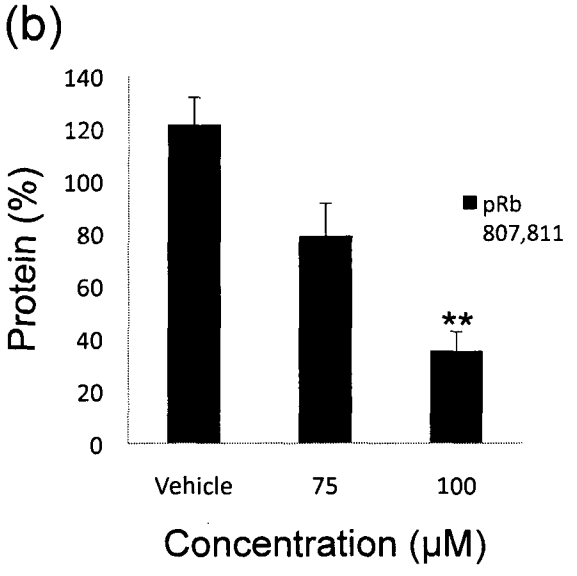
(b)
Protein (%)
140
120
100
80
60
40
20
0
pRb 807,811
**
Vehicle
75
100
Concentration (µM)

Figure 10.

Figure 11. Piperine Upregulates p21 Protein Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on p21 protein expression. Data are representative of 3 independent experiments. (b) p21 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

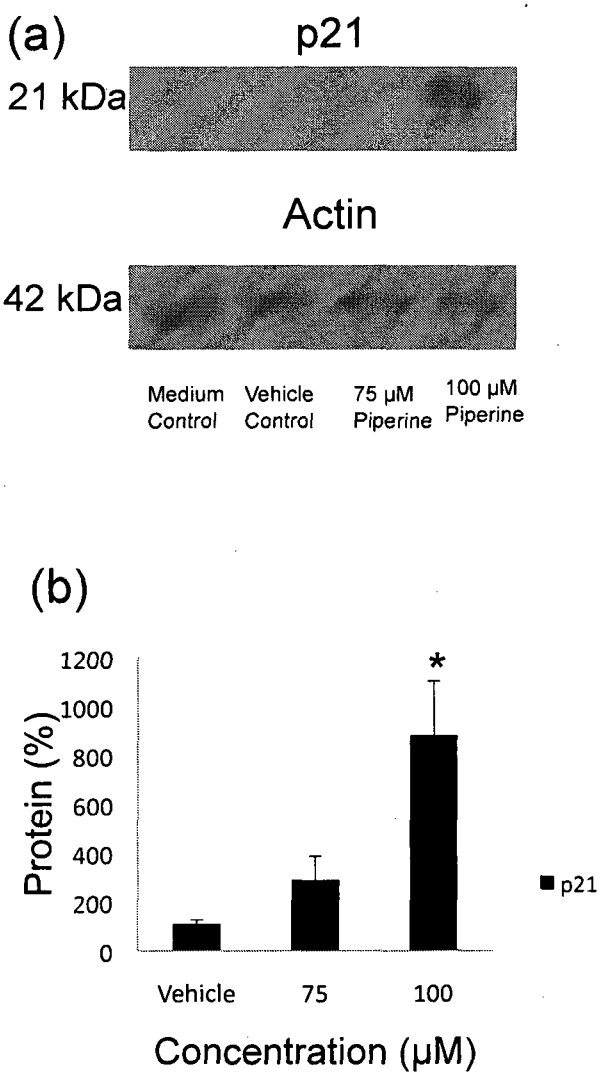
(a)
p21
21 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
1200
1000
800
600
400
200
0
*
p21
Vehicle
75
100
Concentration (μM)

Figure 11.

Figure 12. Piperine Upregulates p27 Protein Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on p27 protein expression. Data are representative of 3 independent experiments. (b) p27 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

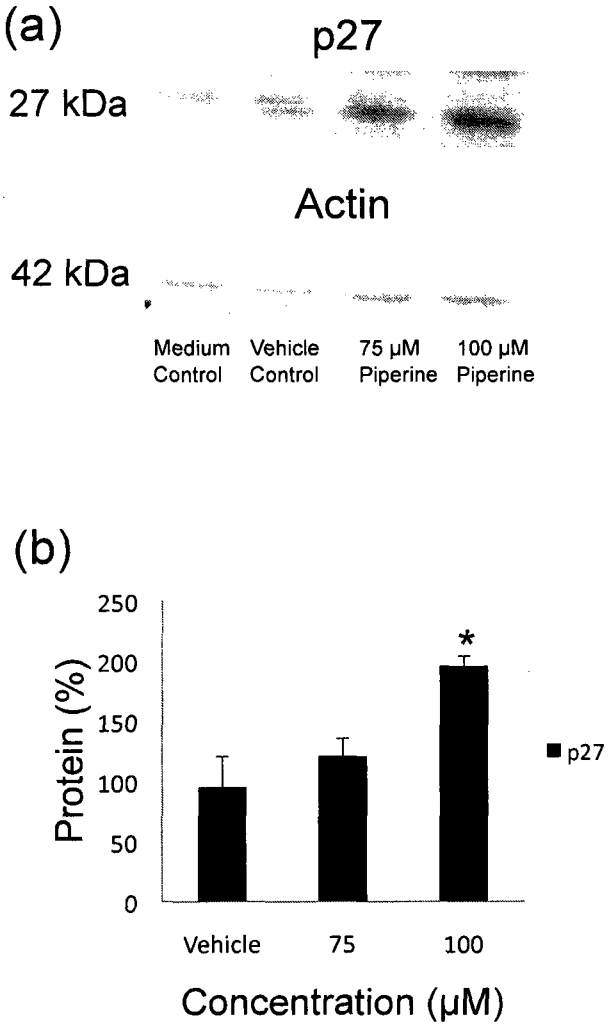
(a)
p27
27 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
250
200
150
100
50
0
Protein (%)
*
p27
Vehicle
75
100
Concentration (µM)

Figure 12.

Figure 13. Piperine Reduces AKT Phosphorylation in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on phospho-AKT as well as total AKT protein expression. Data represent the mean of 3 separate experiments ± standard error. (b) phospho-AKT protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$. (c) Total AKT protein expression relative to actin was also quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Data was not statistically significant in comparison to vehicle control as determined by the Bonferroni multiple comparisons test.

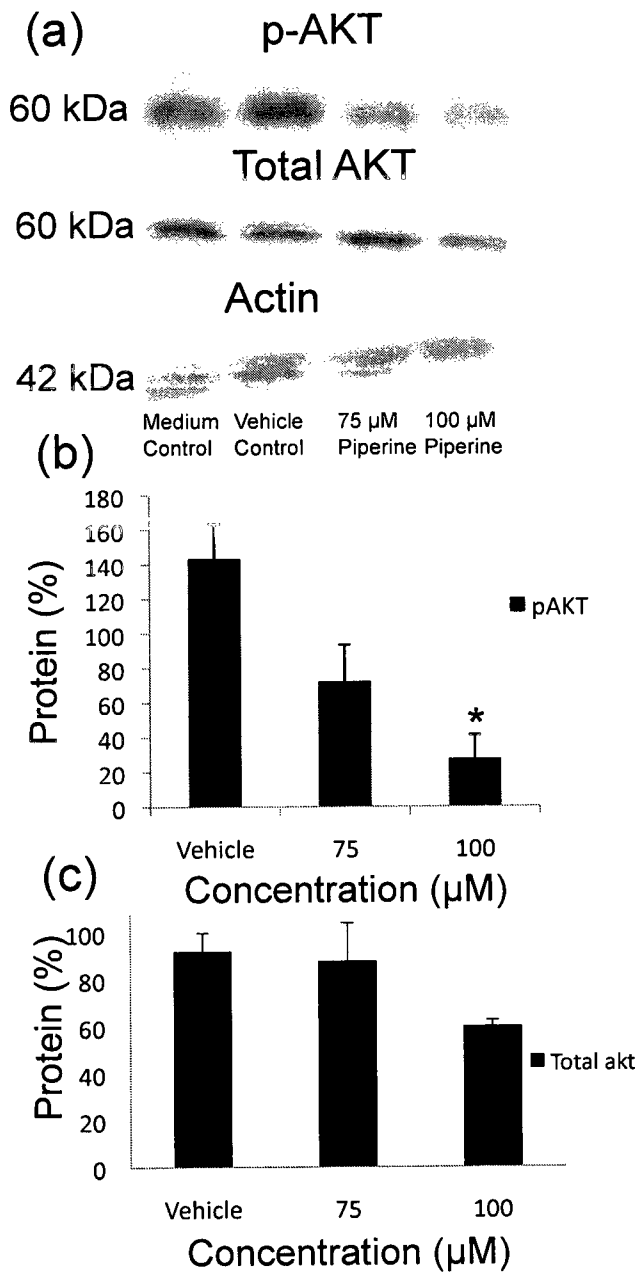
(a)
p-AKT
60 kDa
Total AKT
60 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
180
160
140
120
100
80
60
40
20
0
pAKT
*
Vehicle
75
100
Concentration (μM)
(c)
Protein (%)
100
80
60
40
20
0
Total akt
Vehicle
75
100
Concentration (μM)

Figure 13.

Figure 14. Piperine-Mediated G1 Phase Cell Cycle Arrest Does Not Affect p15 Protein Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on p15 protein expression. Data represent the mean of 3 separate experiments ± standard error. (b) p15 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

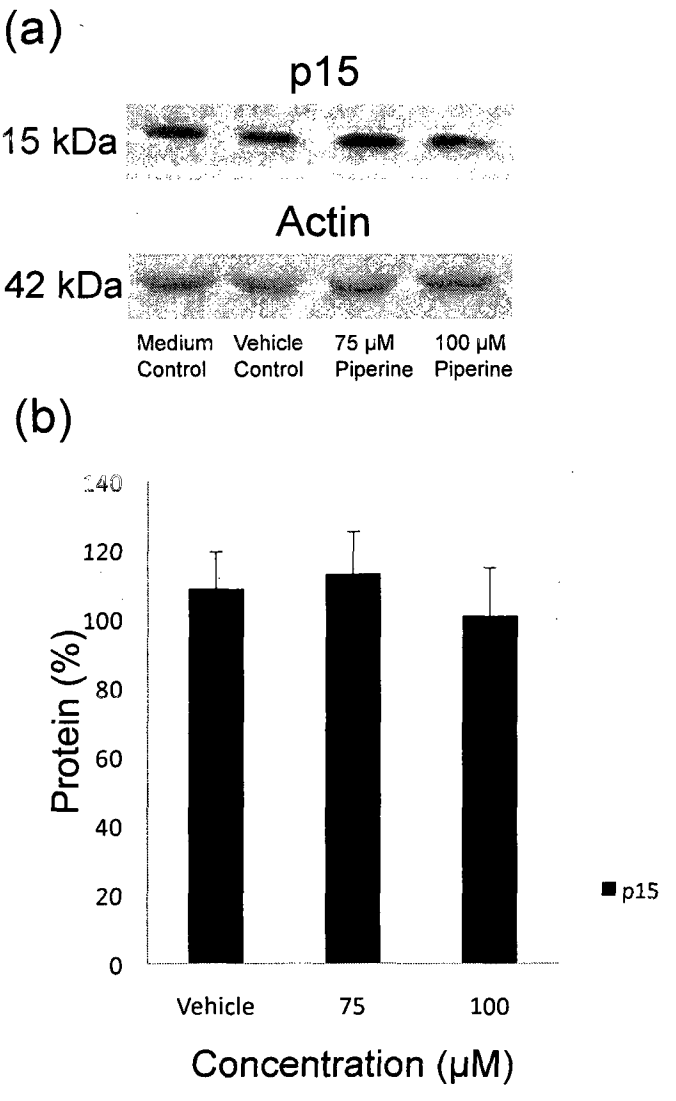
(a)
p15
15 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
140
120
100
80
60
40
20
0
Vehicle
75
100
Concentration (µM)
p15

Figure 14.

Figure 15. Piperine is Cytotoxic to HT-29 Colon Cancer Cells. Cells were treated with predetermined concentrations of piperine (10-150 uM), medium alone or DMSO control for different periods of time (24-144 hours). MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 6 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

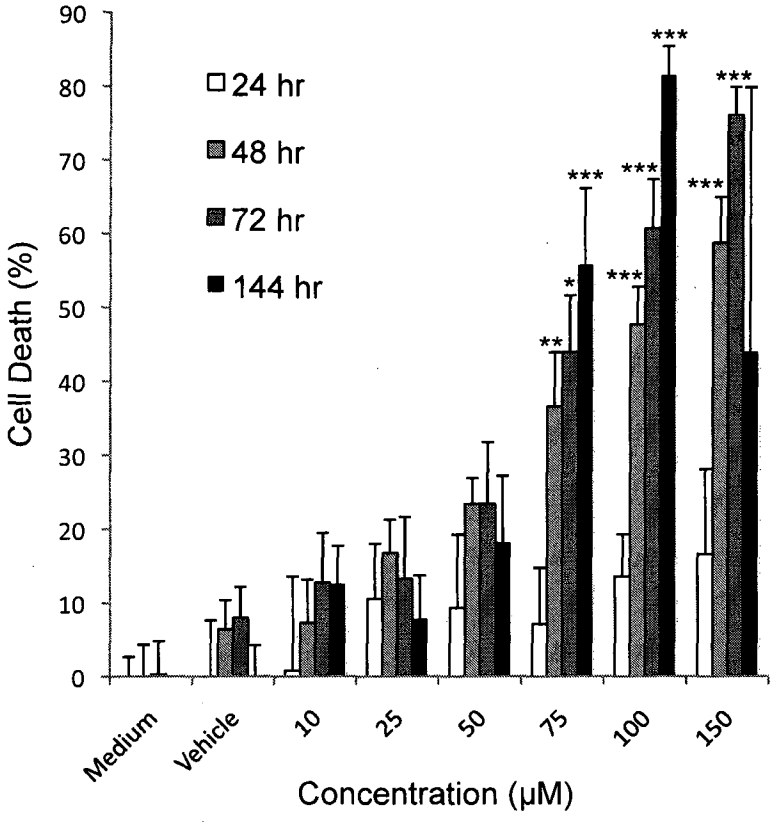
90
80
70
60
50
40
30
20
10
0
Cell Death (%)
24 hr
48 hr
72 hr
144 hr
*
**
***
Medium
Vehicle
10
25
50
75
100
150
Concentration (μM)

Figure 15.

Figure 16. Piperine is Cytotoxic to HRT-18 Rectal Cancer Cells. Cells were treated with predetermined concentrations of piperine (10-150 μM), medium alone or DMSO control for different periods of time (24-144 hours). MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 7 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

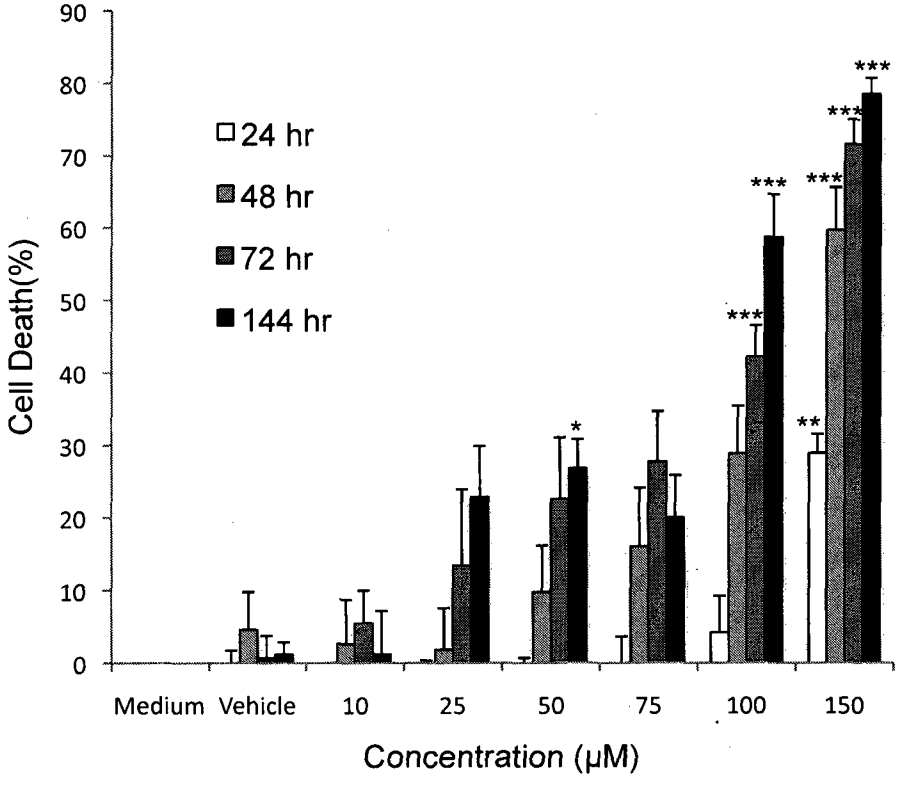
90
80
70
60
50
40
30
20
10
0
Cell Death(%)
24 hr
48 hr
72 hr
144 hr
*
**
***
Medium
Vehicle
10
25
50
75
100
150
Concentration (μM)

Figure 16.

Figure 17. Piperine is Cytotoxic to CaCo-2 Colon Cancer Cells. Cells were treated with predetermined concentrations of piperine (10-100 μM), medium alone or DMSO control for different periods of time (24-144 hours). MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $^{**}p < 0.01$, $^{***}p < 0.001$.

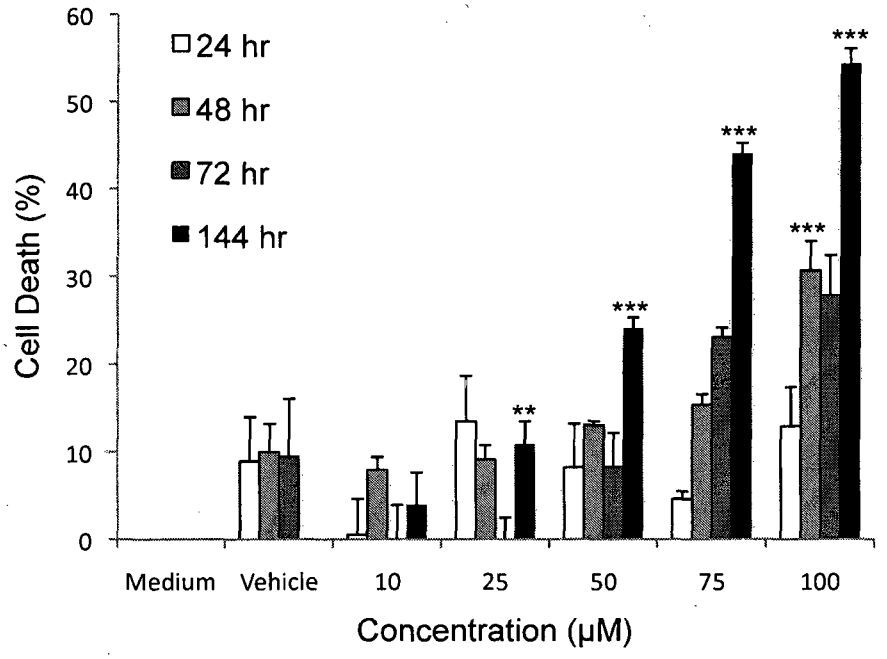
60
50
40
30
20
10
0
Cell Death (%)
24 hr
48 hr
72 hr
144 hr
**
***
***
***
***
Medium
Vehicle
10
25
50
75
100
Concentration (μM)

Figure 17.

Figure 18. Piperine Inhibits Colony Formation by HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 24 hours and plated into 6-well plates. Cells were grown for 13 days, then stained for 15 minutes with 0.4% crystal violet dissolved in methanol. The plates were washed with water and dried for 24 hours. Colonies were counted and compared to cell numbers plated. Data represent the mean of 7 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $^{**}p < 0.01$.

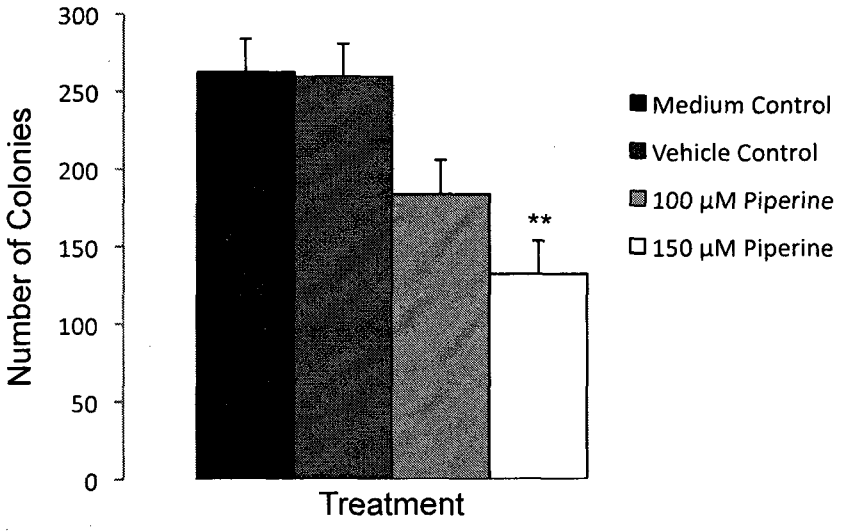
300
250
200
150
100
50
0
Number of Colonies
Treatment
**
Medium Control
Vehicle Control
100 µM Piperine
150 µM Piperine

Figure 18.

Figure 19. Piperine has Reduced Cytotoxicity to HMECs. Cells were treated with predetermined concentrations of piperine (50-150 μM), medium alone or DMSO control for different periods of time (24-72 hours). MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $*p < 0.05$.

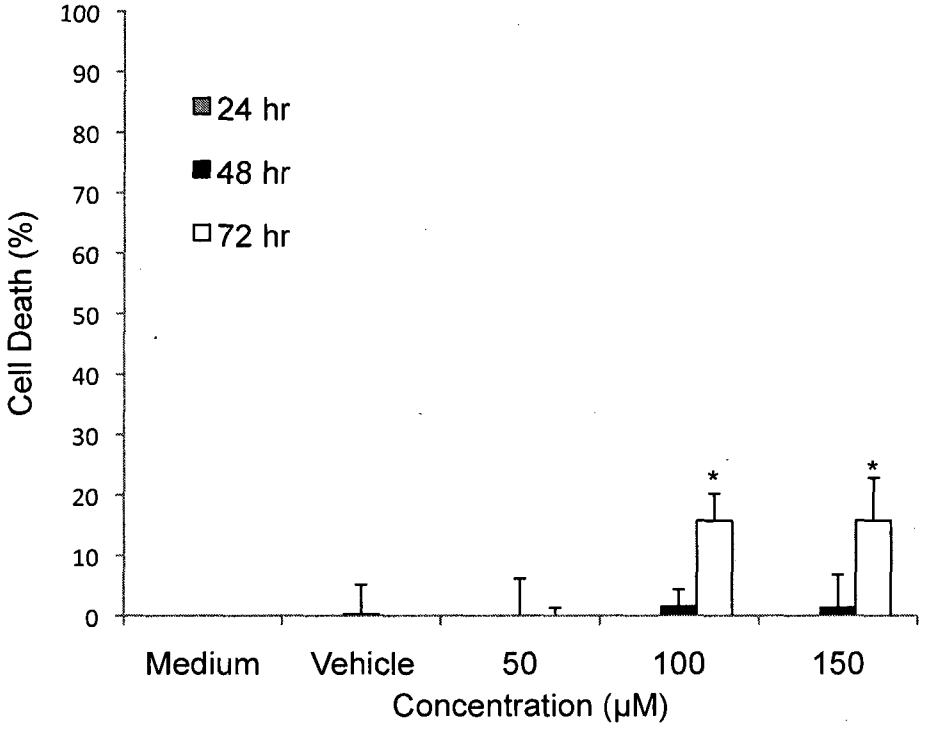
100
90
80
70
60
50
40
30
20
10
0
Cell Death (%)
24 hr
48 hr
72 hr
*
*
Medium
Vehicle
50
100
150
Concentration (μM)

Figure 19.

Figure 20. Piperine Reduces the Number of Viable HT-29 Cells. Medium alone, DMSO control, and piperine-treated cells were harvested after 24-72 hours of culture. Cells were suspended in trypan blue dye for 30 seconds and counted. Data are represent the mean of 3 separate experiments ± standard error. Statistical significance relative to the control was determined by the Bonferroni multiple comparisons test; $*p < 0.05$, $***p < 0.001$.

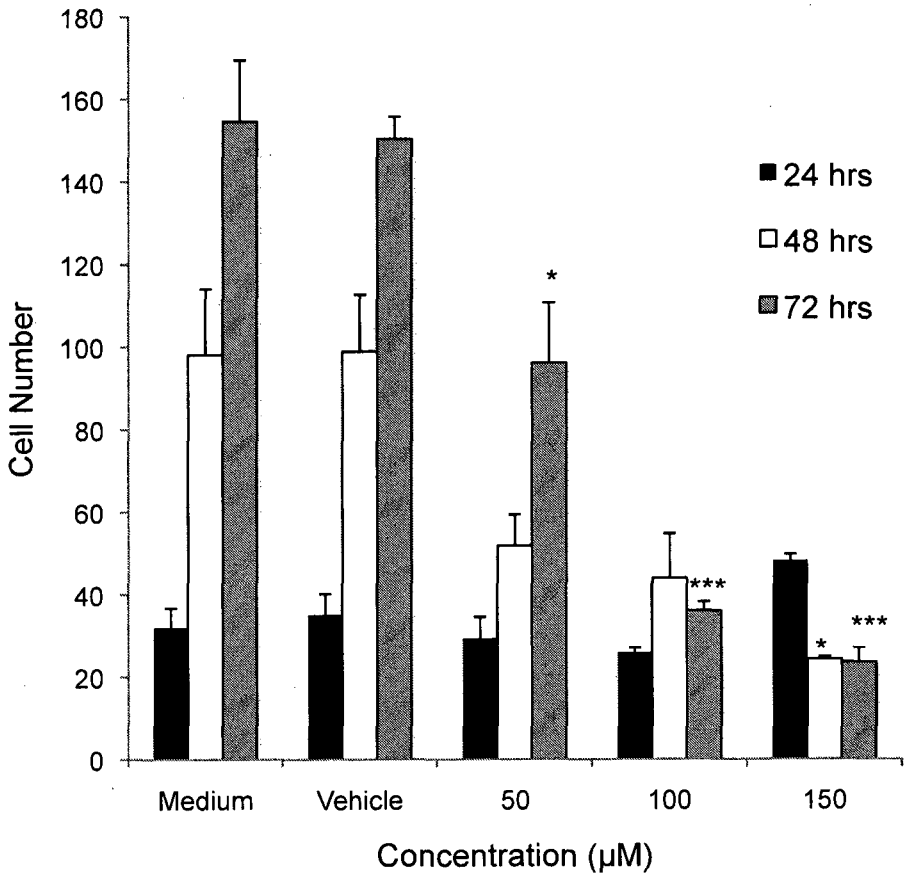
180
160
140
120
100
80
60
40
20
0
Cell Number
24 hrs
48 hrs
72 hrs
*
***
*
***
Medium
Vehicle
50
100
150
Concentration (μM)

Figure 20.

Figure 21. Piperine Induces Apoptosis in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 72 hours of culture. Unstained, Annexin-V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry was then used to determine the relative staining of Annexin-V and propidium iodide to establish the population of viable cells in comparison to those in early apoptosis versus late apoptosis or necrosis. Data are representative of 9 separate experiments. Statistical significance relative to the vehicle control was determined by the Student's t-test using the mean ± standard error of 9 pooled experiments; $p < 0.0001$.

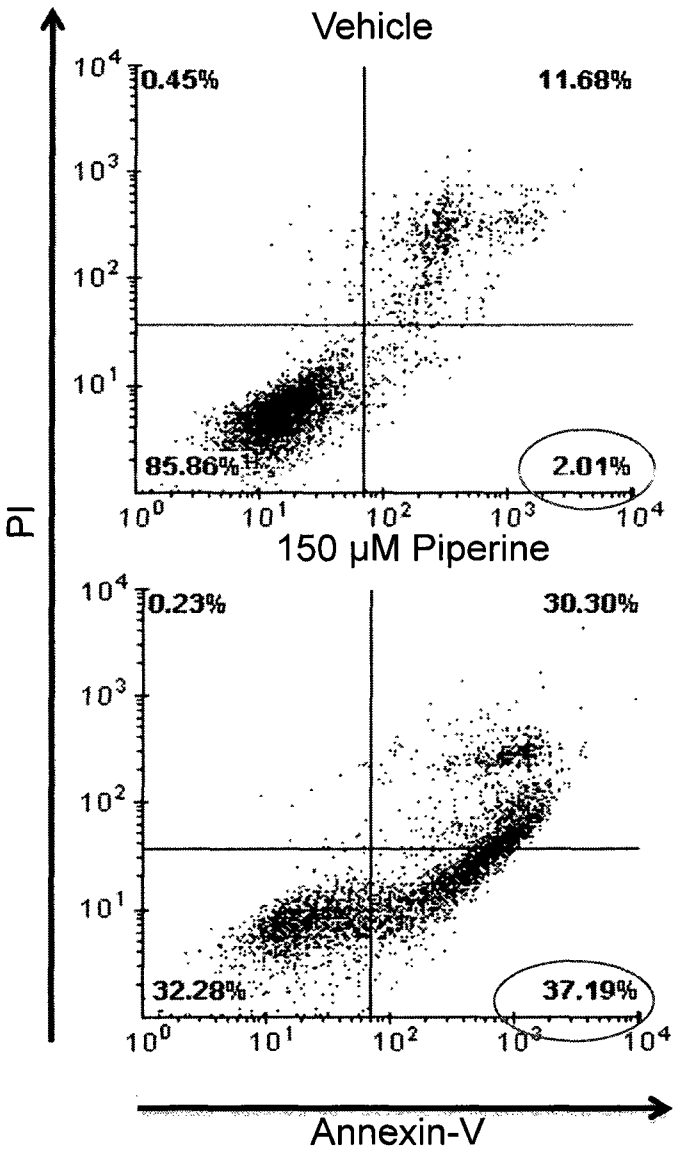
Vehicle
0.45%
11.68%
85.86%
2.01%
150 µM Piperine
0.23%
30.30%
32.28%
37.19%
PI
Annexin-V

Figure 21.

Figure 22. Piperine Induces Apoptosis in HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells were harvested after 72 hours of culture. Unstained, Annexin-V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry was then used to determine the relative staining of Annexin-V and propidium iodide to establish the population of viable cells in comparison to those in early apoptosis versus late apoptosis or necrosis. Data are representative of 6 independent experiments. Statistical significance relative to the vehicle control was determined by the Student's t-test using the mean ± standard error of 6 pooled experiments; p =0.0007

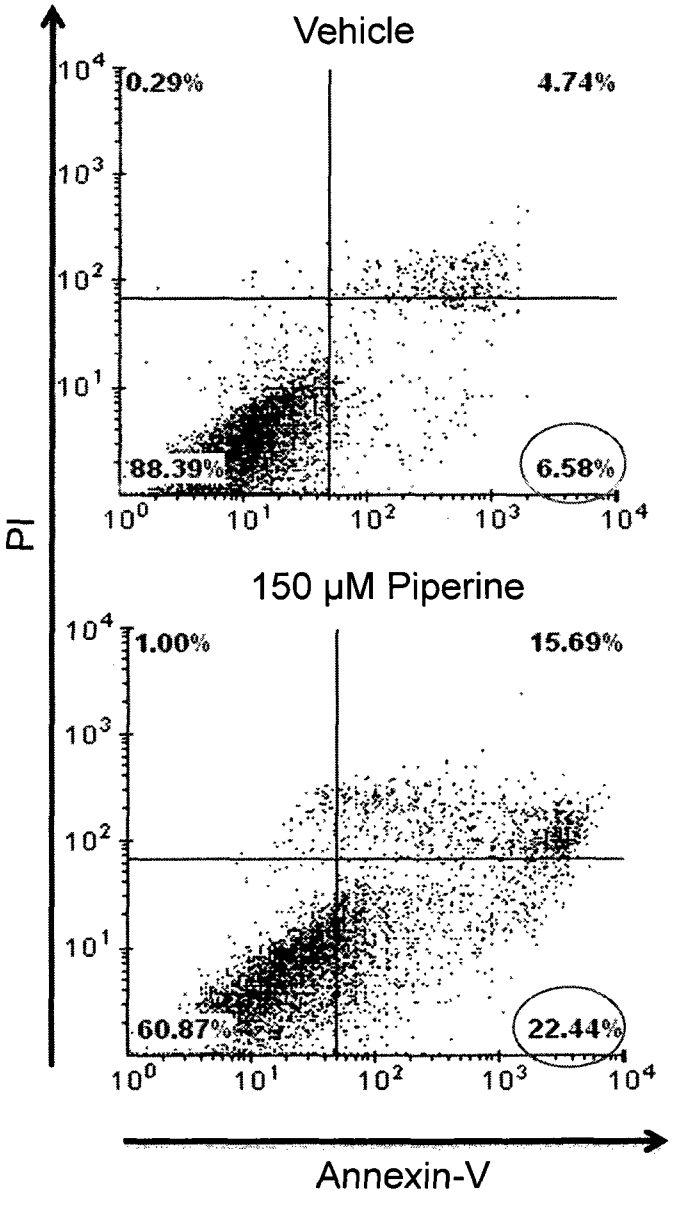
Vehicle
0.29%
4.74%
88.39%
6.58%
150 μM Piperine
1.00%
15.69%
60.87%
22.44%
PI
Annexin-V

Figure 22.

Figure 23. Piperine Causes Minimal Superoxide Anion Production in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. Cells were stained with DHE and analyzed by flow cytometry. Superoxide anion production is typically indicated by a rightward shift as compared to control populations. Data are representative of 6 independent experiments.

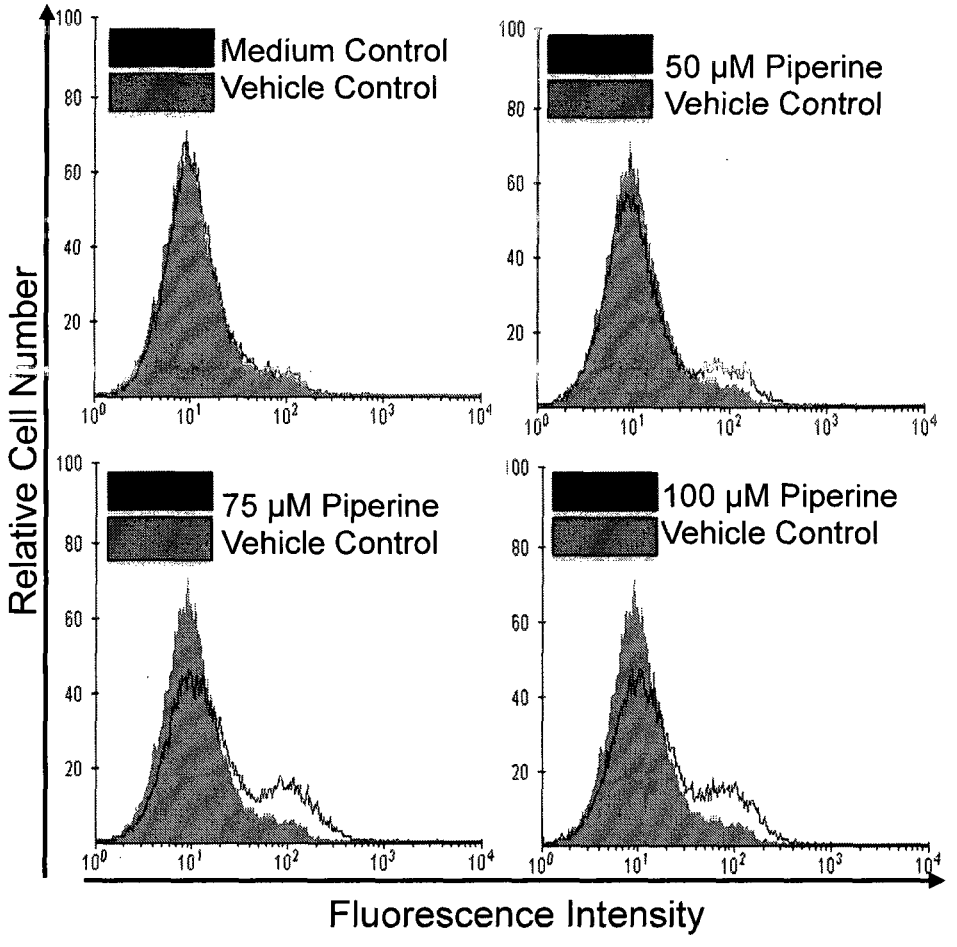
Medium Control
Vehicle Control
50 µM Piperine
Vehicle Control
75 µM Piperine
Vehicle Control
100 µM Piperine
Vehicle Control
Relative Cell Number
Fluorescence Intensity

Figure 23.

Figure 24. Piperine Does Not Cause Glutathione-Sensitive Superoxide Anion Production in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells in the presence or absence of 10mM glutathione pretreatment were harvested after 48 hours of culture. Cells were stained with DHE and analyzed by flow cytometry. Superoxide anion production is typically indicated by a rightward shift as compared to control populations. Data are representative of 6 independent experiments.

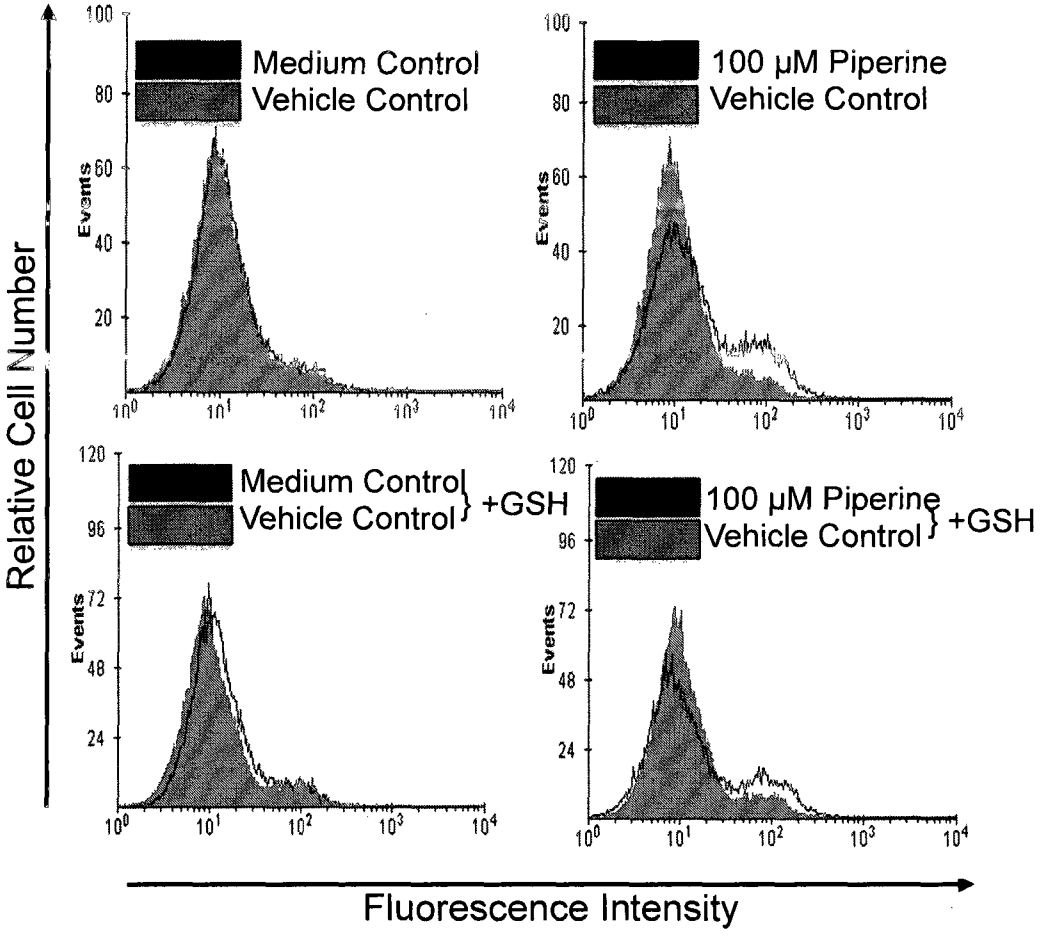
Relative Cell Number
Medium Control
Vehicle Control
100 µM Piperine
Vehicle Control
Medium Control
Vehicle Control
+GSH
100 µM Piperine
Vehicle Control
+GSH
Events
Fluorescence Intensity

Figure 24.

Figure 25. Piperine Causes Minimal Superoxide Anion Production in HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. Cells were stained with DHE and analyzed by flow cytometry. Superoxide anion production is typically indicated by a rightward shift as compared to control populations. Data are representative of 5 independent experiments.

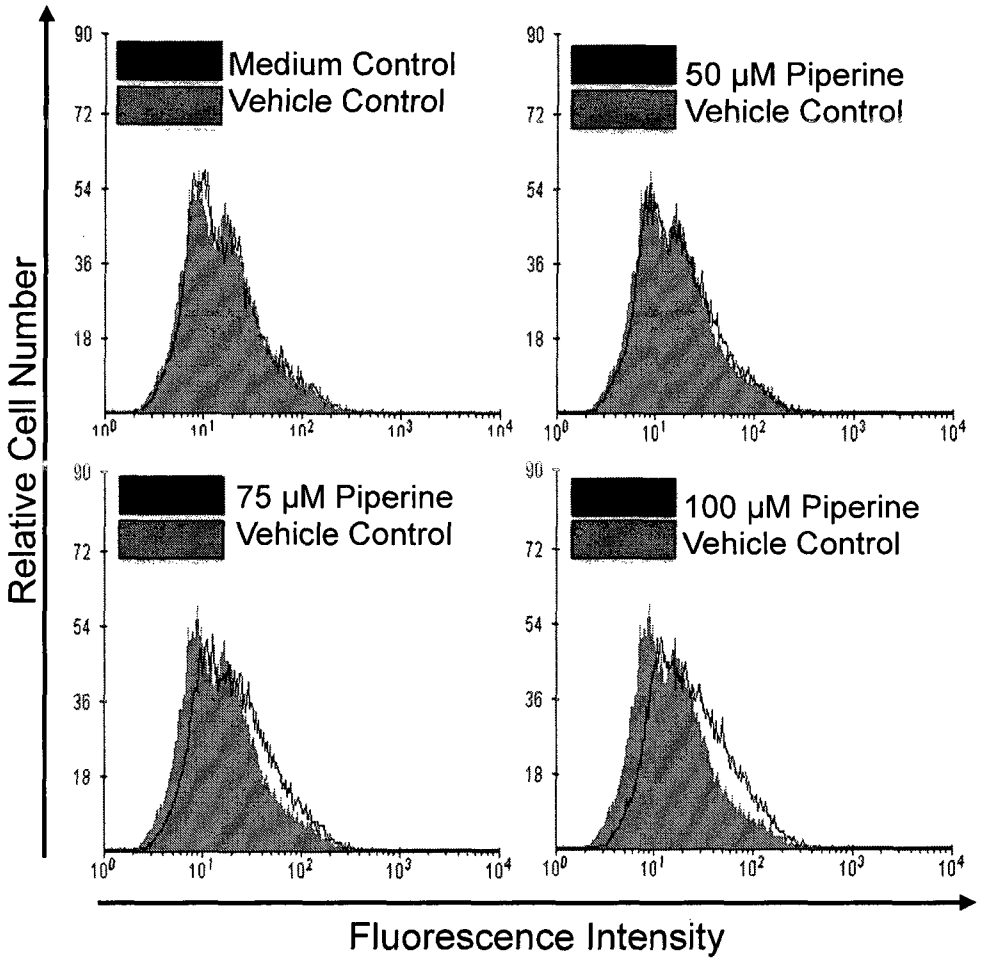
Relative Cell Number
90
72
54
36
18
Medium Control
Vehicle Control
50 µM Piperine
Vehicle Control
75 µM Piperine
Vehicle Control
100 µM Piperine
Vehicle Control
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity

Figure 25.

Figure 26. Piperine Does Not Cause Glutathione-Sensitive Superoxide Anion Production in HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated cells, in the presence or absence of 10mM glutathione pretreatment were harvested after 48 hours of culture. Cells were stained with DHE and analyzed by flow cytometry. Superoxide anion production is typically indicated by a rightward shift as compared to control populations. Data are representative of 5 independent experiments.

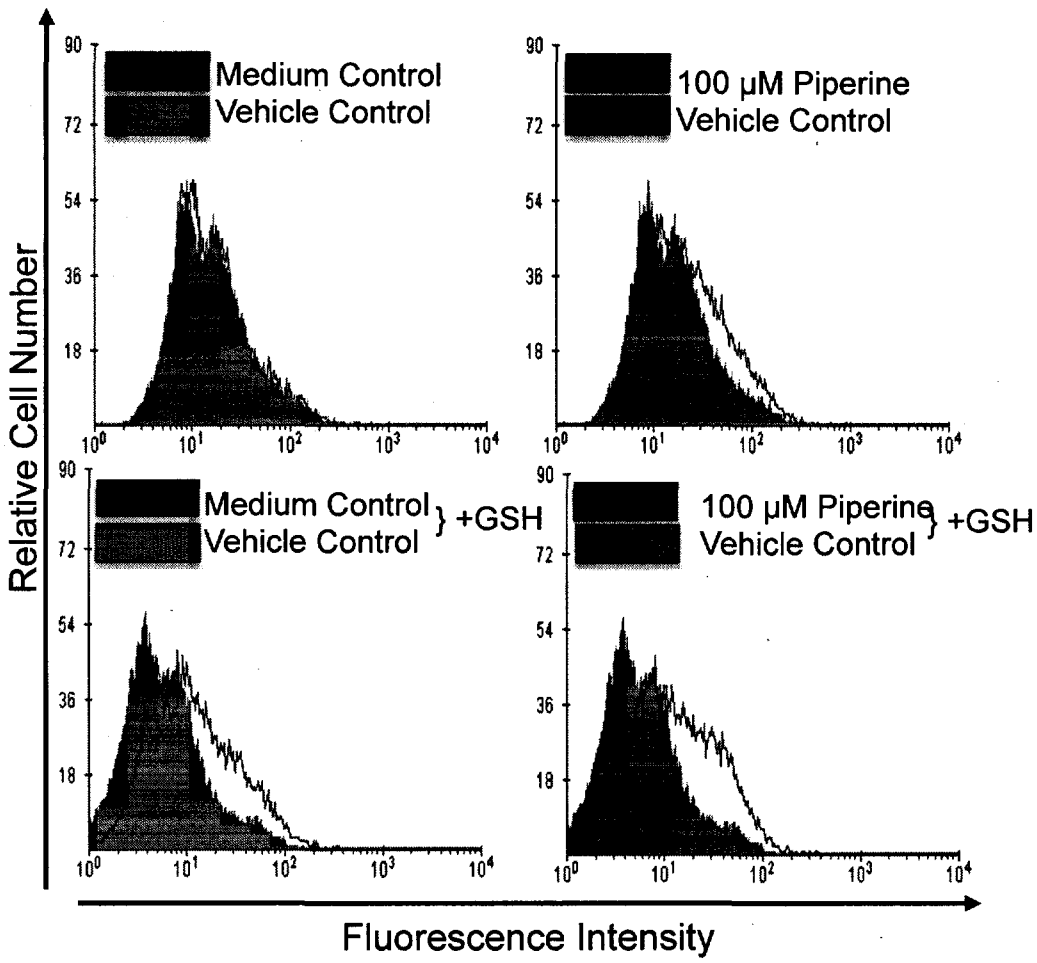
Relative Cell Number
Medium Control
Vehicle Control
100 µM Piperine
Vehicle Control
Medium Control
Vehicle Control
} +GSH
100 µM Piperine
Vehicle Control
} +GSH
90
72
54
36
18
10^0
10^1
10^2
10^3
10^4
Fluorescence Intensity

Figure 26.

Figure 27. Piperine Induces Hydroxyl Radical Production in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. Cells were stained with DCFH-DA and analyzed by flow cytometry. Hydroxyl radical production is typically indicated by a rightward shift as compared to control populations. Data are representative of 3 independent experiments. Statistical significance relative to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 3 pooled experiments; $p < 0.001$.

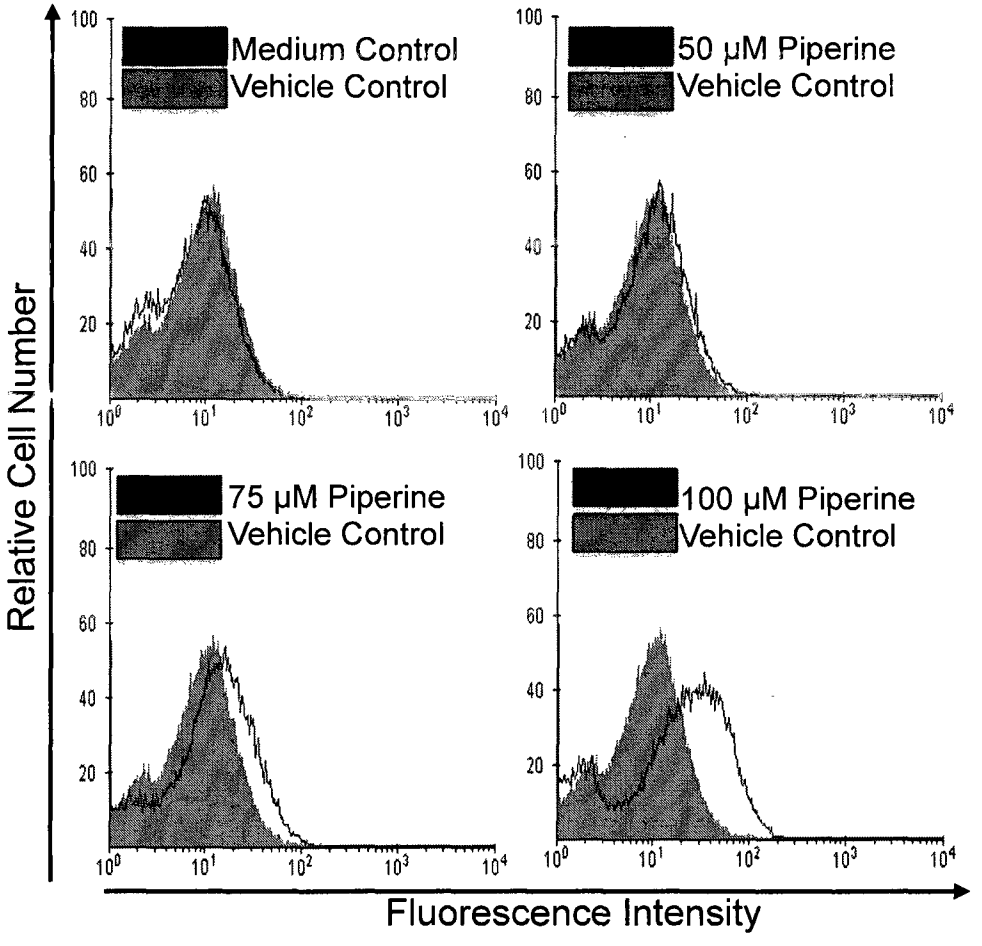
Medium Control
Vehicle Control
50 µM Piperine
Vehicle Control
75 µM Piperine
Vehicle Control
100 µM Piperine
Vehicle Control
Relative Cell Number
Fluorescence Intensity

Figure 27.

Figure 28. Piperine Induces Glutathione-Sensitive Hydroxyl Radical Production in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells in the presence or absence of 10mM glutathione pretreatment were harvested after 48 hours of culture. Cells were stained with DCFH-DA and analyzed by flow cytometry. Hydroxyl radical production is typically indicated by a rightward shift as compared to control populations. Data are representative of 3 independent experiments.

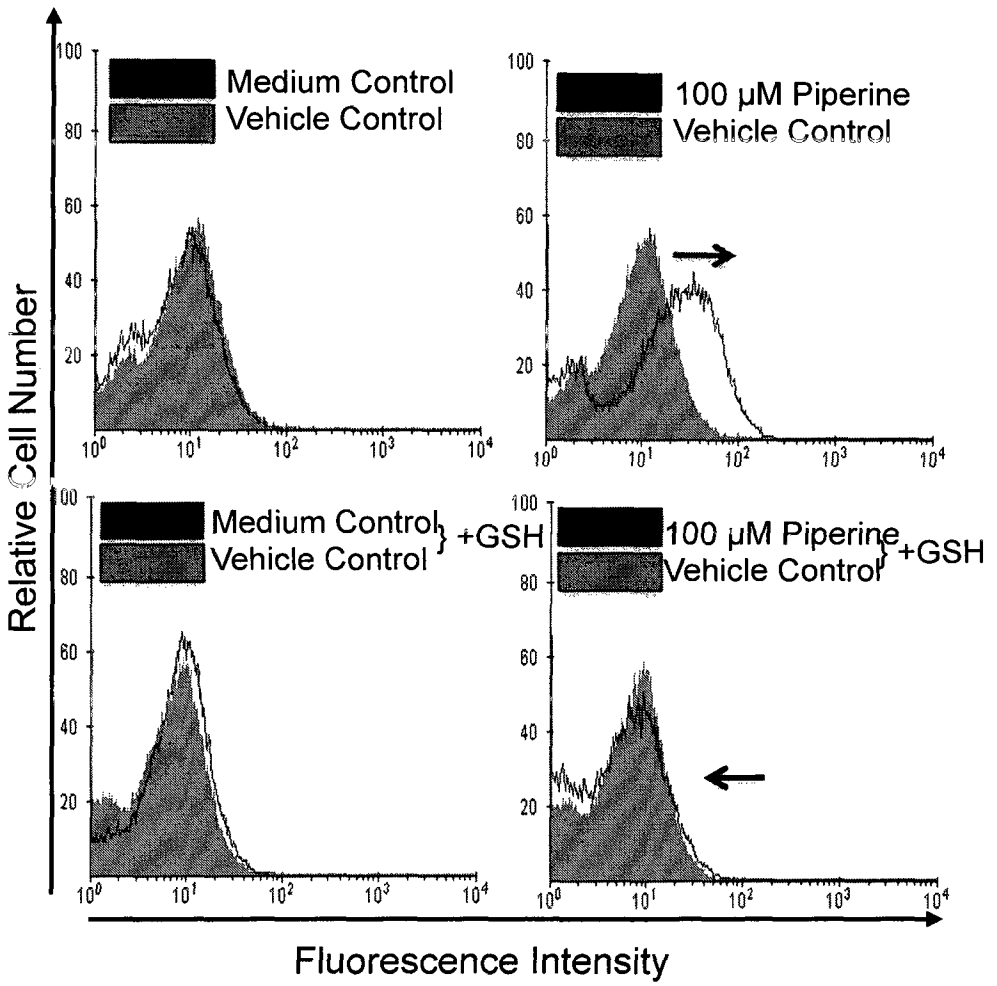
Medium Control
Vehicle Control
100 µM Piperine
Vehicle Control
Medium Control
Vehicle Control
+GSH
100 µM Piperine
Vehicle Control
+GSH
Relative Cell Number
Fluorescence Intensity

Figure 28.

Figure 29. Piperine Induces Hydroxyl Radical Production in HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells were harvested after 48 hours of culture. Cells were stained with DCFH-DA and analyzed by flow cytometry. Hydroxyl radical production is typically indicated by a rightward shift as compared to control populations. Data are representative of 3 independent experiments. Statistical significance relative to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 3 pooled experiments; $p < 0.05$.

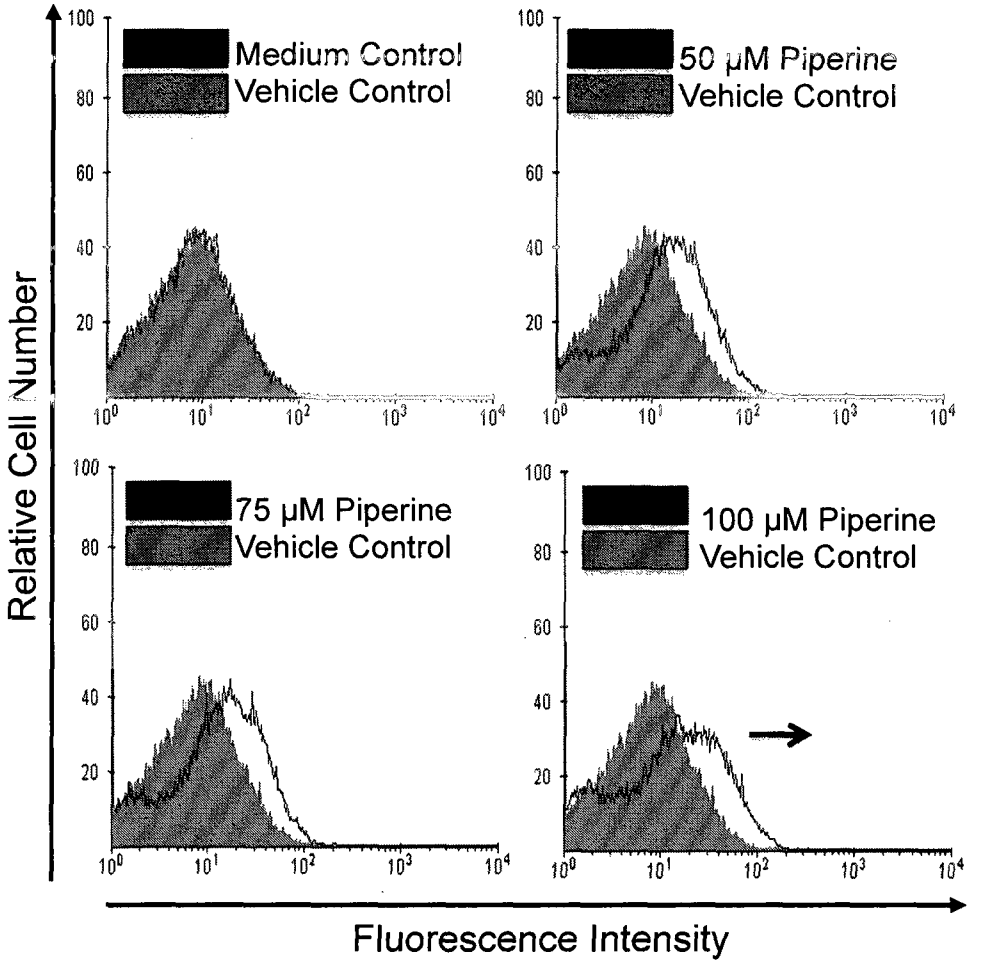
Relative Cell Number
Fluorescence Intensity
Medium Control
Vehicle Control
50 µM Piperine
Vehicle Control
75 µM Piperine
Vehicle Control
100 µM Piperine
Vehicle Control

Figure 29.

Figure 30. Piperine Induces Glutathione-Sensitive Hydroxyl Radical Production in HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells in the presence or absence of 10mM glutathione pretreatment were harvested after 48 hours of culture. Cells were stained with DCFH-DA and analyzed by flow cytometry. Hydroxyl radical production is typically indicated by a rightward shift as compared to control populations. Data are representative of 3 independent experiments.

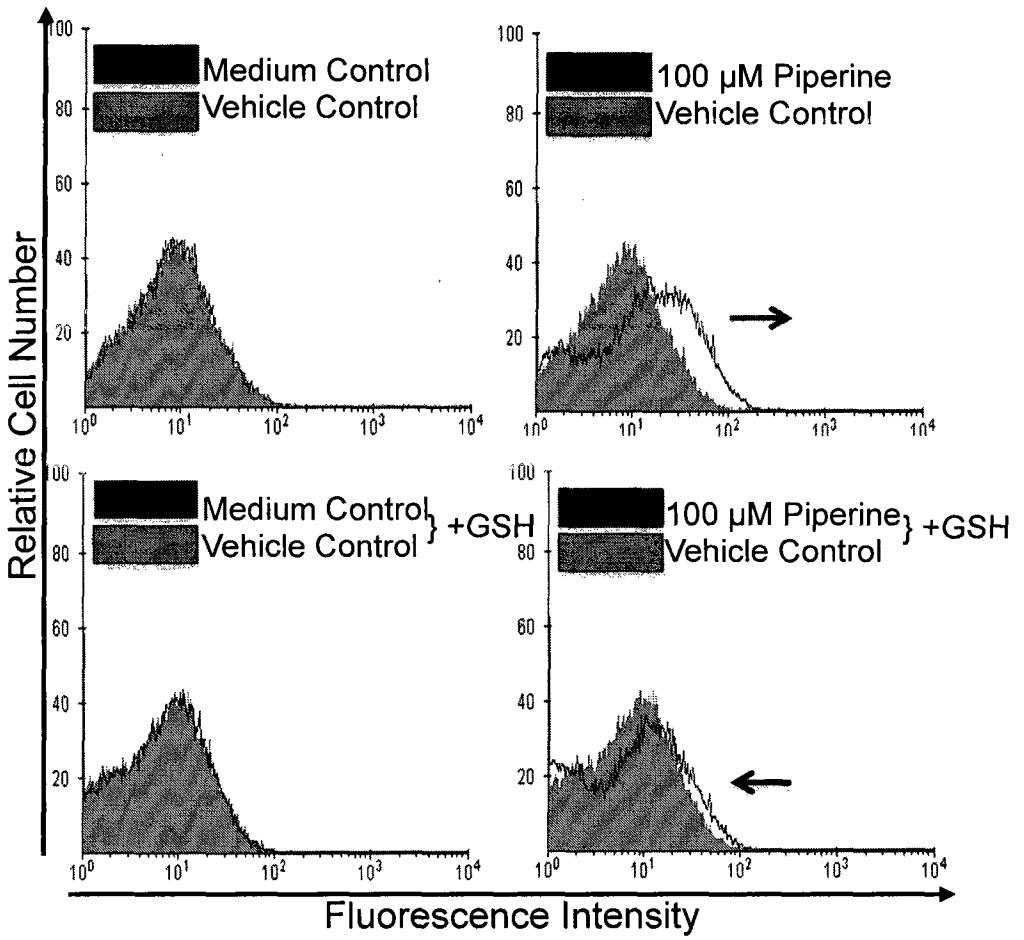
Relative Cell Number
Fluorescence Intensity
Medium Control
Vehicle Control
100 µM Piperine
Vehicle Control
Medium Control
Vehicle Control
} +GSH
100 µM Piperine
Vehicle Control
} +GSH

Figure 30.

Figure 31. Piperine-Mediated Apoptosis is Partially Reversed by Glutathione Pretreatment of HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells pretreated with or without 10 mM glutathione were harvested after 72 hours of culture. Unstained, Annexin-V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry data are representative of 6 independent experiments. Histograms show the mean of 6 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 3 pooled experiments; $*p < 0.05$, $***p < 0.001$.

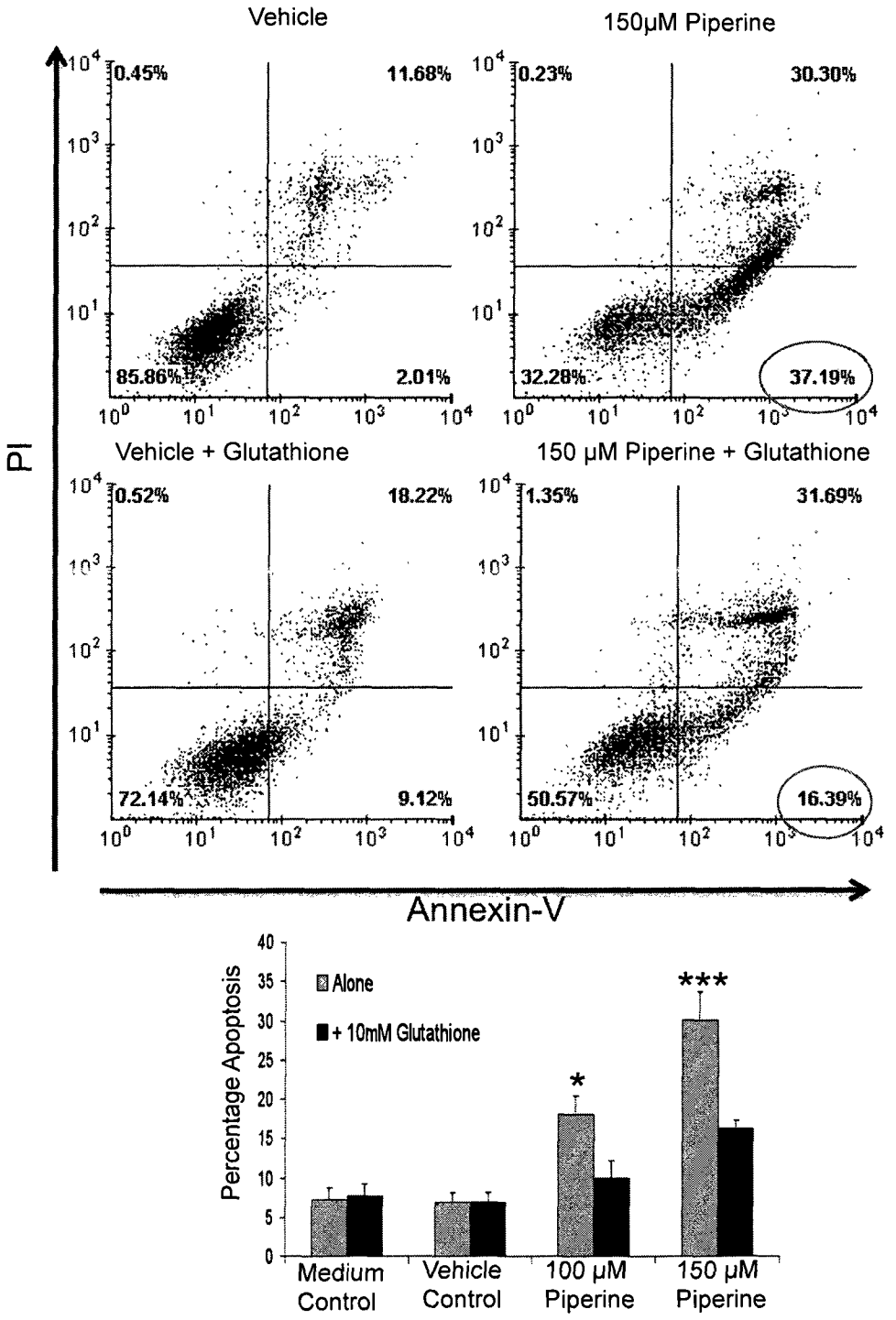
Vehicle
150μM Piperine
0.45%
11.68%
85.86%
2.01%
0.23%
30.30%
32.28%
37.19%
Vehicle + Glutathione
150 μM Piperine + Glutathione
0.52%
18.22%
72.14%
9.12%
1.35%
31.69%
50.57%
16.39%
PI
Annexin-V
Percentage Apoptosis
Alone
+ 10mM Glutathione
*
***
Medium Control
Vehicle Control
100 μM Piperine
150 μM Piperine

Figure 31.

Figure 32. Piperine-Mediated Apoptosis is Partially Reversed by N-acetylcysteine Pretreatment of HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells pretreated with or without 10mM N-acetylcysteine were harvested after 72 hours of culture. Unstained, Annexin V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry data are representative of 3 independent experiments. Histograms show the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 3 pooled experiments; $*p < 0.05$, $**p < 0.01$.

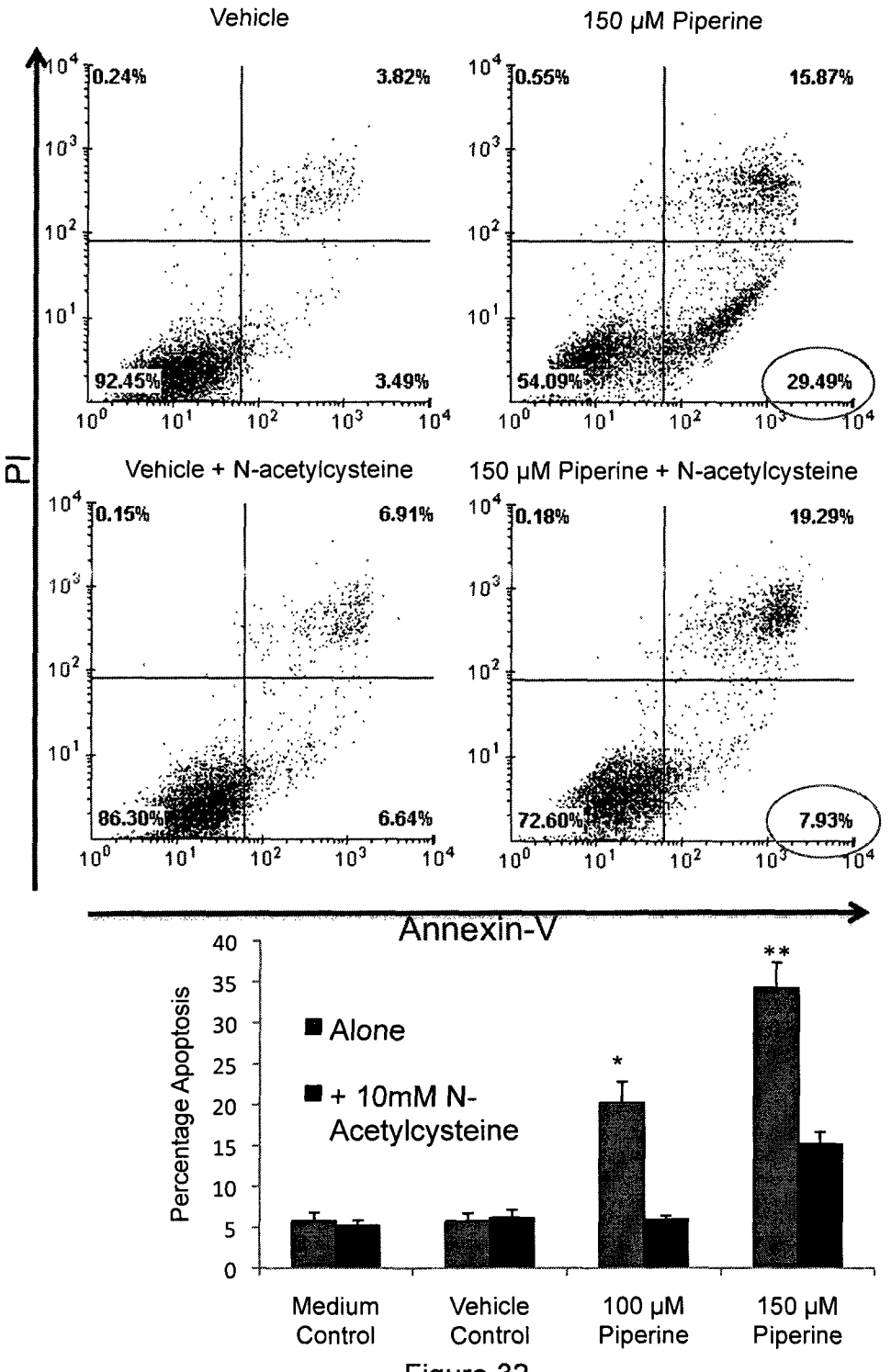
Vehicle
150 μM Piperine
0.24%
3.82%
92.45%
3.49%
0.55%
15.87%
54.09%
29.49%
Vehicle + N-acetylcysteine
150 μM Piperine + N-acetylcysteine
0.15%
6.91%
86.30%
6.64%
0.18%
19.29%
72.60%
7.93%
PI
Annexin-V
Percentage Apoptosis
Alone
+ 10mM N-Acetylcysteine
Medium Control
Vehicle Control
100 μM Piperine
150 μM Piperine

Figure 32.

Figure 33. Piperine-Mediated Apoptosis is Not Reversed by Glutathione Pretreatment of HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells pretreated with or without 10mM glutathione were harvested after 72 hours of culture. Unstained, Annexin-V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry data are representative of 4 independent experiments. Histograms show the mean of 4 separate experiments ± standard error.

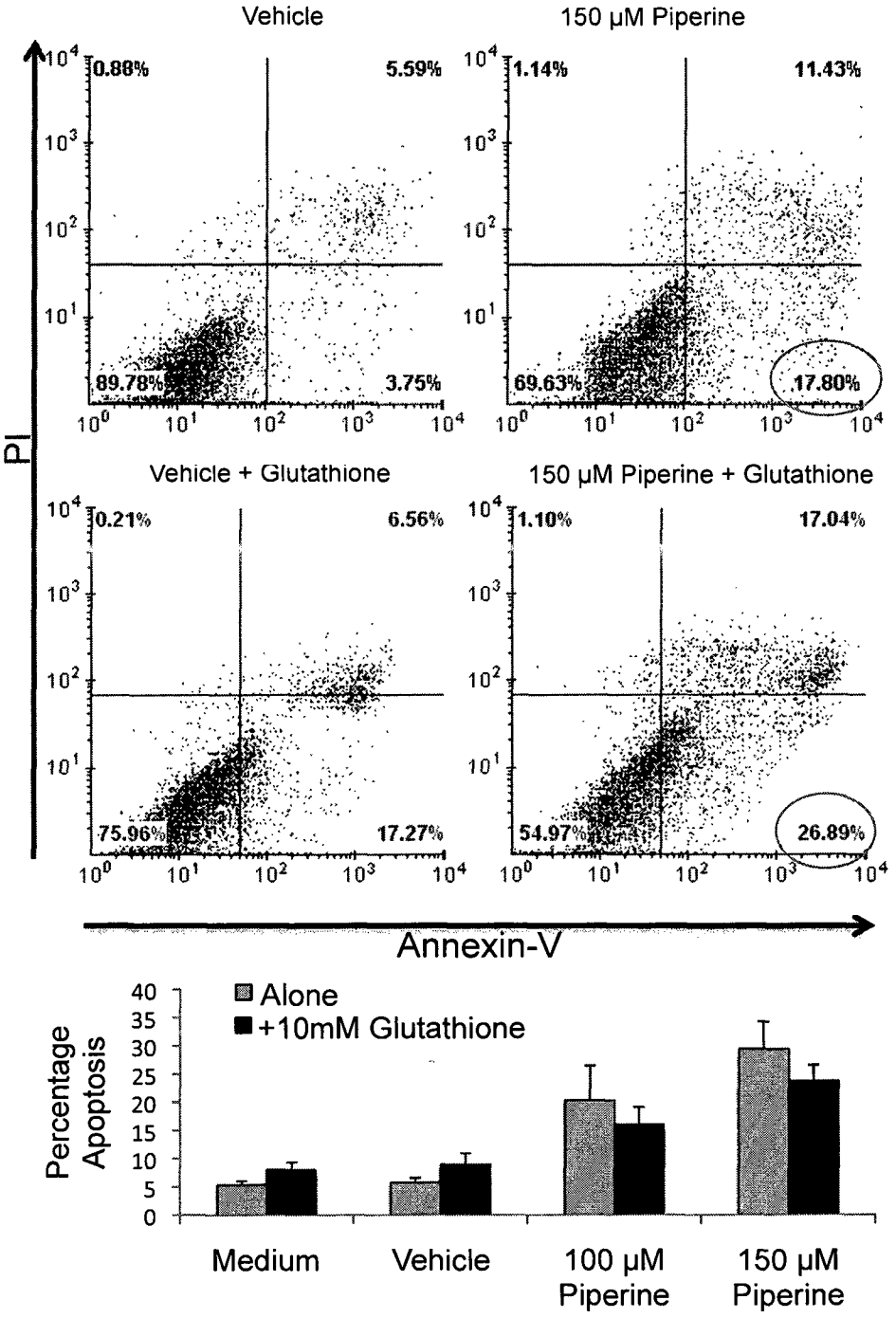
Vehicle
150 μM Piperine
0.88%
5.59%
89.78%
3.75%
1.14%
11.43%
69.63%
17.80%
Vehicle + Glutathione
150 μM Piperine + Glutathione
0.21%
6.56%
75.96%
17.27%
1.10%
17.04%
54.97%
26.89%
PI
Annexin-V
Alone
+10mM Glutathione
Percentage Apoptosis
Medium
Vehicle
100 μM Piperine
150 μM Piperine

Figure 33.

Figure 34. Piperine-Mediated Apoptosis is Partially Reversed by N-acetylcysteine Pretreatment of HRT-18 Rectal Cancer Cells. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells pretreated with or without 10 mM N-acetylcysteine were harvested after 72 hours of culture. Unstained, Annexin-V-stained and propidium iodide-stained controls were used to establish quadrants, for comparison of the control and treatment groups. Flow cytometry data are representative of 3 independent experiments. Histograms show the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 3 pooled experiments; $*p < 0.05$, $**p < 0.01$.

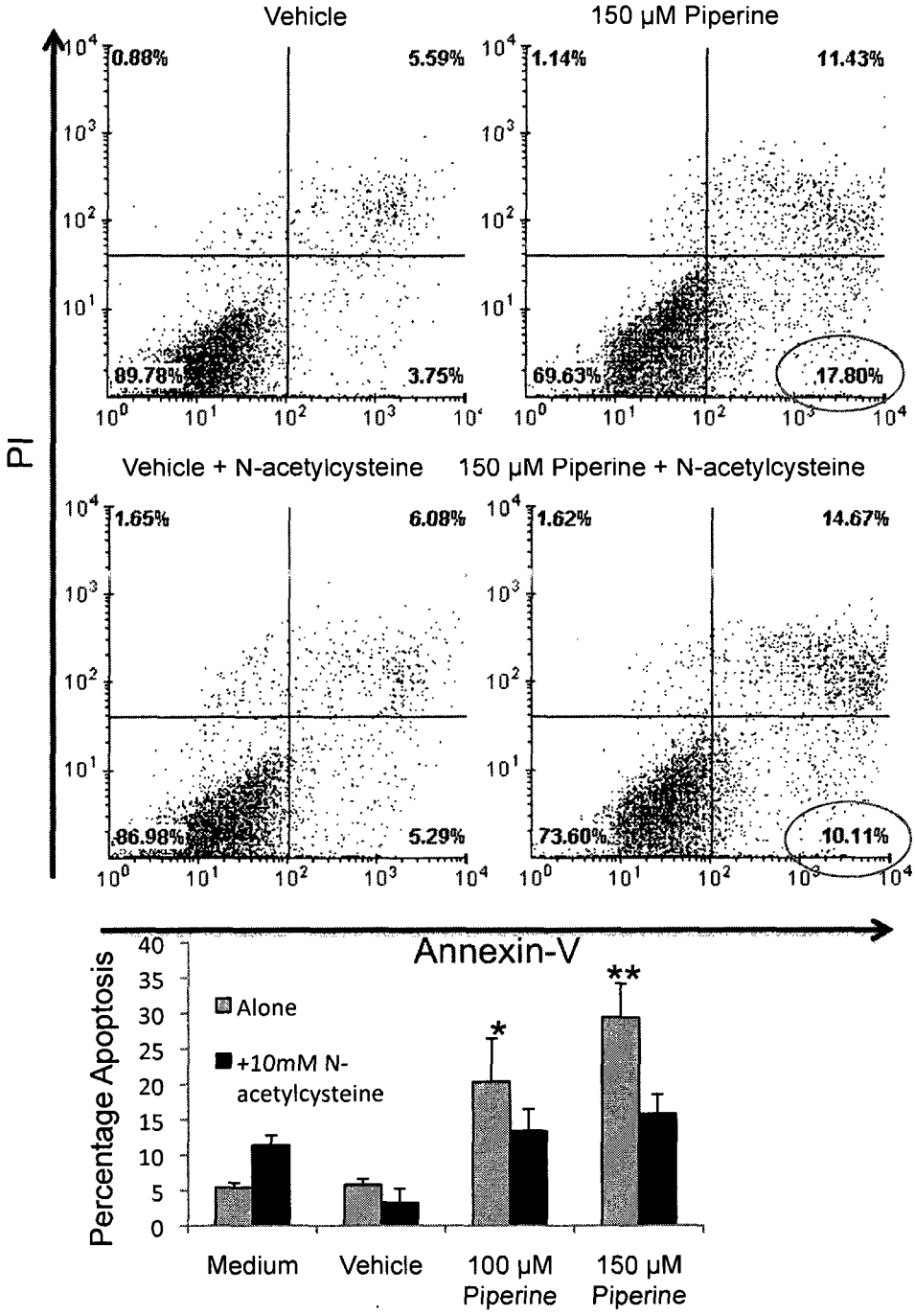
Vehicle
150 µM Piperine
PI
0.88%
5.59%
89.78%
3.75%
1.14%
11.43%
69.63%
17.80%
Vehicle + N-acetylcysteine
150 µM Piperine + N-acetylcysteine
1.65%
6.08%
86.98%
5.29%
1.62%
14.67%
73.60%
10.11%
Annexin-V
Percentage Apoptosis
Alone
+10mM N-acetylcysteine
Medium
Vehicle
100 µM Piperine
150 µM Piperine

Figure 34.

Figure 35. Piperine-Mediated Cytotoxicity in HT-29 Colon Cancer Cell Cultures is Sensitive to N-acetylcysteine. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells with or without 10mM N-acetylcysteine pretreatment were harvested after 72 hours of culture. Cells were stained with 0.4% crystal violet for 15 minutes. Stain was subsequently washed off the cells and the plates were left to dry overnight. Colony counting was then performed. Data are representative of 4 independent experiments. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test using the mean ± standard error of 4 pooled experiments; $*p < 0.05$, $**p < 0.01$.

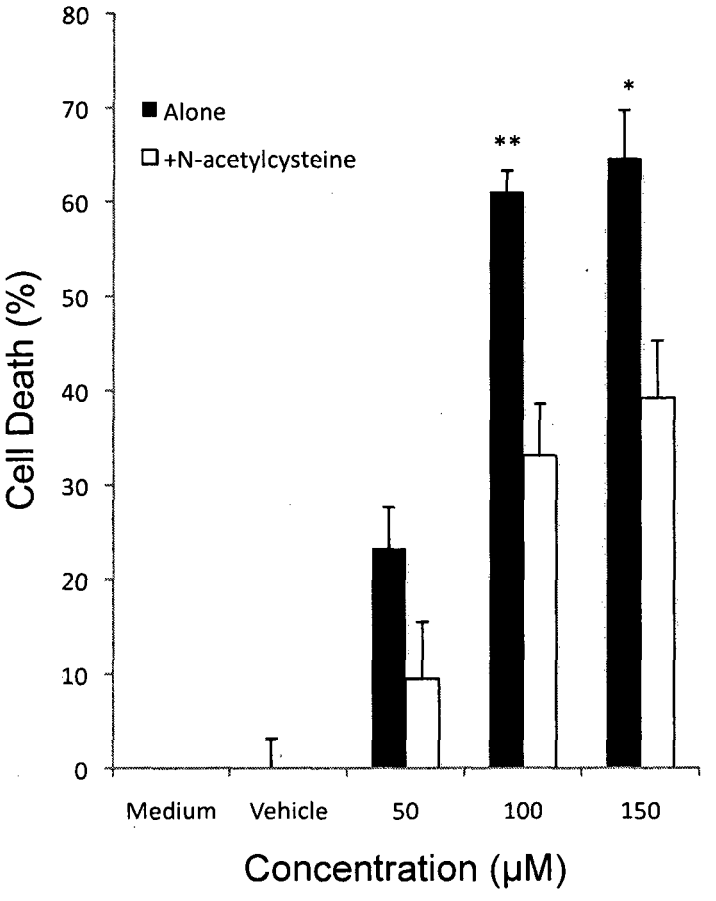
80
70
60
50
40
30
20
10
0
Cell Death (%)
Alone
+N-acetylcysteine
**
*
Medium
Vehicle
50
100
150
Concentration (μM)

Figure 35.

Figure 36. Piperine-Mediated Cytotoxicity in HT-29 Colon Cancer Cell Cultures is Sensitive to Glutathione. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells with or without10mM glutathione pretreatment were harvested after 72 hours of culture. Cells were stained with 0.4% crystal violet for 15 minutes. Stain was subsequently washed off the cells and the plates were left to dry overnight. Colony counting was then performed. Data represent 5 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; $*p < 0.05$, $***p < 0.001$.

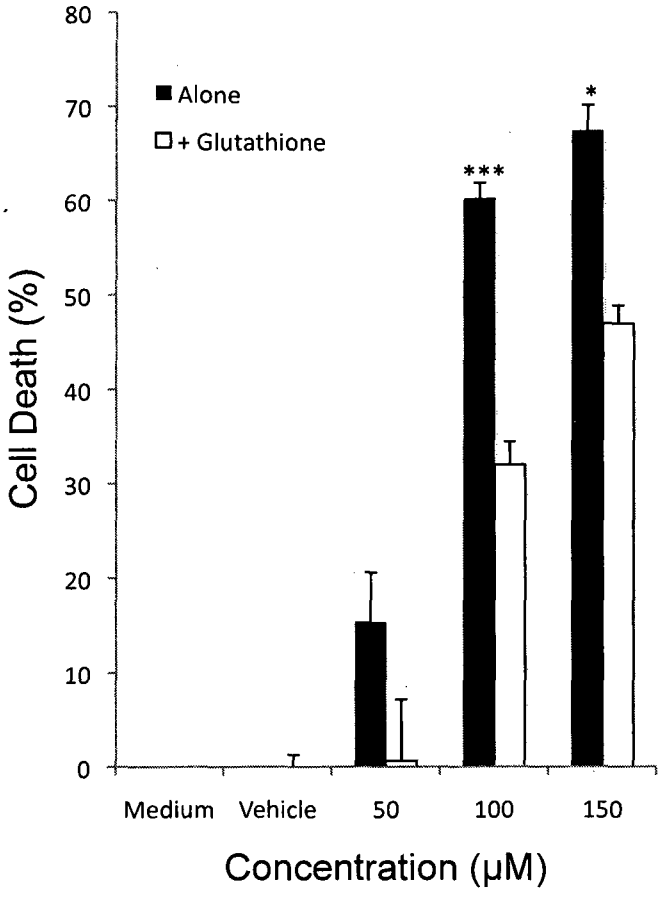
80
70
60
50
40
30
20
10
0
Cell Death (%)
Alone
+ Glutathione
***
*
Medium
Vehicle
50
100
150
Concentration (μM)

Figure 36.

Figure 37. Piperine-Mediated Cytotoxicity in HRT-18 Rectal Cancer Cell Cultures is Not Sensitive to N-acetylcysteine. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells with or without 10mM N-acetylcysteine pretreatment were harvested after 72 hours of culture. Cells were stained with 0.4% crystal violet for 15 minutes. Stain was subsequently washed off the cells and the plates were left to dry overnight. Colony counting was then performed. Data represent 4 separate experiments ± standard error.

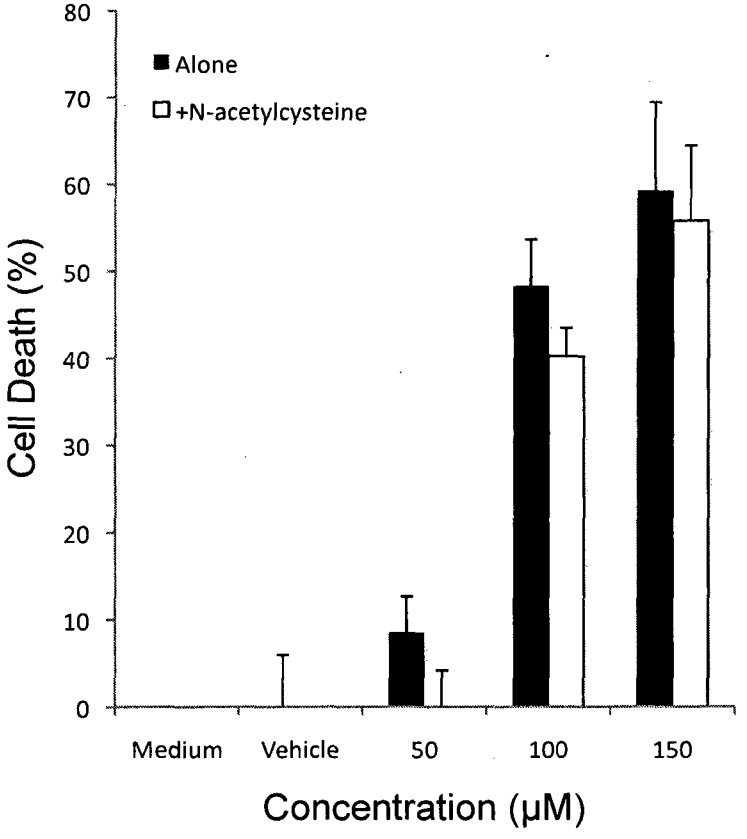
80
70
60
50
40
30
20
10
0
Alone
+N-acetylcysteine
Cell Death (%)
Medium
Vehicle
50
100
150
Concentration (μM)

Figure 37.

Figure 38. Piperine Causes Cytochrome c Release in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 and 72 hours of culture. (a, c) Digitonin lysis was performed to collect the cytosolic fraction of the cell and western blotting of protein lysates was performed to determine the effect of piperine on cytochrome c protein levels in the cytoplasm. Data are representative of 3 independent experiments. (b, d) Cytochrome c protein levels relative to actin were quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

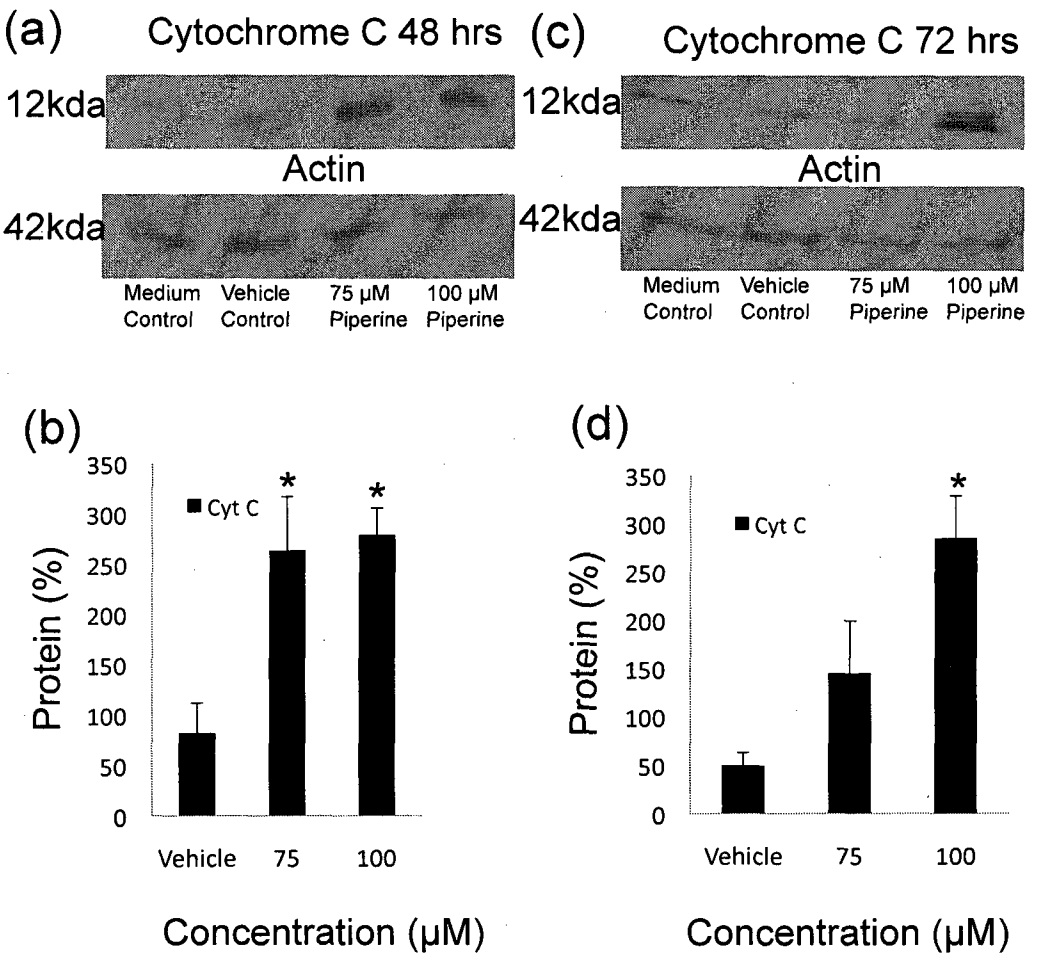
(a)
Cytochrome C 48 hrs
12kda
Actin
42kda
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(c)
Cytochrome C 72 hrs
12kda
Actin
42kda
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
350
300
250
200
150
100
50
0
Cyt C
*
*
Vehicle
75
100
Concentration (µM)
(d)
Protein (%)
350
300
250
200
150
100
50
0
Cyt C
*
Vehicle
75
100
Concentration (µM)

Figure 38.

Figure 39. Piperine-Mediated Cytotoxicity is Caspase-Independent in HT-29 Colon Cancer Cells. Medium alone, DMSO control, and piperine-treated HT-29 cells with or without Boc-D-fmk pretreatment were cultured for 72 hours. MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error.

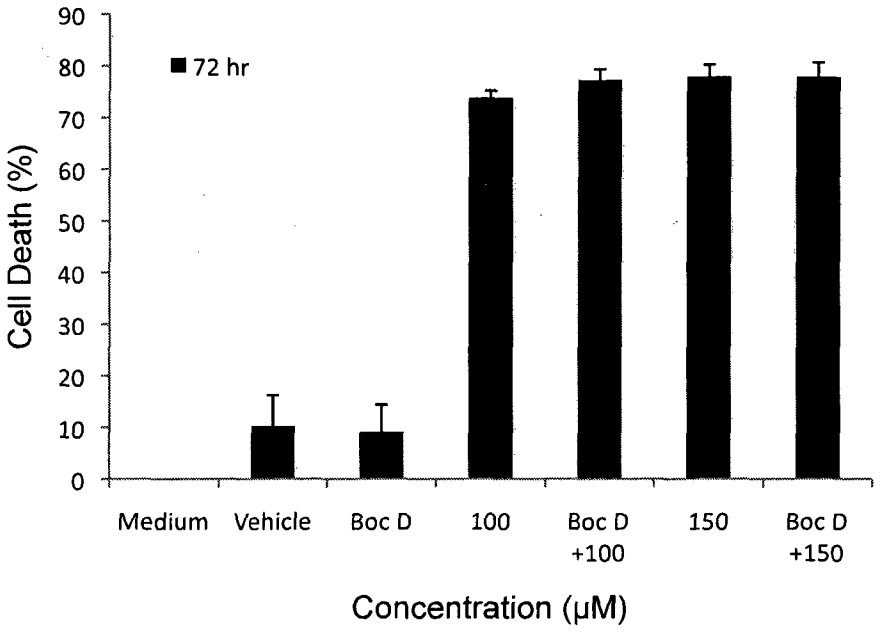
90
80
70
60
50
40
30
20
10
0
Cell Death (%)
72 hr
Medium
Vehicle
Boc D
100
Boc D +100
150
Boc D +150
Concentration (μM)

Figure 39.

Figure 40. Piperine Does Not Reduce Procaspase-3 Levels in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of treatment of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on procaspase-3 protein expression. Data are representative of 3 independent experiments. (b) Procaspase-3 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

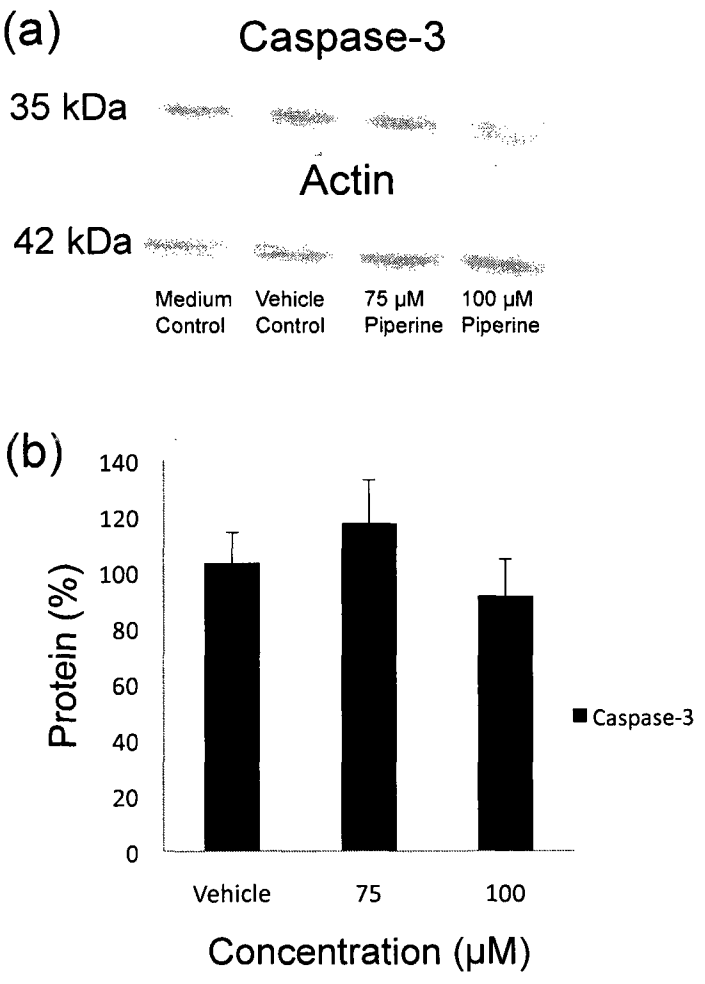
(a)
Caspase-3
35 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
140
120
100
80
60
40
20
0
Vehicle
75
100
Concentration (μM)
Caspase-3

Figure 40.

Figure 41. Piperine Does Not Reduce Procaspase-9 Levels in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of treatment. (a) Western blotting of protein lysates was performed to determine the effect of piperine on protein expression of procaspase-9. Data are representative of 3 independent experiments. (b) Procaspase-3 protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

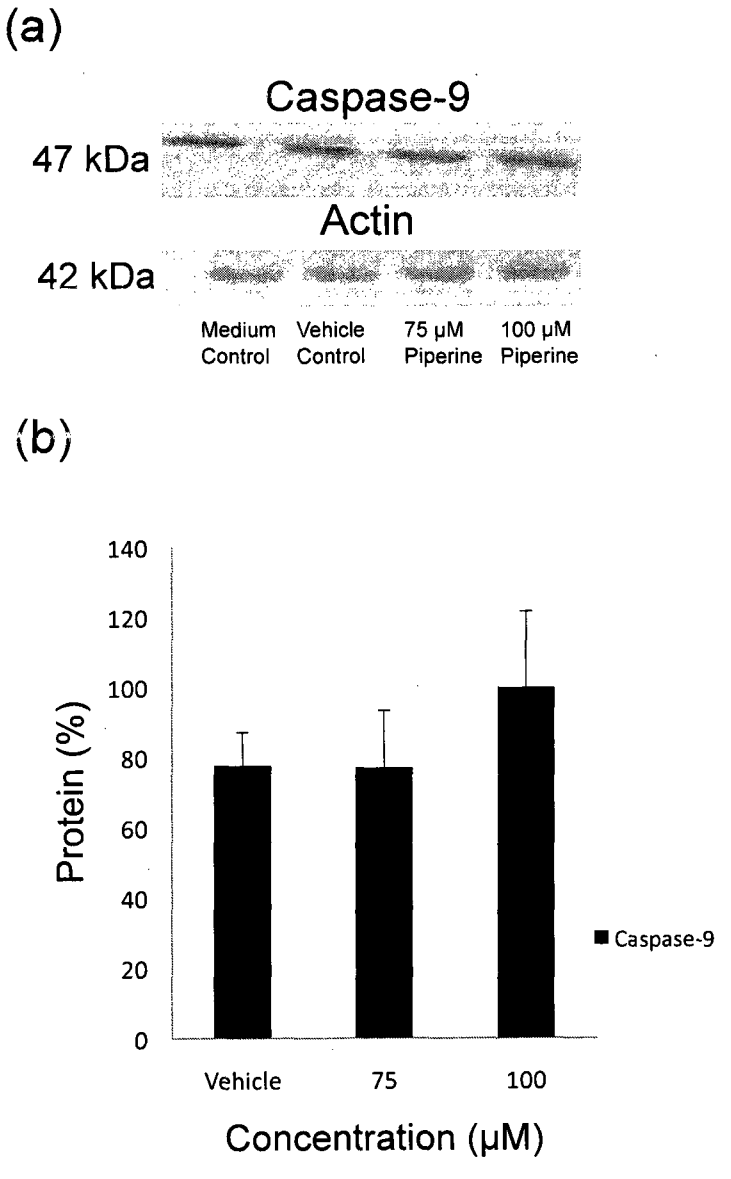
(a)
Caspase-9
47 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
140
120
100
80
60
40
20
0
Protein (%)
Caspase-9
Vehicle
75
100
Concentration (μM)

Figure 41.

Figure 42. Piperine Causes Survivin Downregulation in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on survivin protein expression. Data are representative of 3 independent experiments. (b) Survivin protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

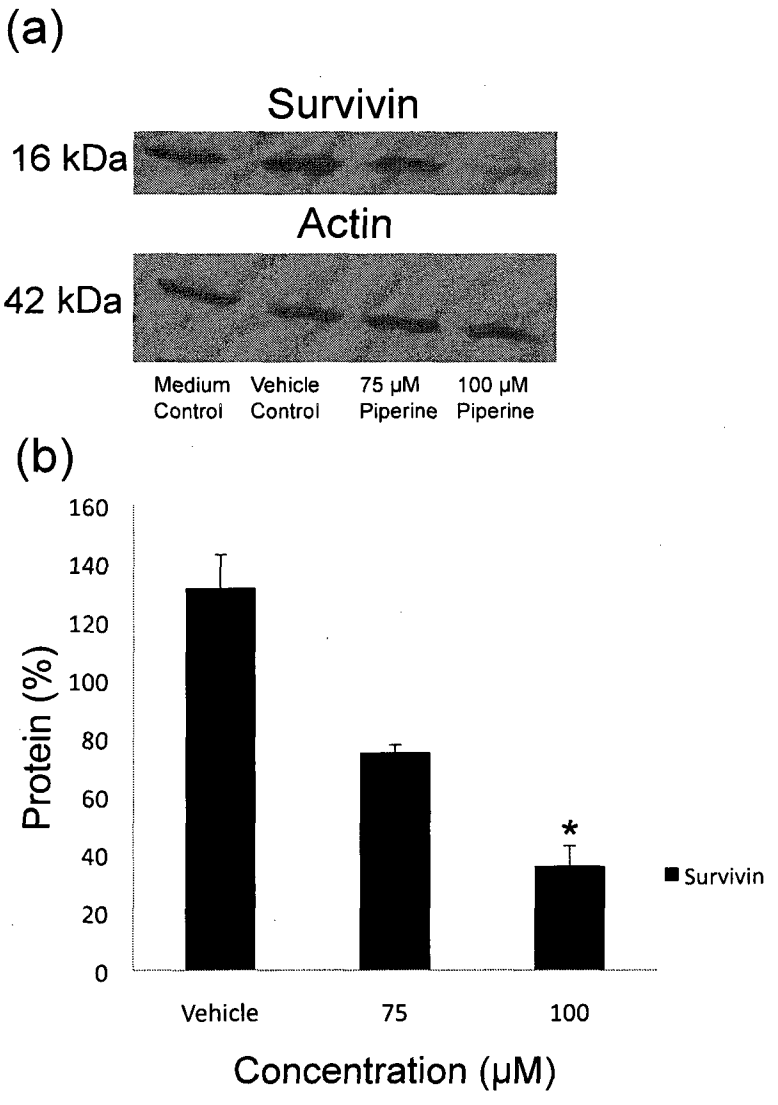
(a)
Survivin
16 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
160
140
120
100
80
60
40
20
0
*
Survivin
Vehicle
75
100
Concentration (μM)

Figure 42.

Figure 43. Piperine Causes PARP Cleavage in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 72 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on PARP protein expression and cleavage. Data are representative of 3 independent experiments. (b) PARP protein expression and cleavage relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

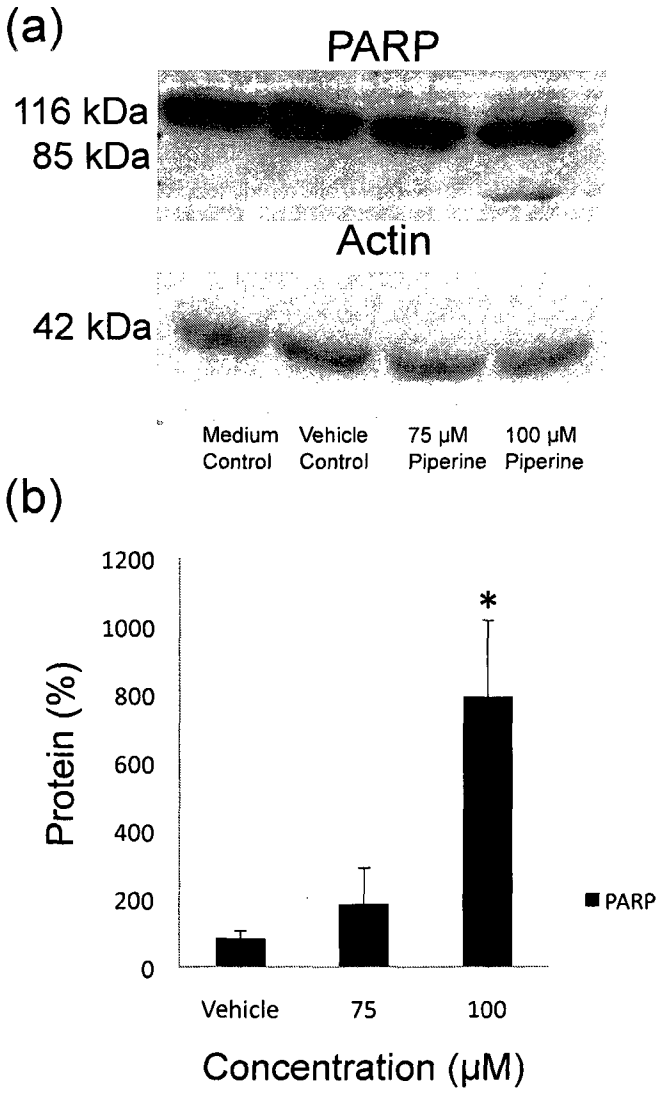
(a)
PARP
116 kDa
85 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 µM Piperine
100 µM Piperine
(b)
Protein (%)
1200
1000
800
600
400
200
0
*
Vehicle
75
100
Concentration (µM)
PARP

Figure 43.

Figure 44. Piperine Induces JNK Phosphorylation in HT-29 Cells. Medium alone, DMSO control and piperine-treated HT-29 cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on phospho- and total JNK protein expression. Data are representative of 3 independent experiments. (b-e) phospho- and total JNK protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p <$ 0.05, ** $p <$0.01.

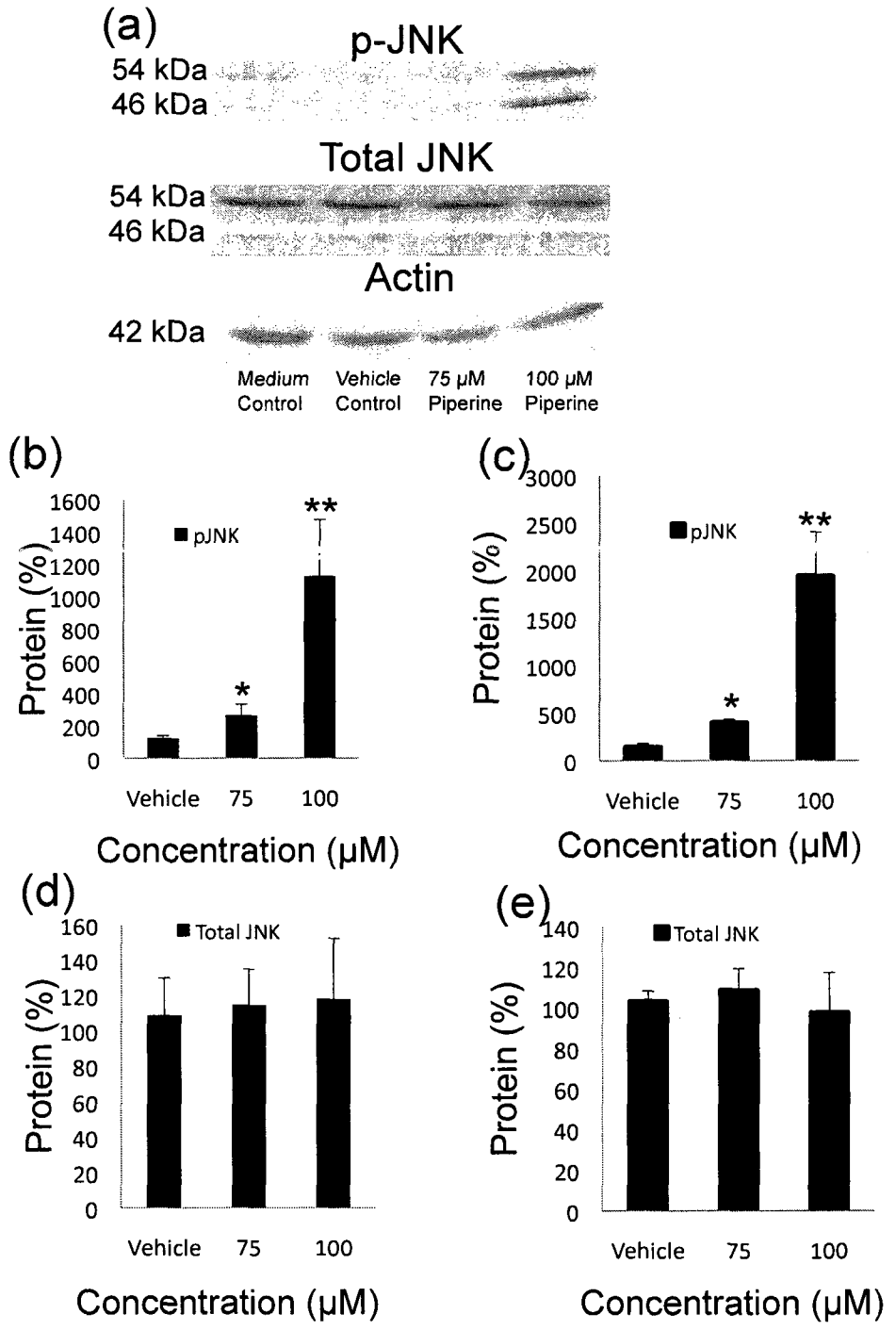
(a)
p-JNK
54 kDa
46 kDa
Total JNK
54 kDa
46 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
pJNK
Protein (%)
Vehicle
75
100
Concentration (μM)
(c)
pJNK
Protein (%)
Vehicle
75
100
Concentration (μM)
(d)
Total JNK
Protein (%)
Vehicle
75
100
Concentration (μM)
(e)
Total JNK
Protein (%)
Vehicle
75
100
Concentration (μM)

Figure 44.

Figure 45. Piperine Induces p38 MAPK Phosphorylation in HT-29 Cells. Medium alone, DMSO control and piperine treated HT-29 colon cancer cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on phospho- and total p38 MAPK. Data are representative of 3 independent experiments. (b, c) phospho- and total p38 MAPK protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

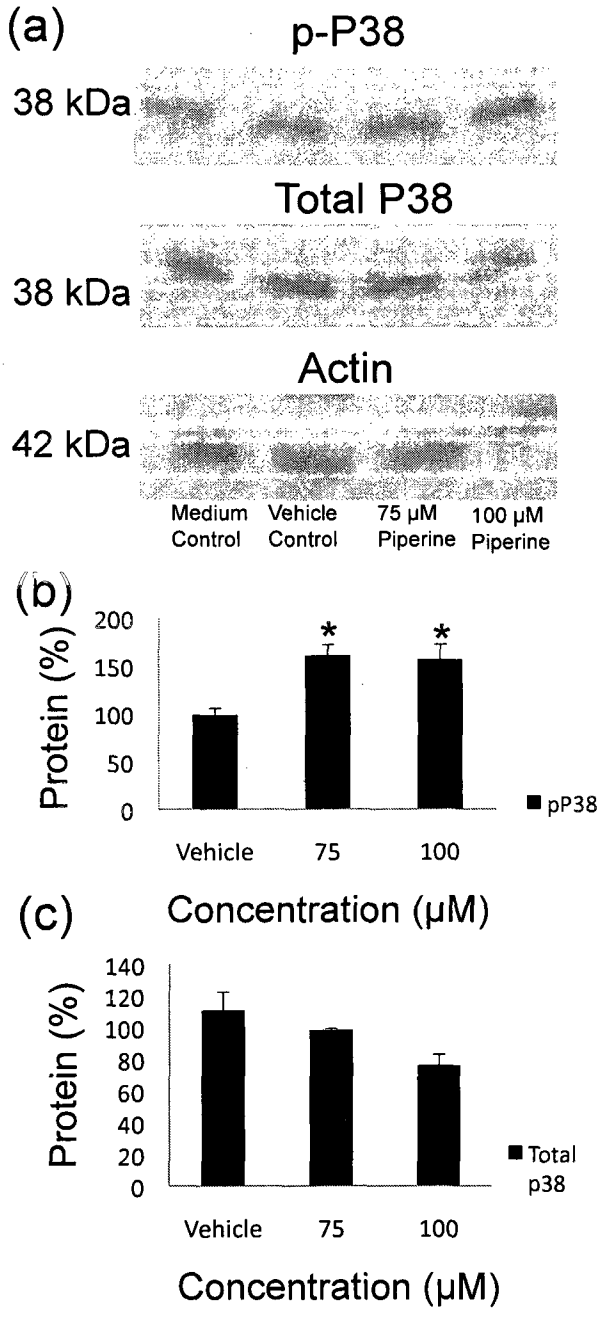
(a)
p-P38
38 kDa
Total P38
38 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
200
150
100
50
0
*
*
pP38
Vehicle
75
100
Concentration (μM)
(c)
Protein (%)
140
120
100
80
60
40
20
0
Total p38
Vehicle
75
100
Concentration (μM)

Figure 45.

Figure 46. Piperine Upregulates IRE1α Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on IRE1α protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) IRE1α protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

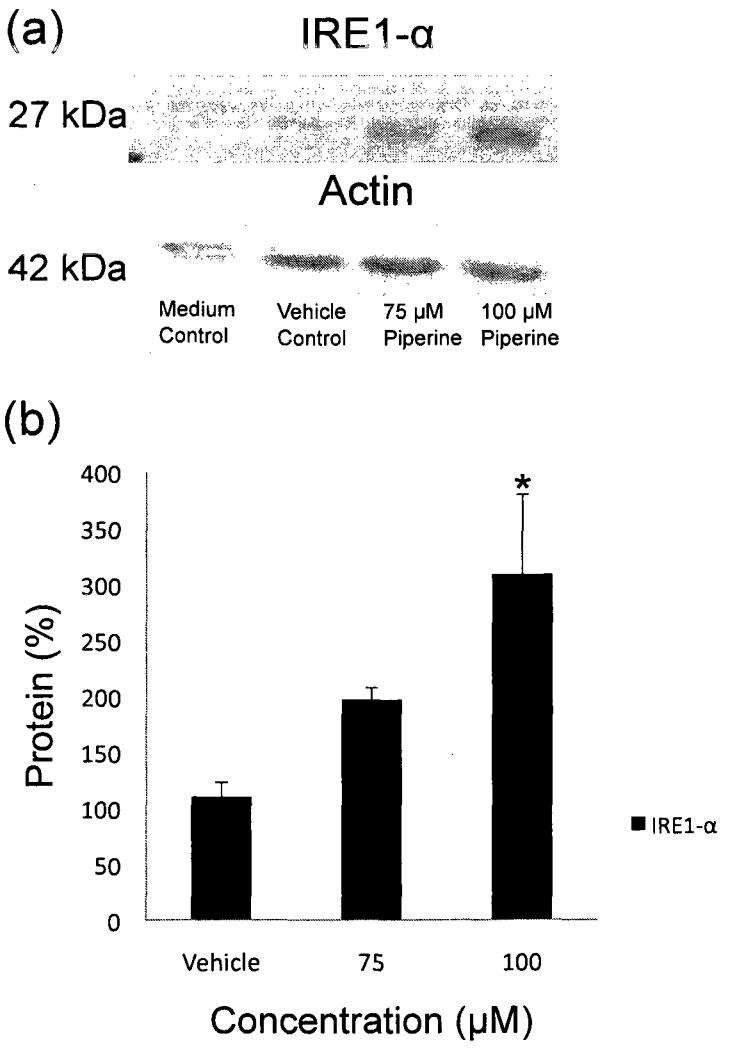
(a)
IRE1-α
27 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
400
350
300
250
200
150
100
50
0
*
Vehicle
75
100
Concentration (μM)
IRE1-α

Figure 46.

Figure 47. Piperine Upregulates CHOP Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on CHOP protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) CHOP protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; *** $p < 0.001$.

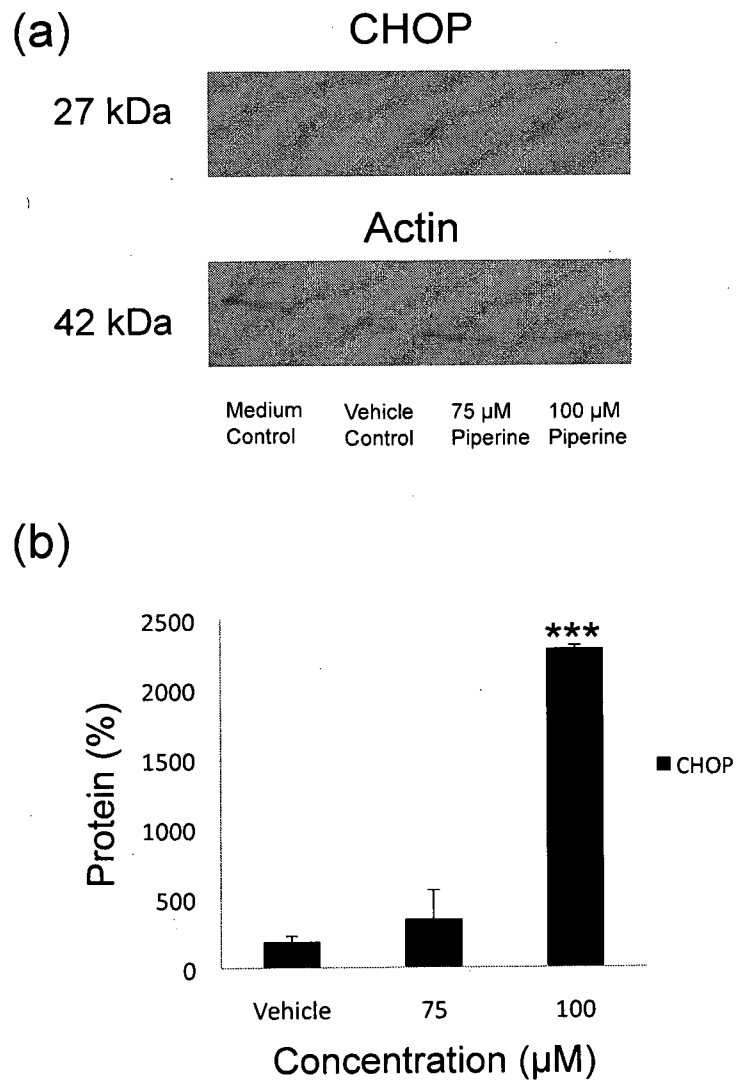
(a)
CHOP
27 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
2500
2000
1500
1000
500
0
Protein (%)
***
CHOP
Vehicle
75
100
Concentration (μM)

Figure 47.

Figure 48. Piperine Upregulates Bip Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. Western blotting of protein lysates was performed to determine the effect of piperine treatment on Bip protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) Bip protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error. Statistical significance in comparison to the vehicle control was determined by the Bonferroni multiple comparisons test; * $p < 0.05$.

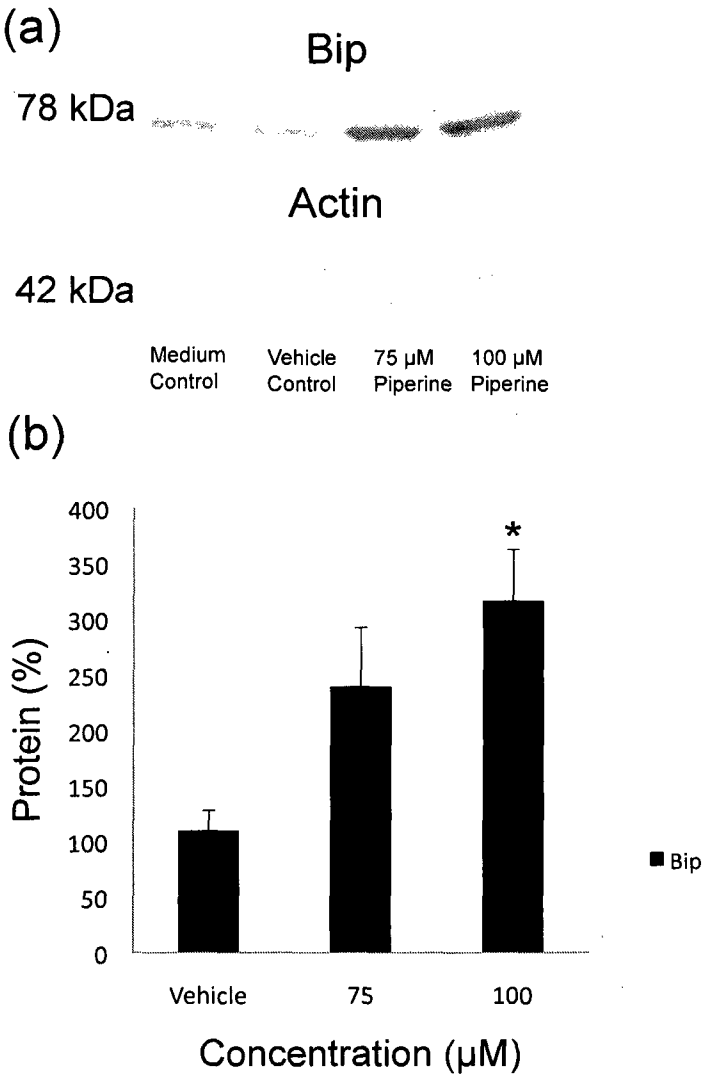
(a)
Bip
78 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
Protein (%)
400
350
300
250
200
150
100
50
0
*
Vehicle
75
100
Concentration (μM)
Bip

Figure 48.

Figure 49. Piperine Does Not Affect Calnexin Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on calnexin protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) Calnexin protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

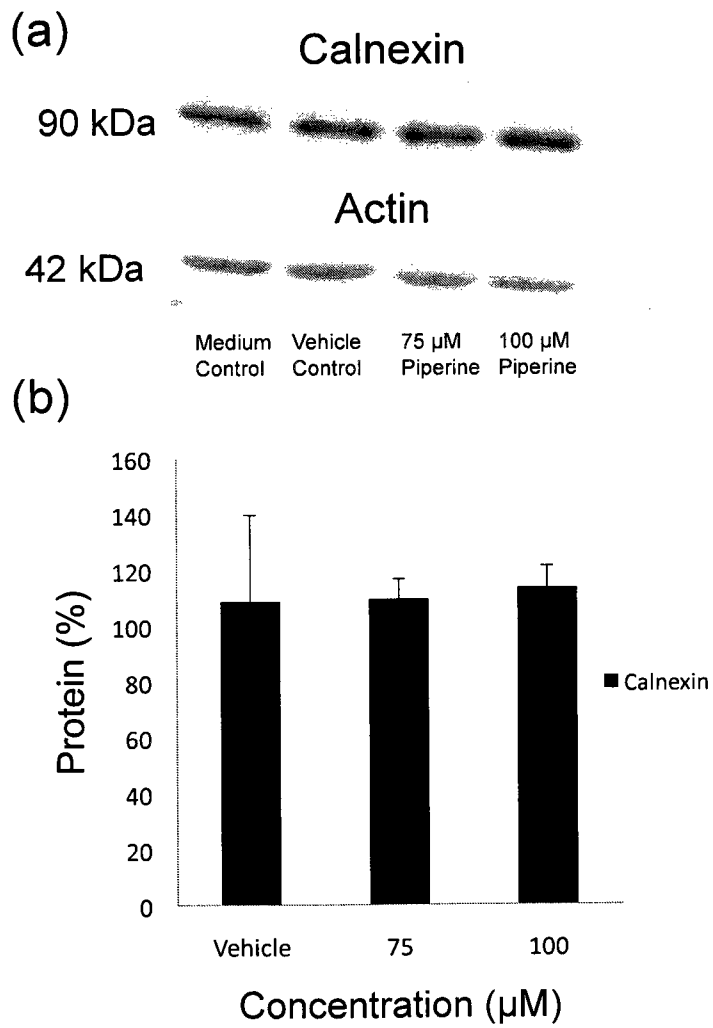
(a)
Calnexin
90 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
160
140
120
100
80
60
40
20
0
Protein (%)
Calnexin
Vehicle
75
100
Concentration (μM)

Figure 49.

Figure 50. Piperine Does Not Affect ERo1-Lα Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on ERo1α protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) ERO1-Lα protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

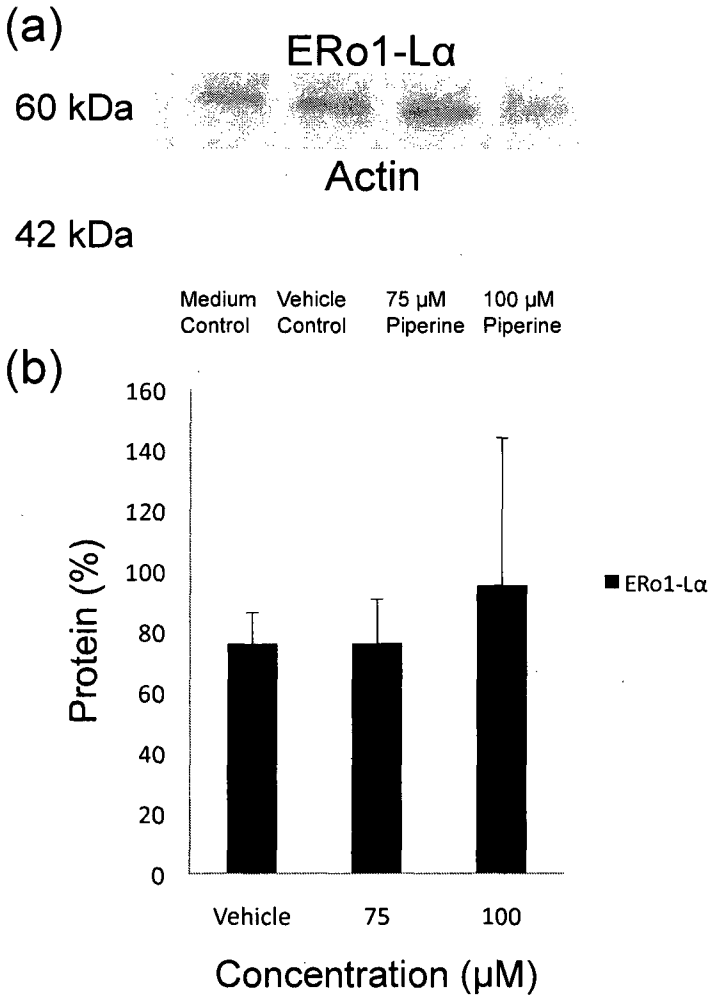
(a)
ERo1-Lα
60 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
160
140
120
100
80
60
40
20
0
Protein (%)
ERo1-Lα
Vehicle
75
100
Concentration (μM)

Figure 50.

Figure 51. Piperine Does Not Affect PDI Expression in HT-29 Colon Cancer Cells. Medium alone, DMSO control and piperine-treated cells were harvested after 48 hours of culture. (a) Western blotting of protein lysates was performed to determine the effect of piperine treatment on PDI protein expression in HT-29 colon cancer cells. Data are representative of 3 independent experiments. (b) PDI protein expression relative to actin was quantified by densitometry and expressed in arbitrary units. Data represent the mean of 3 separate experiments ± standard error.

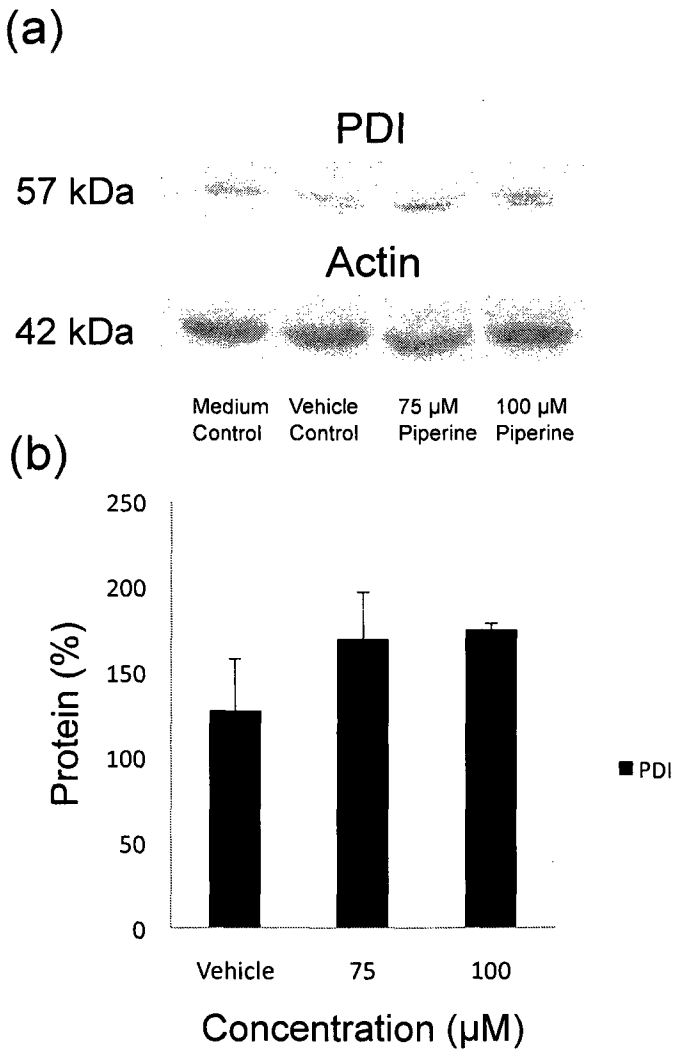
(a)
PDI
57 kDa
Actin
42 kDa
Medium Control
Vehicle Control
75 μM Piperine
100 μM Piperine
(b)
250
200
150
100
50
0
Protein (%)
PDI
Vehicle
75
100
Concentration (μM)

Figure 51.

Figure 52. Piperine Killing of HT-29 Colon Cancer Cells is Not Affected by Blockade of the TRPV1 Receptor with Capsazepine. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells pretreated with or without 10 μM capsazepine were harvested after 72 hours of culture. MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error.

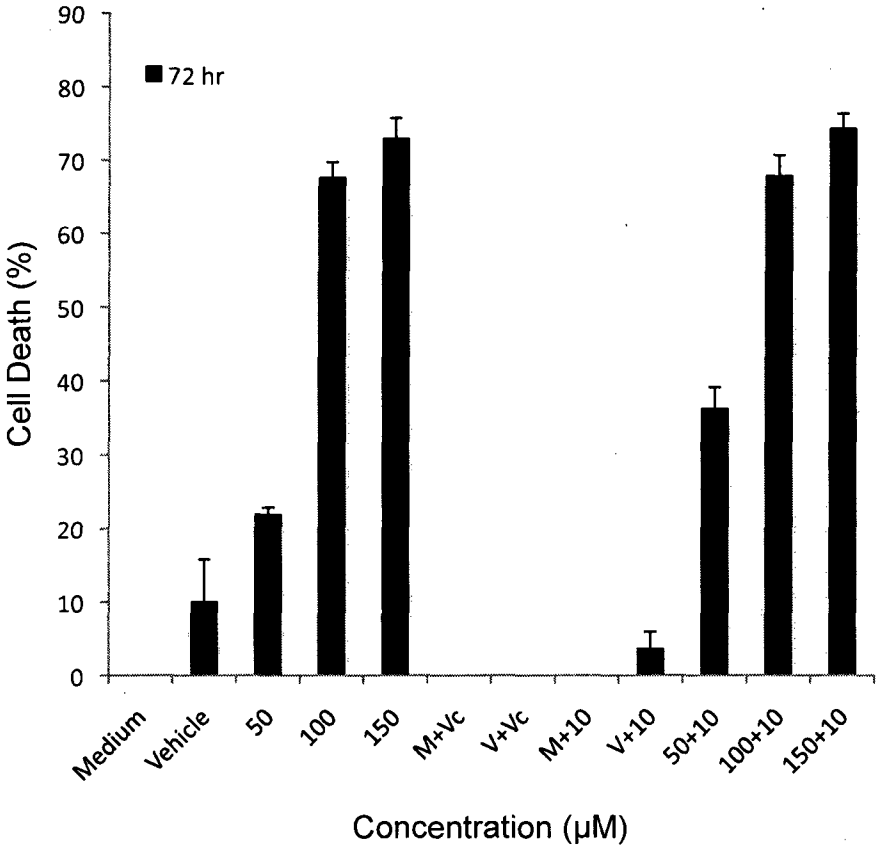
90
80
70
60
50
40
30
20
10
0
72 hr
Cell Death (%)
Medium
Vehicle
50
100
150
M+Vc
V+Vc
M+10
V+10
50+10
100+10
150+10
Concentration (μM)

Figure 52.

Figure 53. Piperine Killing of HRT-18 Rectal Cancer Cells is Not Affected by Blockade of the TRPV1 Receptor with Capsazepine. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells pretreated with or without 10 μM capsazepine were harvested after 72 hours of culture. MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error.

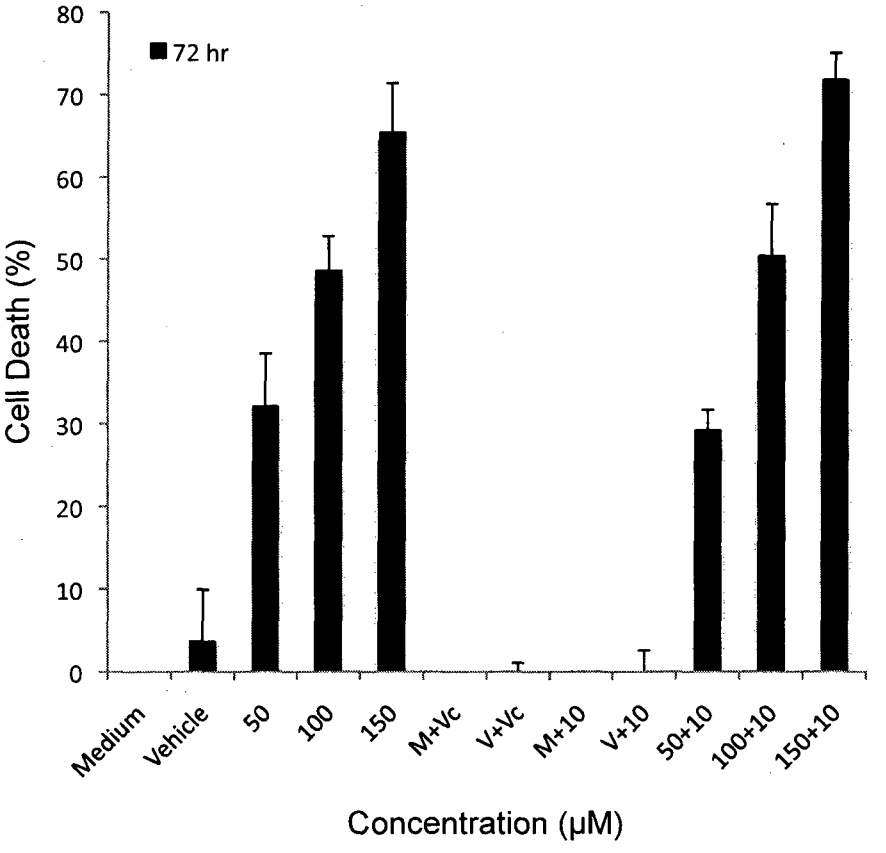
72 hr
Cell Death (%)
80
70
60
50
40
30
20
10
0
Medium
Vehicle
50
100
150
M+Vc
V+Vc
M+10
V+10
50+10
100+10
150+10
Concentration (μM)

Figure 53.

Figure 54. Piperine Killing of HT-29 Colon Cancer Cells is Not Affected by Blockade of the TRPV1 Receptor with SB366791. Medium alone, DMSO control and piperine-treated HT-29 colon cancer cells pretreated with or without 2.5 μM SB366791 were harvested after 72 hours of culture. MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error.

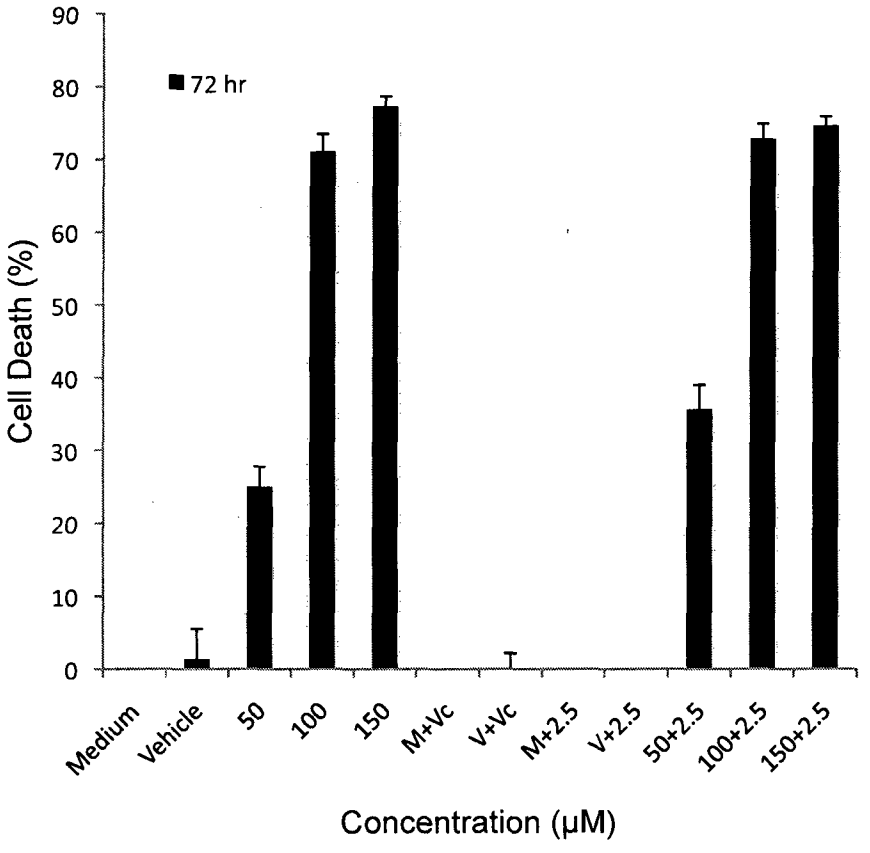
90
80
70
60
50
40
30
20
10
0
72 hr
Cell Death (%)
Medium
Vehicle
50
100
150
M+Vc
V+Vc
M+2.5
V+2.5
50+2.5
100+2.5
150+2.5
Concentration (μM)

Figure 54.

Figure 55. Piperine Killing of HRT-18 Rectal Cancer Cells is Not Affected by Blockade of the TRPV1 Receptor with SB366791. Medium alone, DMSO control and piperine-treated HRT-18 rectal cancer cells pretreated with or without 2.5 μM SB366791 were harvested after 72 hours of culture. MTT (455μg/ml) was added 2 hours before cells were dissolved in DMSO. Data represent the mean of 3 separate experiments ± standard error.

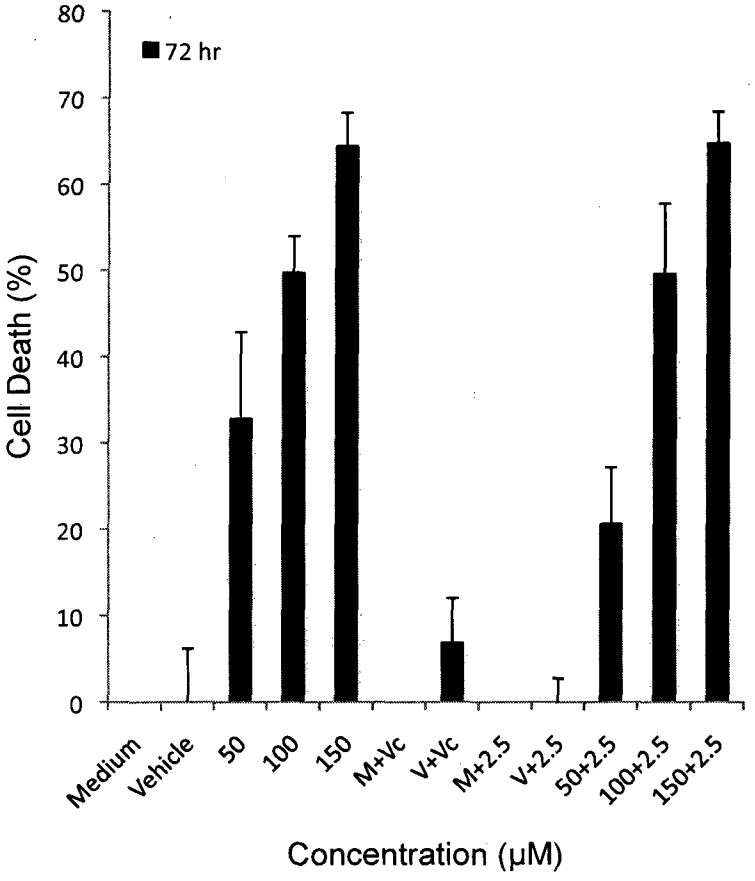
80
70
60
50
40
30
20
10
0
72 hr
Cell Death (%)
Medium
Vehicle
50
100
150
M+Vc
V+Vc
M+2.5
V+2.5
50+2.5
100+2.5
150+2.5
Concentration (μM)

Figure 55.

Chapter 4.0 Discussion

4.1 Piperine Treatment Arrests Proliferation of HT-29 Colon and HRT-18 Rectal Cancer Cells

Previous studies have identified piperine as an anti-carcinogenic and tumour-inhibitory phytochemical using models as diverse as Ehrlich ascites carcinoma, Dalton's lymphoma ascites, L929, B16-F10, and Sarcoma 180 cells (Bezerra et al 2008, Pradeep & Kuttan 2002, Sunila & Kuttan 2004, Unnikrishnan & Kuttan 1990). Piperine's effect was also studied in Benzo(α)pyrene-induced experimental lung cancer and the Wing Somatic Mutation and Recombination Test in a *Drosophila melanogaster* model (El Hamss et al 2003, Selvendiran et al 2004). However, the precise mechanism of action remains poorly understood and determination of this mechanism is the focus of the research presented.

Piperine treatment at 150 μM inhibited both HT-29 colon and HRT-18 rectal cancer cell proliferation using the Oregon Green 488 flow cytometry assay (Figures 1 and 2). HRT-18 cell proliferation was also inhibited significantly following treatment with 100 μM piperine. The Oregon Green 488 flow cytometry assay has not been previously used in the study of either HT-29 or HRT-18 cancer cells, therefore it is difficult to compare the effect of piperine to other phytochemicals in these cells using this assay.

The Oregon Green-488 flow cytometric assay performed uses a fluorescein molecule with two acetate moieties and a succinimidyl ester functional group. It is membrane permeable and non-fluorescent in this form. The compound diffuses into the cell, where endogenous esterases remove acetate groups. This makes the compound fluorescent and membrane impermeable. The succinimidyl ester also reacts with free

amines of intracellular proteins to form dye-protein adducts. Staining persists due to binding to cytoskeletal components, which have low turnover. There is an exponential reduction in fluorescence observed with linear increases in the number of rounds of proliferation due to dilution of the relative intracellular concentration of the fluorescent probe. Up to 10 cycles of cell division can be detected through this method (Lyons 2000, Lyons & Doherty 2004). Therefore, flow cytometry is an ideal means of detecting the anti-proliferative effect of piperine. The protocol used is similar to that published by MacMillan et al in 2009 to study T-cell proliferation (MacMillan et al 2009). Other methods to study proliferation include tritiated-thymidine incorporation (Choi et al 2009). Oregon Green 488 flow cytometry was chosen to study proliferation due to the inability to lyse HT-29 and HRT-18 cells in order to measure tritiated-thymidine incorporation.

The data derived from flow cytometry represents an average of the proliferative ability of the cells being tested. I concluded from the experiments in HT-29 colon and HRT-18 rectal cancer cells that piperine exerts an anti-proliferative effect in these cells. Other investigators studied the antiproliferative effects of piperine on DLD-1 colon cancer cells using a crystal violet staining assay, which showed a trend towards an antiproliferative effect at 24 hours, with statistical significance evident at 48-72 hours at concentrations of 100-200 μM piperine (Duessel et al 2008). Combined with my data, this strongly supports an antiproliferative effect of piperine on human colorectal cancer cells.

4.2 The Antiproliferative Effect of Piperine is Due to a G1 Phase Cell Cycle Arrest in HT-29 Colon Cancer Cells

Treatment of HT-29 colon cancer cells with 100 μM piperine was associated with a statistically significant G_1 phase cell cycle arrest, with corresponding reductions in the percentage of cells in S and G_2 phases (Figure 3). Similar G_1 phase cell cycle arrest was shown by Khan et al in LNCaP prostate cancer cells treated with the flavonoid fisetin (Khan et al 2008). In comparison, the same investigators studied delphinidin and found that it induced a G_2/M phase arrest in HCT-116 colon cancer cells (Yun et al 2009). Therefore, different phytochemicals induce differential cell cycle phase arrest in different cell lines.

In contrast, HRT-18 rectal cancer cells treated with piperine demonstrated a much smaller effect on cell cycle progression (Figure 4). HRT-18 cells tend to aggregate, which causes difficulty in counting and increases intra- and inter-experimental variability. This may account to an extent for the lack of statistical significance observed in these experiments. While there were fewer cells in G_2 phase, there was no significant change in the percentage of cells in G_0/G_1 or S phase of the cell cycle in HRT-18 cells. This likely represents a different form of cell cycle arrest than that observed with HT-29 cells. The G_2 phase population reduction supports a cell cycle arrest prior to G_2. Nevertheless, cell cycle analysis of both HT-29 colon cancer and HRT-18 rectal cancer cells following treatment with 100 μM piperine is consistent with the antiproliferative effect seen in the Oregon Green 488 flow cytometric assays.

In cell cycle analysis, cells were serum-starved to synchronize them to G_0/G_1 phase of the cell cycle. Piperine treatment was applied for 48 hours and the cells were

harvested and fixed in 70% ethanol. The cells were treated with DNase-free RNase A and propidium iodide on the day of analysis. Propidium iodide is a fluorescent dye whose binding to DNA is directly proportional to DNA content. Flow cytometric analysis can then determine the number of cells with different amounts of DNA. The percentage of cells in each phase of the cell cycle is determined through generation of DNA histograms. Histogram peak amplitudes define the relative percentages of cells within a given phase of the cell cycle. The x-axis defines the relative distribution of DNA content. Therefore, there will be greater DNA content in cell populations with a greater value on the x-axis. G_1 phase contains cells with 2N DNA content, in contrast to S phase with intermediate DNA content and G_2/M phase with 4N DNA content.

One of the limitations of cell cycle analysis by flow cytometry is its reliance on controlled cell collection by the flow cytometer. As the assay utilizes propidium iodide staining of DNA to determine the distribution of the cells according to the content of DNA histograms, if the collection rate is too rapid, 2N DNA could be interpreted as 4N and therefore overestimate the percentage of cells in G_2/M phase of the cell cycle. This could underestimate the cell cycle arrest observed. Extensive care was taken to control the collection rate to approximately 15-60 cells collected per second.

The G_1 phase cell cycle arrest was further studied in HT-29 colon cancer cells using western blotting to study protein expression. Downregulation of Cyclins D1 and D3 (Figures 5 and 6), Cdk 4 and 6 (Figures 7 and 8), and the phosphorylated isoforms of Rb protein (795, 807/811) (Figures 9 and 10) following 100 μM piperine treatment further supports a G1 phase cell cycle arrest. Additionally, phospho-Rb (795) is downregulated following treatment with 75 μM piperine. Upregulation of inhibitors of

Cdks (CKIs) p21 and p27 (Figures 11 and 12) is also likely involved in G_1 phase arrest. Similar results were obtained with fisetin treatment of prostate cancer cells (Khan et al 2008).

HT-29 colon cancer cells were chosen as the cell line of study for western blotting due to the more significant data seen on cell cycle analysis experiments using HT-29 cells. The lack of effect on p15 INK4B (Figure 14) protein expression simply indicates that this protein is likely not involved in cell cycle progression in HT-29 colon cancer cells. I conclude from the western blot data that piperine induces a G1 phase cell cycle arrest, which accounts for the antiproliferative effect of piperine observed on Oregon Green 488 flow cytometry.

AKT protein phosphorylation was also studied as this protein is important in cell survival and apoptosis. AKT, also known as protein kinase B, is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation of and degradation of cyclin D1 (Diehl et al 1998). AKT also negatively regulates Cdk inhibitors p27 and p21 (Gesbert et al 2000, Zhou et al 2001). AKT activation has also been shown to overcome G_1 and G_2 phase cell cycle arrest (Kandel et al 2002). I found that 100 μM piperine treatment was associated with reduced phosphorylation of AKT, with minimal and statistically insignificant reduction in total AKT protein expression (Figures 13a-c). This supports piperine's role in cell cycle arrest. Similar effects have been observed with other phytochemicals (Ahmad et al 2001, Khan et al 2008, Yim et al 2005).

4.3 Piperine is Cytotoxic to HT-29 and CaCo-2 Colon and HRT-18 Rectal Cancer Cells

To determine the mechanism of piperine's anti-proliferative effect and cell cycle arrest, MTT cytotoxicity assays were performed on HT-29 and CaCo-2 colon cancer, HRT-18 rectal cancer, and normal HMECs. MTT assays were similarly used to study the cytotoxic effect of delphinidin on HCT-116 colon cancer cells by Yun et al (Yun et al 2009). I concluded from MTT assays that piperine exerts a cytotoxic effect on HT-29 colon cancer cells at concentrations between 75 μM and 150 μM at 24-72 hours (Figure 15). The effect was not observed consistently at 144 hours with 150 μM piperine, likely due to a portion of the surviving cells proliferating in some experiments due to the extended duration of the treatment interval.

In the MTT assays performed using HRT-18 rectal cancer cells, 150 μM piperine was found to be cytotoxic to these cells at all time points (Figure 16). In contrast, 100 μM piperine was cytotoxic at only 72-144 hours. It is unclear as to the reason for cytotoxicity of 50 μM piperine at 144 hours, in absence of cytotoxicity at 75 μM piperine at the same time point. This may be due to interexperimental variability resulting from the more aggregative nature of HRT-18 cells in comparison to HT-29 cells.

CaCo-2 cells were treated with piperine up to 100 μM, though cytotoxicity was observed predominantly at 144 hours (Figure 17). It is unclear as to the reason for statistically significant cytotoxicity of 100 μM piperine at 48 hours in exclusion of cytotoxicity at 72 hours, other than that CaCo-2 cells are extremely aggregative in nature, which makes their counting unreliable and contributes to a large amount of intra-and inter-experimental variability.

MTT assays were performed on normal HMECs because of the need to compare the effect of piperine on a similar histological cell type to typical colorectal cancer (adenocarcinoma), which is of glandular epithelial origin. Furthermore, human colonic epithelial cells are not commercially available, thus mammary epithelial cells seemed a reasonable surrogate cell line. Despite a statistically significant cytotoxic effect on HMECs at 72 hours (Figure 19), this is significantly lower than that seen in transformed cells at similar doses, likely due to the lower proliferation rate of normal HMECs. This data supports the safety profile of piperine, which like other phytochemicals, has relatively low toxicity.

One of the criticisms of the MTT assay is its dependence on the activity of mitochondrial succinate dehydrogenase as an index for viability. Cells may remain viable, and the effects observed may represent growth inhibition. MTT represents mitochondrial metabolic rate and thus, indirectly reflects viable cell numbers. Furthermore, the colour intensity of formazan crystals correlates with the number of viable cells, although several phytochemicals and other compounds interact with MTT directly, or alter the activity of succinate dehydrogenase (Wang et al 2010). Therefore, additional viability assays were chosen to provide supporting data.

Trypan blue dye exclusion assays were performed as a further measure of piperine's cytotoxic effect on HT-29 colon cancer cells (Figure 20). Piperine's cytotoxic effect was significant at both 48 and 72 hours using a 150 μM concentration and at 72 hours alone using 50 μM and 100 μM concentrations, which supports the MTT cell viability data. Trypan blue dye exclusion has been used by many other investigators as a measure of cell viability (Voutsadakis et al 2010). However, this assay is limited in use

due to its time requirements to perform and manual counting inaccuracies. The MTT assay has been reported to underestimate the antiproliferative effect of (-)-epigallocatechin-3-gallate (EGCG) in comparison to measures of ATP or DNA, or trypan blue dye uptake (Wang et al 2010). All cell viability assays have limitations, although in combination, piperine's cytotoxic effect can be better appreciated.

Colony-forming, or clonogenic assays were performed to determine the effect of piperine treatment on subsequent formation of cancer cell colonies. Colony-forming assays represent another form of cell viability assay that may be used to screen for chemotherapeutic effect, which has clinical utility (Shoemaker et al 1985). Piperine treatment at 150 μM resulted in a 50% reduction in the number of colonies formed following a 24-hour treatment interval. Problems associated with colony-forming assays in the past have included difficulty preparing cell suspensions from solid tumours and variability in the estimates of growth from assay to assay, which may affect drug effect measurement (Shoemaker et al 1985). In contrast, the methodology used in this study involved growth of an adherent cell line in 6-well plates. It was anticipated that piperine would inhibit colony formation, therefore, greater numbers of piperine-treated cells were plated as compared to wells containing medium or vehicle control cells. This allowed for counting in a more acceptable range of approximately 100 cells per well (Shoemaker et al 1985). I concluded that piperine inhibits colony-formation in HT-29 colon cancer cells (Figure 18).

4.4 Piperine-Mediated Cytotoxicity is Due to Apoptosis

Annexin-V/ propidium iodide flow cytometry assays were performed to determine the presence of early apoptosis versus late apoptosis or necrosis in cultures of piperine-treated cancer cells. The Annexin-V/ propidium iodide flow cytometry assay was also used by Yun et al (2009) in their study of the apoptosis-inducing effect of delphinidin on HCT-116 colon cancer cells. I concluded that exposure to150 μM piperine was associated with a statistically significant increase in early apoptosis in both HT-29 and HRT-18 cells (Figures 21 and 22). The 72-hour time point was chosen to correspond with the optimal cytotoxic effect of piperine, as seen in cell viability assays. However, use of this later time point likely also accounts for the increased late apoptotic/necrotic HT-29 colon and HRT-18 rectal cancer cell populations observed in these experiments.

Annexin-V, purified and expressed in *Escherichia coli*, was initially determined to be a vascular protein with potent anticoagulant properties. It is now known as a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserines (Andree et al 1990, Reutelingsperger et al 1985). This phosphatidylserine translocation is an early event in apoptosis, in which the cell membrane remains intact. Furthermore, preferential binding of Annexin-V, which can be conjugated to the fluorochrome FITC, allows differentiation of apoptosis from necrosis. Performed in coordination with dye exclusion of the non-vital dye propidium iodide (PI), which readily diffuses across disrupted cell membranes, as associated with necrotic cell death, can discriminate between viable, apoptotic, and necrotic cells (Vermes et al 1995).

A number of different techniques exist to study cell death, although the most convenient method is flow cytometry. These methods determine results quantitatively and independently of visual determination of apoptotic events. Therefore, operational bias is minimal and data acquisition is rapid. Further, the Annexin-V assay does not require prior fixation of cells and is specific for an early event in apoptosis that occurs before DNA fragmentation and nuclear breakdown. Annexin-V can be conjugated with several fluorecent and non-fluorescent labels. One of the criticisms of flow cytometric cell death studies is the need for a large number of cells. Specific to the Annexin-V/ propidium iodide flow cytometry assay, which requires exposure to phosphatidylserine, Annexin-V may bind to intracellular phosphatidylserine in the event of plasma membrane permeabilization, which occurs in late apoptosis or necrosis. Exposure of phosphatidylserine can prepare cells for phagocytic removal, which is independent of apoptosis. However, it is important to note that phosphatidylserine exposure may also occur independently of apoptosis (Galluzzi et al 2009).

4.5 Piperine-Mediated Apoptosis is Due to Production of Reactive Oxygen Species

I concluded that piperine treatment did not induce significant superoxide anion production (Figures 23-26) in HT-29 colon and HRT-18 rectal cancer cells because of minimal DHE staining. In contrast, piperine treatment was associated with significant hydroxyl radical production, as determined by DCFH-DA staining (Figures 27-30), which is consistent with existing literature (Mittal & Gupta 2000). Furthermore, cancer cells have constitutive elevated production of hydrogen peroxide. Hydrogen peroxide accumulation above a threshold level results in cell cycle arrest and/or apoptosis (Loo 2003). Piperine has never been demonstrated to generate superoxide anions, although

one investigator showed that piperine did not scavenge superoxide anions (Krishnakantha & Lokesh 1993).

To further support the finding that piperine mediates its cytotoxic effect via hydroxyl radical production, Annexin-V/ propidium iodide flow cytometry assays were performed with glutathione and N-acetylcysteine treatment of cells prior to exposure to piperine. Glutathione and N-acetylcysteine significantly reduced the early apoptotic cell population in HT-29 colon cancer cells treated with 100 μM and 150 μM piperine (Figures 31 and 32). The reason for statistically significant reductions in the HRT-18 rectal cancer cell early apoptotic populations following N-acetylcysteine pre-treatments, in the absence of similar effects following glutathione pre-treatments (Figures 33 and 34), is not clear at this time but could be attributed to the dual role of N-acetylcysteine in scavenging free radicals and maintenance of cellular glutathione levels (Kerksick & Willoughby 2005). N-acetylcysteine may therefore provide a relatively greater antioxidant effect than occurs with glutathione. N-acetylcysteine provides a source of glutathione since cysteine is the rate-limiting precursor for glutathione synthesis (Berk et al 2008).

Crystal violet staining assays were used to corroborate the Annexin-V/ propidium iodide data using a viability assay, in which glutathione and N-acetylcysteine could be tested. MTT assays are not amenable to experimentation with these antioxidants due to effects on the mitochondria that lead to false positive results. HT-29 colon cancer cells had improved viability in crystal violet staining assays with both glutathione and N-acetylcysteine pre-treatments (Figures 35 and 36), which was shown as well with the apoptosis assay. Therefore, I concluded that reactive oxygen species production and

specifically hydroxyl radical production results in apoptosis in HT-29 cells treated with 100 μM and 150 μM concentrations of piperine. In contrast, N-acetylcysteine pre-treatment of the HRT-18 rectal cancer cells did not result in any improvement in cell viability (Figure 37), which suggests that reactive oxygen species production is less important as a mechanism of apoptosis in these cells. The reason for early apoptotic cell death identified on Annexin-V staining being significantly reduced in the presence of N-acetylcysteine pre-treatments is not clear. However, there are a number of limitations of Annexin-V/ propidium iodide staining, as explained above.

Another group reported a reversal of the antiproliferative effects and G_2/M phase cell cycle arrest in HT-29 colon cancer cells induced by exposure to benzyl isothiocyanate, diallyl disulfide, dimethyl fumarate, sodium butyrate, or buthione sulfoxamine after pre-treatment with N-acetylcysteine (Odom et al 2009). The authors concluded that N-acetylcysteine is important in restoring the intracellular equilibrium of glutathione from the oxidized to the reduced form. This was not demonstrated in lycopene in this study, indicating that different phytochemicals work through different mechanisms.

Phenolic phytochemicals such as piperine have antioxidant activity due to the phenolic hydroxyl group, which provides hydrogen atoms needed for scavenging reactive oxygen species. However, these same phytochemicals have pro-oxidant activities under certain conditions (Loo 2003). Chronic oxidative stress in cancer cells appears to sensitize these cells to stress or apoptotic effects of chemotherapeutics, which typically function via reactive oxygen species production. Cancer cells are more susceptible in this

regard as they are already close to a threshold for tolerating reactive oxygen species (Benhar et al 2001).

4.6 Piperine-Mediated Apoptosis Involves Mitochondrial Membrane Destabilization

Western blotting of the cytosolic fractions of HT-29 colon cancer cells obtained by digitonin lysis identified cytochrome c release, more significantly at 48 hours, which was seen with both 75 μM and 100 μM concentrations of piperine (Figures 38a, b). Cytochrome c release was also seen at 72 hours with 100 μM piperine treatment (Figures 38c, d), which likely indicates that the mitochondrial destabilization associated with piperine-mediated apoptosis is an event occurring earlier than 72 hours. By 72 hours, a number of processes associated with apoptotic cell death have already occurred, including reactive oxygen species production. This further supports the Annexin-V/propidium iodide data discussed above in which there is an incomplete reversal of apoptosis at 72 hours with antioxidant pre-treatments. Therefore, multiple mechanisms are most likely involved in piperine-induced apoptosis, including reactive oxygen species production and mitochondrial membrane destabilization. Many other phytochemicals also induce mitochondrial cytochrome c release. Loss or disruption of the mitochondrial membrane potential results in initiation and activation of apoptotic cascades that trigger cytochrome c release and other molecules, such as Smac/DIABLO from the mitochondria to the cytosol. Cytochrome c then binds to Apaf-1 and initiates the caspase cascade (Longpre & Loo 2008, Meeran et al 2008, Shyur et al 2004). The method of digitonin lysis is well explained in a paper by Waterhouse et al (Waterhouse et al 2004).

4.7 Piperine-Mediated Apoptosis is Caspase-Independent

I concluded that piperine-mediated apoptosis occurs independently of caspase activation, based on data from MTT (Figure 39) and western blotting (Figures 40a, b and 41 a, b) assays. Boc-D-fmk, a pan-caspase inhibitor with activity reported between 20-50 μM (Gupta et al , Uchide et al 2009), did not reduce piperine-induced cytotoxicity. Western blotting for caspase-3 and -9 protein expression did not show reduced levels or cleavage of caspase proforms, indicating that caspase activation did not occur at 48 hours after piperine treatment. This finding is consistent with those of other investigators (Arimura et al 2003, Chen et al 2009, Vene et al 2008). Caspase-independent apoptosis is increasingly being recognized as a central pathway in phytochemical-mediated cytotoxicity, though it remains poorly understood.

Piperine-mediated apoptosis is further supported by downregulation of survivin protein expression seen with 100 μM piperine treatment (Figures 42a, b). Survivin is known to provide a link between the cell cycle and apoptosis, which further supports piperine's pro-apoptotic effect.

PARP cleavage was studied due to its important role in both caspase-dependent and independent apoptotic cell death. Due to PARP cleavage, as evidenced by a cleavage product associated with 100 μM piperine treatment (Figures 43a, b), I concluded that PARP is involved in piperine-mediated apoptosis, in a caspase-independent manner. AIF released by damaged mitochondria may mediate caspase-independent apoptosis caused by piperine, although further experiments are needed to further substantiate this claim.

Western blotting of stress-activated protein kinase/JUN-amino-terminal kinase (SAPK/JNK) (Figures 44a-e) and p38 MAPK (Figures 45a-c) showed increased

phosphorylation, supporting a possible role of oxidative stress in piperine's mechanism of action. In another study, U937 human leukemic cells exposed to low concentrations of exogenous hydrogen peroxide demonstrated upregulated phosphorylation of p38 MAPK, which induced a cell cycle arrest. Pre-treatment with N-acetylcysteine prevented cell cycle arrest, JNK activation, and apoptosis induction. Furthermore, p38 MAPK was determined to be essential for apoptosis (Kurata 2000). In a separate study, JNK was found to be essential to apoptosis induction in HT-29 colon cancer cells exposed to hydrogen peroxide, with additional activation of ERK and p38 MAPK also being observed (Salh et al 2000).

4.8 Piperine-Mediated Apoptosis Involves ER Stress

I concluded that ER stress is a pathway involved in piperine-mediated apoptosis, since IRE1α (Figure 46a, b), CHOP (Figure 47a, b) and Bip (Figure 48a, b) expression is upregulated following piperine treatment, despite unchanged calnexin (Figure 49a, b), ERO1-Lα (Figure 50a, b), and PDI (Figure 51a, b) protein expression. IRE1α is an enzyme with dual activity, via a serine-threonine kinase domain and an endoribonuclease domain(Szegezdi et al 2006). Activation of the endonuclease activity of this enzyme removes an intron from XBP1 mRNA, which typically has pro-survival effects. However, overexpression of IRE1α, has been shown to be pro-apoptotic in HEK293T cells (Wang et al 1998). IRE1α may exert its pro-apoptotic effects via downstream activation of the MAP Kinase pathway through JNK and p38 (Nishitoh et al 1998).

CHOP, also known as Growth-arrest-and DNA-damage-inducible gene 153 (GADD153), is transcriptionally activated by all three arms of the UPR (Zinszner et al

1998). CHOP is also post-translationally regulated by p38 (Zinszner et al 1998). Like other ER stress proteins, Bip responds to unfolded proteins in the endoplasmic reticulum (Kohno et al 1993). I therefore conclude that ER stress plays a role in piperine's cytotoxic effects.

4.9 Piperine-Mediated Apoptosis is p53-Independent

p53 mutations are very important in colorectal cancers for transition between large adenomas and invasive cancers (Vogelstein et al 1988). HT-29 colon cancer cells have mutated p53 (Ravizza et al 2004). CaCo-2 cells are also known to be p53-mutated (Perathoner et al 2005), although the p53 status of HRT-18 rectal cancer cells is controversial (Perathoner et al 2005). Nevertheless, since piperine killed both HT-29 and CaCo-2 cells, the cytotoxic activity of this phytochemical does not depend on p53. As p53 mutations are so common in colorectal cancer, it is prudent that chemotherapeutic agents function via p53-independent mechanisms.

4.10 Piperine-Mediated Effects on Human Colon and Rectal Cancer Cells are Independent of TRPV1 Receptor Binding

Blockade of TRPV1 receptors in HT-29 colon cancer cells with capsazepine (Figure 52) and SB366791 (Figure 54) revealed that piperine-mediated cytotoxicity is independent of TRPV1 receptor binding. Therefore, the TRPV1 receptor is not involved in piperine-mediated cytotoxicity against HT-29 cells. Similar results were obtained using the TRPV1 receptor antagonists on piperine-treated HRT-18 rectal cancer cells (Figures 53 and 55). Since piperine is lipid-soluble, it likely diffuses across the plasma

membrane but whether the phytochemical remains in the cell membrane or trafficks to other cell compartments is not known. TRPV1 blockade likely bears greater significance in the context of the adverse "burning" aftertaste sensation induced by piperine consumption (McNamara et al 2005). To enable piperine ingestion as a route of administration, blockade of the TRPV1 receptor would be advantageous, as long as TRPV1 blockade does not reduce piperine-induced cytotoxicity to malignant cells.

4.11 Conclusions

Piperine exhibited a dose-dependent inhibitory effect on the proliferation of HT-29 colon and HRT-18 rectal cancer cells. HT-29 colon cancer cell growth inhibition was attributed to G_1 phase cell cycle arrest, while piperine exerted a much smaller effect on cell cycle progression in HRT-18 rectal cancer cells. HT-29 and CaCo-2 colon and HRT-18 rectal cancer cell growth inhibition was also attributed to a cytotoxic effect by piperine. Furthermore, piperine-mediated cytotoxicity resulted from induction of apoptosis in a dose-dependent manner, which was mediated by hydroxyl radical production. Piperine- mediated apoptosis involved membrane destabilization and occurred in a caspase-independent manner. This effect was also associated with activation of the p38 and JNK MAPK pathways. In addition, piperine-mediated apoptosis involved ER stress. Piperine-mediated apoptosis is p53-independent and is independent of TRPV1 receptor binding. Overall, piperine exerted an anti-proliferative and apoptosis-inducing effect on colorectal cancer cells that may have utility in colorectal cancer treatment.

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