Chapter 1: Introduction

1.1 Renal Cell Carcinoma

Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for 80-85% of all malignant tumors of the kidney [1]. It originates most commonly in the epithelial cells lining the proximal convoluted tubules of the nephrons (clear cell renal RCC), and sometimes in the cells of the collecting duct [2]. Other forms of kidney cancer include transitional-cell carcinoma of the renal pelvis, and Wilm's tumor in children.

The incidence of renal cancer has been rising steadily over the last few decades. The American Cancer Society estimates that in 2010 there were 58,240 new cases and 12,980 deaths from the disease in the US [3]. RCC is a highly lethal cancer with overall five-year survival rate less than 50% [4]. This is because it is a highly metastatic disease: 30% of RCC patients have metastatic disease at the time of diagnosis and an additional 20-30% with localized disease at diagnosis will develop metastatic disease after surgical removal of all or part of the kidney, which is the primary initial treatment [5]. Metatstatic RCC carries a dismal prognosis with 5-year survival less than 10% [4]. RCC is generally unresponsive to conventional radiation and chemotherapy. Immunotherapies such as interleukin-2 and interferon-alpha have been effective but only in a small proportion of patients and often with severe side-effects [6, 7]. As such, the development of new chemoprevention and chemotherapy strategies is of vital importance in combating RCC.

Risk factors for RCC include cigarette smoking [8] and obesity [9, 10] which have repeatedly been shown to have strong associations. Other risk factors are family history, hypertension, diabetes and diet [1, 11]. Many dietary factors have been considered in relation to RCC. Several case-control and prospective cohort studies have shown an increased risk with meat consumption [12, 13] and a reduced risk with intake of fruits and vegetables [14, 15]. These findings are consistent with the dietary compositions of different regions of the world and the RCC incidence rates: the "Western" diet is high in meat and low in fruits and vegetables and this is accompanied with some of the highest incidence rates of kidney cancer in Europe and North American countries.

Studies that looked at sub-groups within fruits and vegetables have found one sub-group: cruciferous vegetables to have a consistent negative association with RCC risk [16, 17]. Notable among them is a Los Angeles population based case-control study with 1,204 RCC patients and an equal number of controls which revealed a significant inverse association of RCC risk with consumption of cruciferous vegetables with an odds ratio of 0.53 between the highest and the lowest intake quintiles [18].

1.2 Cruciferous Vegetables and Isothiocyanates

Cruciferous vegetables are vegetables belonging to the family Cruciferae (or Brassicaceae). Common examples include broccoli, Brussels sprouts, cabbage, cauliflower, mustard, bok choy, horseradish, wasabi and watercress. These vegetables are unique in their high content of glucosinolates. When these vegetables are cut, crushed or cooked, the glucosinolates are hydrolyzed to isothiocyanates (ITCs), the bioactive products, by the enzyme myrosinase, which is released from the plant's cellular

compartments. Myrosinase activity has also been confirmed in intestinal bacteria, which may be important when we ingest intact glucosinolates.

ITCs are sulfur containing compounds with the general formula R-NCS (Fig. 1*A*). We now know of more than 20 different naturally occurring ITCs and several synthetic analogs. Different cruciferous vegetables are now known to be rich sources of different ITCs [19]. The typical ITC content of various agriculturally important cruciferous vegetables can range from approximately 0.1 to 1 mg/g [20]. Some of the commonly studied ITCs are sulforaphane (SFN) found in broccoli, allyl isothiocyanate (AITC) also found in broccoli, phenethyl isothiocyanate (PEITC) richly available in watercress and benzyl isothiocyanate (BITC) found in cabbage.

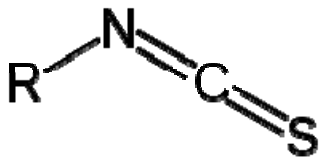


Fig. 1.1*A*: General structure of ITC

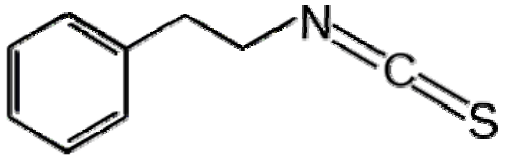


Fig. 1.1*B*: Structure of PEITC

1.3 Cruciferous Vegetables and Cancer: Epidemiological Data

Due to their high content of various unique phytochemicals, cruciferous vegetable consumption has been widely studied for their effect on human cancer risk. There is a large amount of epidemiological data to suggest that consumption of cruciferous vegetables reduce the risk of various cancers. An extensive review of 87 case-controlled studies showed that majority (67%) of the studies found an inverse association between

cruciferous vegetable intake and various cancer risks [21]. A few of these studies are mentioned here.

There have been quite a few epidemiological studies looking at cruciferous vegetable intake and lung cancer risk. A number of case-control studies found that patients diagnosed with lung cancer had significantly lower consumption of cruciferous vegetables than people in cancer-free control groups [17]. There have also been prospective cohort studies of men and women in Netherlands [22], women in US [23] and Finnish men in the CARET study [24] that show higher intakes of cruciferous vegetables associated with significantly reduced risks of lung cancer. In the case of colorectal cancer a number of case-control studies have found association between low intake of cruciferous vegetables and increased risk of colorectal cancer [25-27]. A prospective study of men and women in Netherlands also found that those with the highest intakes of cruciferous vegetables were significantly less likely to develop colon cancer than those with the lowest intakes [28]. There have been similar case-control and prospective studies that show association of high intake of cruciferous vegetables and reduced risk of breast [29, 30], prostate [31, 32] and pancreatic cancer [33]. For our study we are interested in the association between cruciferous vegetable and kidney cancer risk. There are two case-control studies that have shown negative correlation between intake of cruciferous vegetables and kidney cancer risk. Moore *et al.* found a significant association between cruciferous vegetable intake and reduced kidney cancer risk, with an overall odds ratio (OR) of 1.29 between lowest and highest intake groups [34]. In a population based case-control studies with non-Asians of Los Angeles, Yuan *et al.* showed a significant inverse association between intake of cruciferous vegetables and

RCC risk [18]. These are some of the epidemiological evidence that suggest protective effect of cruciferous vegetable consumption for various types of cancer.

However, there are also many case-control and prospective studies that fail to show any significant association between cruciferous vegetable consumption and various cancers [35-37]. One of the challenges with these epidemiological studies is that people with high consumption of cruciferous vegetables also have a generally higher intake of fruits and vegetables, so it is difficult to separate out the benefits of cruciferous vegetables by themselves. Increased consumption of fruits and vegetables in general is known to reduce risk of almost all types of cancer. Most of the epidemiological studies looking at cruciferous vegetable intake relies on self-reports and dietary questionnaires which can be somewhat unreliable.

There is also emerging evidence that individual genetic variations can affect the extent of protective effect of cruciferous vegetables [38]. One such genetic factor is variations in the genes encoding Glutathione S-Transferases (GSTs). GSTs are phase II enzymes that metabolize various compounds including ITCs for elimination from the body. Polymorphisms in the various GSTs including null variants are quite frequent and vary widely among various ethnic populations; individuals homozygous for the null variant cannot produce the corresponding GST enzyme. For example the frequency of *GSTT1*-null individuals within various populations range from 20% among Caucasians to 60% among Asians [39]. The other common GST polymorphism is *GSTM1* with frequency of *GSTM1*-null individuals ranging between 20 to 50% [40]. Because GSTs are involved in ITC metabolism and elimination from body, individuals lacking particular GSTs may have slower elimination and hence longer exposure to the beneficial effects of ITCs. In support of this idea, several epidemiological studies have found that inverse

associations between cruciferous vegetable intake and the risk of various cancers were more pronounced in *GSTM1*-null and/or *GSTT1*-null individuals [41-44]. In the case-control study by Moore *et al.* the inverse association between cruciferous vegetable consumption and kidney cancer risk was modified by GST polymorphism: the overall Odds Ratio (OR) between low and high intake was 1.29, however the protective effect was even stronger for individuals with *GSTT1*-null genotype (OR = 1.86) and with both *GSTT1/M1*-null genotypes (OR = 2.49). Among the various GST variants, *GSTT1* may be most important in the context of kidney cancer and ITCs. Toxicological studies suggest that the *GSTT1* enzyme is the most active GST in the kidneys [45, 46]. In terms of ITC metabolism, studies suggest that the *GSTT1* enzyme plays an important role in ITC conjugation compared with the other GSTs examined: urinary ITC excretion differed significantly between individuals with *GSTT1* active and null genotypes[47, 48]. GST variation can thus be an important compounding variable for the various epidemiological studies that just looked at cruciferous vegetable intake and various cancer risks. GST polymorphisms can also be an important factor in determining which individuals benefit most in terms of reduced cancer risk from consumption of cruciferous vegetables.

1.4 Phenethyl Isothiocyanate

1.4 .1 Dietary Sources

Phenethyl Isothiocyanate (PEITC) is one of the most-studied members of the ITC family of compounds. The chemical structure of PEITC is shown in Fig. 1B. It is converted from its natural glucosinolate precursor gluconasturtiin by myrisonase activity. Richest sources of PEITC, include watercress and horseradish. Fresh watercress has a

gluconasturtiin content of 0.72 mg/g which is equivalent to a PEITC content of 0.25 mg/g [49].

1.4.2 Bioavailability and Pharmacokinetics

The bioavailability and pharmacokinetics of PEITC has been studied in animals and humans. Once absorbed into the blood, ITCs, like many other xenobiotics are conjugated to glutathione (GSH) mainly in the liver [50]. ITCs are among the most rapidly conjugated substrates of glutathione-S-transferase (GST) [51], the enzyme involved in conjugation of ITCs to GSH. The GSH-conjugated ITC then travels to the kidneys where they are further metabolized to the N-acetylcysteine (NAC) conjugate by the mercapturic acid pathway which is then excreted in urine [50]. Pharmacokinetic studies in humans and animals agree with the metabolic pathway of ITCs. Humans fed a meal containing 30 g of watercress excreted up to 67% of the PEITC content of the watercress in urine as its urinary metabolite within a 24 hr period; the peak of the excretion occurred within 2-4 hr of the consumption [49]. This suggests that PEITC is efficiently absorbed and metabolized in the body. In another study, human subjects consumed 100 g watercress, and the plasma PEITC concentration measured over a 24 hr period. The plasma PEITC concentration peaked at 928 nM within 2.6 hrs and the average elimination half-life was 4.9 hrs [52] showing that dietary PEITC is rapidly absorbed into the blood and is also eliminated rapidly. However there was wide variation in elimination rate within the participants and the authors speculated that genetic variations such as GST polymorphism may account for that. Pharmacokinetic studies in animals also support the findings from human studies that PEITC is rapidly and efficiently absorbed. In an animal study, rats were given a single gavage dose of ^{14}C

radiolabeled PEITC [53]. Whole blood ^{14}C peaked within 2.9 hrs with an elimination half life of 21.7 hrs. A high amount of ^{14}C was detected in the liver and kidneys, the two major sites of ITC metabolism, within several hours. Within 48 hr of administration, 89% of the dose was excreted in the urine, and 10% in the feces. In a similar study rats were given PEITC either orally or intravenously (i.v.), and the plasma concentration measured over time. The Area Under Curve (AUC) were not significantly different between oral and i.v. administration suggesting that oral PEITC is almost completely absorbed into the blood. The oral bioavailability was close to 100%. In another study, mice administered radiolabeled PEITC orally, showed increased radioactivity in lungs, liver and kidneys within a few hours. 50% of the administered PEITC was excreted in urine and feces within 24 hr and 80% within 72 hrs. Taken together, these studies show high bioavailability and rapid metabolism and excretion of PEITC in mammals.

1.4.3 Dose and Toxicity

Being a phytochemical occurring naturally in human diet, PEITC can be expected to have low toxicity. Studies in mice show no toxicity with oral PEITC administration at fairly high doses over several week periods. Several xenograft studies with nude mice, where PEITC was administered by oral gavage at doses ranging from 5 to 12 μmol/day for 2 to 4 weeks showed no weight loss or other signs of overt toxicity [54-56]. In another study, oral doses up to 12 μmol in A/J mice showed no toxicity, however higher doses at 18 and 24 μmol showed significant weight loss (~10%) suggesting toxicity [57]. Considering average mice weight of 30 g, a dose of 12 μmol in mice is equivalent to ~400 μmol/kg (or 65 mg/kg) body weight. To put it in perspective, for a 60 kg human that is a dose of ~4000 mg / day. Several Phase I trials with PEITC have either been

completed or is underway, looking at the tolerable doses of PEITC in humans, but the results are not available yet. One of these trials, scheduled to start in June 2011, will look at up to 9 different doses of PEITC, starting at the lowest dose of 40 mg to determine the maximum tolerable dose in humans.

1.5 Phenethyl Isothiocyanate and Cancer

1.5 .1 Evidence from *In Vivo* Studies

PEITC has been shown to be powerful inhibitor of tumorigenesis in various animal models of cancer. Available animal models of cancer can be divided into three categories: 1) chemically induced models where a carcinogen is applied to induce tumor formation (e.g., Dimethylbenz(a)anthracene (DMBA) induced model of breast cancer), 2) xenograft models where cancer cells are injected into immunodeficient or syngeneic mice, and 3) transgenic mice that have a genetic modification that makes it develop spontaneous tumors. All three of these models have been used to study the effects of PEITC in various cancers. Some of the *in vitro* studies demonstrating anti-cancer effects of PEITC in various types of cancer are summarized below.

PEITC has been shown to inhibit NNK and benzo(a)pyrene [B(a)P] induced lung carcinogenesis in multiple studies in mice and rats. NNK and B(a)P are carcinogens found in cigarette smoke, and are believed to play a significant role in causing lung cancer in smokers. In a study by Conaway *et al.* A/J mice were treated for 8 weeks with NNK and B(a)P to induce tumorigenesis followed by 22 weeks of oral PEITC administration through diet between weeks 21 to 42 [58]. A dose of 3mmol/kg of PEITC in diet reduced the incidence of adenoma significantly from 42% to 19%.

Immunohistochemical analysis revealed significant reduction of proliferating cell nuclear antigen (PCNA) and induction of apoptosis in the adenomas of PEITC treated group. In another study Boysen *et al.* showed that PEITC significantly reduced the levels of DNA adducts of NNK in rats treated with NNK and B(a)P and also reduced the levels of Hemoglobin adducts of NNK in blood [59]. The group also made a similar observation in a separate study with mice [60]. Studies show that the NAC-conjugates of ITCs can also be just as effective as the ITCs in their anti-cancer effects. Yang *et al.* showed inhibition of B(a)P induced lung tumorigenesis by PEITC-NAC in mice, which was associated with induction of apoptosis, activation of p38 and ERK1/2 MAPK, and activation of p53 [61].

The tumor inhibiting potential of PEITC has also been studied in animal models of prostate cancer. Chiao *et al.* administered PEITC-NAC orally by diet supplementation (3 μmol/kg diet) to nude mice xenografted with PC-3 prostate cancer cells [62]. Nine week treatment resulted in a 50% reduction in tumor weight and this was associated with up-regulation of p21 and p27, down-regulation of cyclins D and E and an induction of apoptosis in the tumor tissue. Xiao *et al.* administered PEITC by oral gavage at a dose of 12 μmol/day to PC-3 xenograft mice and showed a 50% reduction in tumor volume that was associated with increased apoptosis and induction of Bax and Bid proteins [55]. In another study, the authors used a transgenic spontaneous tumor model of prostate cancer: TRAMP mice were fed a diet supplemented with 3 μmol/kg PEITC for 19 weeks. PEITC significantly reduced incidence of tumor from 58% to 22% and was associated with an induction of autophagy and over-expression of E-cadherin in the prostate. In two other studies PEITC was used in a combination treatment. Khor *et al.* showed PEITC and curcumin synergistically inhibited PC-3 xenograft in mice [63], and Xiao *et al.* showed

that PEITC sensitizes PC-3 xenograft to docetaxel induced tumor growth inhibition and induction of apoptosis [64].

PEITC has also been shown to inhibit tumorigenesis in animal models of breast, esophageal, colon and hepatic cancer. These include inhibition of DMBA-induced mammary tumorigenesis in rats [65]; inhibition of colon adenomas in Apc (Min/+) transgenic mice that is associated with induction of apoptosis and p21 upregulation [66]; inhibition of colon tumorigenesis in azoxymethane (AOM) induced mouse model of colon cancer [67]; inhibition of NMBA-induced esophageal carcinogenesis in rats [68, 69] inhibition of diethylnitrosamine-induced liver foci and hepatocellular adenomas in mice [70]. However, thus far, there have been no studies looking at the potential chemoprotective effect of PEITC in animal models of renal carcinoma.

1.5.2 Evidence from *In Vitro* Mechanistic Studies

Epidemiological studies show inverse association between human consumption of ITC-rich cruciferous vegetables and cancer risk; and animal studies show dietary administration of PEITC to inhibit or reduce tumorigenesis. But in order to understand the molecular mechanisms that are involved in the anti-cancer effects of PEITC, researchers rely on *in vitro* models of cancer. There have been quite a few studies that have looked at the effect of PEITC on various cancer cell lines and the mechanism of action involved. Here we summarize what we know so far about the mechanisms of chemoprotection by PEITC based on the findings from various *in vitro* studies:

Inhibition of Phase I Enzymes

Cytochrome P450s (CYP), also known as Phase I enzymes, are drug metabolizing enzymes that are involved in the 1st step of detoxification of xenobiotics including carcinogens. More than 50 different CYPs have been identified in humans. In the process of metabolizing carcinogens, CYPs convert them into more reactive electrophilic metabolites that can form DNA adducts and cause DNA damage. Because of this effect, CYPs have been implicated in the bioactivation of carcinogens (conversion of pro-carcinogens to carcinogens). One of the ways PEITC and other ITCs are thought to exert their chemopreventive effects is by inhibiting the enzymatic activity of various CYPs. PEITC has been shown to competitively inhibit activity of CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 [71]. By inhibiting CYPs, PEITC may be blocking the activation of pro-carcinogens and thus having a chemopreventive effect.

Induction of Phase II Enzymes

Whereas Phase I enzymes convert pro-carcinogens and other xenobiotics to reactive metabolites that can form adducts with DNA and proteins and cause cellular damage, phase II enzymes detoxify these metabolites by conjugating them to endogenous substrates such as glutathione and lead to their excretion and elimination, thus protecting the cell. One such important phase II enzyme is the glutathione-S-transferase (GST) superfamily of enzymes. Numerous studies have shown PEITC to induce phase II enzymes both *in vitro* and *in vitro*. In one study, rats fed diet supplemented with PEITC showed increased mRNA and protein levels of various GSTs in the liver, suggesting that PEITC increases expression of hepatic GSTs [72]. In another study, researchers used microarray technology to show increased mRNA expression of various GST isoforms in mice liver in response to PEITC treatment [73]. A recent animal study further also

confirms this observation. Konsue *et al.* showed increased GST activity in the liver of rats treated with dietary PEITC, however there was no evident change in GST activity in the lung and kidney [74]. In an *in vitro* study murine hepatoma and several other cell lines exposed to PEITC showed potent induction of GST and NAD(P)H:quinone reductase, two phase II enzymes [75]. Apart from phase II enzymes, there are also several antioxidant enzymes that have been shown to upregulated in response to PEITC treatment. One such antioxidant enzyme is heme oxygenase-1 which has been shown to be upregulated in PC-3 cells treated with PEITC [76]. In another study, peripheral blood cells exposed to PEITC for 24 hr showed increased expression of superoxide dismutase 2 (SOD2) and gluthatione peroxidase 1 (GPX1), two antioxidant enzymes, as well as increased SOD2 enzymatic activity [77]. The expression of some of these antioxidant enzymes, as well as the phase II enzymes is under the control of Nrf2 transcription factor, a master regulator of cellular antioxidant response. In normal conditions Nrf2 is sequestered in the cytoplasm, but in conditions that elucidate an antioxidant response, Nrf2 translocates into the nucleus and activate transcription of its target genes. PEITC has been shown to increase Nrf2 activation and translocation [78, 79], and this may be the mechanism by which it induces expression of the various phase II and antioxidant enzymes.

Induction of Cell Cycle Arrest

Cell cycle arrest is an important way in which phytochemicals can exert their chemoprotective effects. Most cancer cells maintain uncontrolled proliferation by overcoming some of the key cell cycle checkpoints. The mammalian cell cycle of normally dividing cells consists of four phases: G_1, S, G_2 and M. During G_1 the cell contains the normal diploid amount of DNA, and the biosynthetic activity of the cell is at

its highest with the increased synthesis of enzymes required for DNA synthesis in the S-phase. The G_1 phase is followed by the S-phase during which the cell undergoes DNA replication. By the end of the S-phase the cell contains double the amount of DNA. Once DNA replication is complete the cell enters G_2 another phase of high biosynthetic activity during which the cell synthesizes microtubules and enzymes required for mitosis, or the M-phase. During G_2 the DNA content of the cell remains double even though the cell contains only one nucleus. Once ready for division, the cell enters M-phase, or mitosis during which the daughter chromatids separate and the nucleus and cell divides into two separate cells, completing the process of cell division. Regulation of the cell cycle is crucial to the survival of a cell, including detection and repair of DNA damage, preventing cell division during times of cellular stress or starvation, and preventing uncontrolled replication of cells. The progression through the cell cycle is strictly controlled by regulatory proteins called cyclins and their associated kinases. There are two main checkpoints where most of the cell cycle control occurs: 1) The G_1/S checkpoint that prevents cell from undergoing DNA synthesis when it is not ready, for example during mitogen deprivation or loss of anchorage. 2) The G_2/M checkpoint that prevents the cell from entering mitosis in conditions such as DNA damage or mitogen deprivation. Cyclins D and E control the G_1/S transition, while the G_2/M transition is controlled by Cyclins A and B1.

The cell cycle arrest effect of PEITC was first investigated in 1993. Hasegawa *et al.* showed 2.5 μM PEITC treatment for 16 hrs to arrest HeLa cells at G_2/M phase, inhibiting growth to 41% of control [80]. Since then, several other studies have shown the G_2/M arrest activity of PEITC in various cell lines. 5 μM PEITC treatment for 48 hrs induced a G_2/M arrest in LNCAP prostate cancer cells, and this was associated with a

downregulation of cdc2 and cyclinB1 protein expression [81]. In multiple myeloma (MM) cells, 10 μM PEITC treatment for 24 hrs significantly arrested cells in the G_2/M phase and the authors correlated this with inhibition of proteasome activity by PEITC [82]. In another study, 20 μM PEITC induced G_2/M arrest in PC-3 cells and this was related to protein degradation and reactive oxygen species (ROS) generation by PEITC [83]. In A549 lung cancer cells, 10 μM PEITC treatment for 24 hrs was shown to induce G_2/M arrest by PEITC binding to tubulin [84]. Xiao *et al.* showed G_2/M arrest induction in PC-3 cells by 10 μM PEITC treatment that was associated with a reduction in cdc2 and cdc25C protein levels and inactivation of cdc2 by phosphorylation. The cell cycle arrest and the reduction in cdc2 and cdc25C levels were dependent on proteasome-mediated degradation and was inhibited by treatment with proteasome inhibitor lactacystin [85]. The G_2/M arrest of PEITC may also be mediated through Chk2 activation. Visanji *et al.* showed increased Chk2 phosphorylation and upregulation of p21 to be associated with G_2/M arrest in Caco-2 colon cancer cells treated with PEITC [86]. Although most studies show PEITC to arrest cells in the G_2/M checkpoint, one study by Cheung *et al.* showed a G_1 arrest induced in HT-29 colon cancer cells that was mediated by p38 MAPK activation and downregulation of cyclins A, D and E [87].

Induction of Apoptosis

Apoptosis is the process of programmed cell death by which cells that are ready to be disposed are disposed safely. These can be cells that have suffered damage such as oxidative damage, or extensive DNA damage beyond repair, or they can be cells that are in the process of natural turnoer. Cancer cells often have disrupted apoptotic signaling making them resistant to apoptosis: by overcoming apoptosis they are able to maintain uncontrolled proliferation. Many chemotherapeutic drugs work by inducing apoptosis in

cancer cells. PEITC has been shown to induce apoptosis both *in vitro* and *in vitro*. Here we will summarize some of these studies that looked at the induction of apoptosis by PEITC.

In MCF-7 human breast cancer cells, 10 and 30 μM PEITC treatment was shown to induce apoptosis and this was associated with caspases 7 and 9 activation, decrease in anti-apoptotic Bcl-2 and increase in pro-apoptotic Bax and Bid proteins, increase in cytochrome c, decrease in XIAP protein level and translocation of Smac to the cytosol [88]. In PC-3 cells, apoptosis induced by 10 μM PEITC was associated with caspases 3, 8 and 9 cleavage, and downregulation of Bcl-2 and Bcl-X_L [85]. Use of general caspase inhibitor as well as specific caspase-8 and 9 inhibitors attenuated the apoptosis induction. An alternate mechanism of apoptosis induction by PEITC was proposed by Mukherjee *et al.* [89]. Using HeLa cervical cancer cells they showed that up to 5 μM PEITC downregulated the anti-apoptotic isoforms of protein kinase C (PKC-α, -βII, -ε, and -ζ) while upregulating the pro-apoptotic isoform (PKC-δ). 5 μM PEITC also inhibited telomerase activity in the cells by 65%. In a separate study, the authors also made similar observations in PC-3 prostate cancer cells [90]. PKC and telomerase activity is increased in various cancers and they play an important role in the proliferation of cancer cells and the progression of cancer [91], so their downregulation by PEITC in association with apoptosis induction may be important. Xiao *et al.* showed ERK 1/2 and JNK to be involved in the apoptosis induction by PEITC in DU145 and LNCAP human prostate cancer cells [92].

Based on the *in vitro* data, PEITC can thus be considered a potent inducer of cell cycle arrest and apoptosis, and some of the signaling pathways involved during the cell cycle arrest or apoptotic response is now known. But how PEITC initiates these

responses and the sequence of events leading to cell cycle arrest and death is not clearly understood. There are several studies that suggest that PEITC induces its effect by generating reactive oxygen species (ROS) [55, 93]. PEITC is known to have a high affinity for glutathione (GSH) [94], and is one of the strongest substrates of the enzyme GST [51]. The GSH content of cells is important for their antioxidant defense: GSH rapidly binds to and neutralizes ROS thereby protecting the cell. By binding to and depleting cellular GSH, PEITC may make the cell more susceptible to ROS. Xiao *et al.* showed that the PEITC-induced apoptosis in PC-3 cells is mediated by generation of ROS, disruption of mitochondrial membrane potential and release of cytochrome c and other apoptogenic molecules into the cytosol [55]. Use of the antioxidant Euk134, a combined mimetic of superoxide dismutase (SOD) and catalase, attenuated the apoptosis induction by PEITC. However in another study it was shown that PEITC was a more potent inducer of apoptosis in A549 cells than sulforaphane (SFN), another ITC, even though SFN is a more potent activator of ROS, suggesting that the apoptosis induction effect of PEITC may not be mediated by its ROS generating activity [84]. Instead the binding of PEITC to tubulin may be the initial trigger that induces apoptosis. Mi *et al.* showed that PEITC binds strongly to tubulin and disrupts microtubule polymerization and this may be the mechanism by which PEITC induces cell cycle arrest and apoptosis [84]. In a follow-up study the authors also showed that during apoptosis, microtubule depolymerization by PEITC was followed by selective degradation of α- and β- tubulins by proteasome [95]. There are many chemotherapeutic drugs such as vinblastine and paclitaxel that work by interfering with microtubule dynamics. PEITC's effect on tubulin dynamics may be important in its chemoprotective effect.

1.6 Caspase signaling and Apoptosis

The process of apoptosis is tightly controlled and executed by a range of cell signaling pathways that initiates with some form of trigger which may initiate extracellularly (extrinsic) such as binding of cytokines to a membrane receptor, or intracellularly (intrinsic) such as in response to a stress [96]. During the process of programmed cell death by apoptosis, the cell goes through a series of characteristic changes that culminate with the elimination of the cell. These changes include chromatin condensation, DNA fragmentation, loss of cell membrane asymmetry and membrane blebbing. Caspases are a group of enzymes that are important in orchestrating many of these events. Based on their activity they can be divided into two groups: 1) The effector caspases that include caspases 3 and 7 are involved in carrying out the final events of apoptosis either by directly cleaving cellular proteins such as nuclear lamins (thereby disrupting nuclear membrane) or by cleaving and activating downstream substrates such as caspase-dependent DNAse (CAD) that are involved in the final events of apoptosis such as DNA fragmentation. 2) The initiator caspases that include caspases 8 and 9 are upstream of the effector caspases and lead to activation of the effector caspases by proteolytic cleavage. Caspases are activated at the post-translational level: they are synthesized as inactive pro-caspases, and during activation they are cleaved to release active caspases. This ensures that they can be rapidly activated.

One of the early initiator caspases is caspase-8. It is activated by death receptors such as DR4/5 and Fas in response to external apoptosis stimulus such as TRAIL and Fas-ligand (FasL) [97]. Activation of caspase-8 is inhibited by the pro-survival molecule FLIP [98]. Once activated, caspase-8 can directly activate the effector caspases (3 and 7)

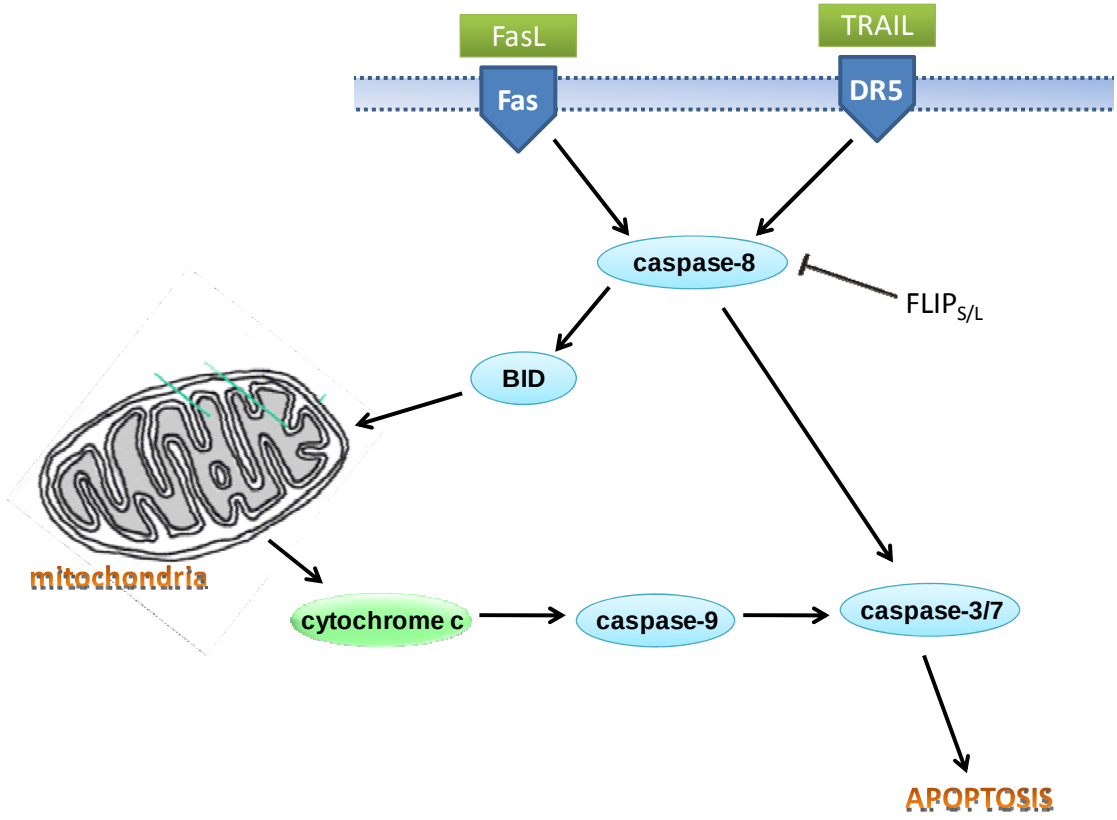


Figure 1.2: Apoptosis pathway mediated by Fas and DR5.

leading to what is considered extrinsic pathway of apoptosis, or it can activate BID leading to activation of the mitochondrial (intrinsic) apoptotic pathway. Activated BID (tBID) activates the mitochondrial apoptotic pathway, that involves a number of pro and apoptotic molecules such as Bcl-2, Bax, Bad, Bcl-X_L, leading ultimately to cytochrome c release into the cytosol [99]. Cytochrome c then binds to and activates Apaf-1 forming the apoptosome. The apoptosome then cleaves and activates caspase-9 which goes on to activate the effector caspases 3 and 7 [100]. There are a number of inhibitors of apoptosis (IAPs) that can block the apoptosis signaling at this late stage, by either blocking

activation of caspase-9 or by blocking subsequent activation of caspase-3 and 7. These include cIAP-1, cIAP-2 and XIAP [101, 102], while on the other hand pro-apoptotic molecules like Smac do the opposite by inhibiting IAPs [103]. Once the effector caspases 3 and 7 are activated they go on to complete the final events of apoptosis by proteolytically cleaving their downstream substrates which include nuclear lamins, PARP, Rb and caspase-activated DNAse (CAD).

1.7 Death Receptors

Death receptors are cell surface receptors that transmit extracellular "death" signals from specific ligands and turn on the cell's internal apoptosis cascade. They belong to the tumor necrosis factor receptor (TNFR) superfamily, and are composed of an extracellular domain that binds to the ligand and an internal "death domain", region of high homology shared by the different death receptors, that activates the downstream apoptosis pathways. Some of the death receptors important for transmitting extrinsic apoptotic signaling include: 1) Fas, which binds to its ligand FasL; 2) TNFR-1 and 2 which bind to TNF-α; 3) DR3 which binds to TWEAK and 4) DR4 and 5 which bind to TRAIL. Modulating or activating these death receptors can be an important mechanism of targeted therapy to induce apoptosis in cancer cells. In this context Fas and DR5 seems to be the most promising candidates. Systemic TNF-α administration has shown low antitumor activity and higher doses induce a severe inflammatory response similar to septic shock. TWEAK is one of the more recently identified cytokine and its death signaling through DR3 may be important in cancer but has not been studied much. Both FasL and TRAIL can induce strong apoptosis response in cancer cells, making their signaling a potential target for cancer therapeutics. Of the two TRAIL receptors: DR4 and

DR5, differential expression has been reported in various tumor types with DR5 being the most prevalent. In cancer cells that express both death receptors, there is data that suggest that DR5 contributes more to TRAIL induced apoptosis than DR4 [104]. Because of these reasons, there has been a lot of interest lately in utilizing Fas or DR5 mediated apoptosis in targeted cancer therapy.

1.7.1 Fas

The Fas receptor, one of the major death receptors involved in apoptosis signaling, was first cloned and identified in 1991 [105]. It is a 48 kDa membrane bound receptor consisting of an extracellular domain that binds to its ligand, and an intracellular death domain that mediates downstream signaling [106]. The death domain of Fas mediates downstream signaling by protein-protein interaction: upon receptor triggering by binding of FasL, the receptors oligomerize into aggregates [107] and recruits and binds to an adapter molecule Fas-associated death domain (FADD) [108]. FADD contains a death effector domain (DED) that can bind to and cleave procaspase-8. Together, the oligomerized Fas receptors, FADD and procaspase-8 form the Death Inducing Signaling Complex (DISC) that leads to caspase-8 cleavage and activation and triggering of the downstream apoptotic cascade [109].

Treatment with FasL has been shown to induce a strong apoptotic response *in vitro* [110], however systemic administration of FasL as an anti-tumor therapy has not been tried in humans because of its severe liver toxicity in mice [111]. The Fas receptor is abundantly expressed in hepatocytes, and as such, administration of FasL induces significant apoptosis of liver cells. However, recent understanding of Fas receptor

functioning has opened up the possibility of inducing Fas mediated apoptosis, without the need for administering FasL. Contrary to the original paradigm that FasL binding is required for Fas oligomerization and activation, later studies showed that Fas can oligomerize by itself prior to FasL binding [112]. The authors also showed that binding to FasL is not required for the apoptotic signaling by Fas: apoptosis can be triggered in cells expressing Fas that lack the N-terminal ligand binding domain [112]. Whether there are intracellular mechanisms that control Fas activation in the absence of FasL remains to be understood. But one thing is clear: even though Fas/FasL interaction can enhance apoptosis, FasL is not required for Fas activation. A growing number of agents have been shown to induce Fas activation in the absence of FasL. Without the need for FasL administration, upregulation of Fas itself may be enough to enhance Fas mediated apoptosis.

1.7.2 Death Receptor 5

Death receptor 5 (DR5), another member of the TNFR superfamily, is similar in structure and function to Fas receptor. It contains an extracellular ligand binding domain and a cytoplasmic death domain (DD). The ligand responsible for triggering DR5 is TRAIL. Binding of TRAIL to DR5 promotes association of the DD with the adapter molecule FADD, the same adapter molecule that binds to Fas. FADD then recruits and binds to procaspase-8 forming the Death Inducing Signaling Complex (DISC) which leads to cleavage and activation of caspase-8 and initiation of the apoptotic cascade.

Unlike Fas which is expressed in healthy tissues including hepatocytes where it has high expression, DR5 expression seems to be restricted to infected, damaged or

malignant cells [113]. Administration of pro-apoptotic agonists to DR5 has consistently shown relative selectivity for malignant over healthy cells [114]. TRAIL induced apoptosis is an important mechanism by which the body's immune system targets and destroys cancer cells, however tumors also develop various mechanisms to evade anti-tumor activity of the immune system. Studies have shown various cancer cell lines and primary tumor isolates to show resistance to DR5 agonists [115, 116]. Phase I clinical trials have shown TRAIL agonist administration to be generally safe and well-tolerated, however only modest anti-tumor activity has been observed in patients with advanced malignancies [117]. Low receptor density may be responsible for the resistance to TRAIL induced apoptosis. Various agents have been shown to increase sensitivity to TRAIL-induced apoptosis by increasing DR5 expression [118] . Upregulation of DR5 may thus be a potential mechanism for chemotherapeutic targeting of malignancies.

1.8 p38 MAPK signaling

Mitogen Activated Protein Kinase (MAPK) signaling pathways are evolutionarily well-conserved signaling pathways that respond to a variety of extracellular stimuli such as mitogens, osmotic stress, heat shock, oxidative stress, etc. and regulate a variety of cellular processes such as cell cycle regulation, survival/apoptosis, differentiation and proliferation. The various MAPK pathways can be divided into three major groups: 1) the extracellular regulated kinases (ERKs) which respond primarily to growth factors, 2) the c-Jun N-terminal Kinases (JNKs) that are activated by stress stimuli, and 3) p38 MAPKs which are also responsive to stress stimuli. Each MAPK pathway consists of a cascade of three kinases: a MAP kinase, a MAP kinase kinase (MKK or MEK) and a MAP kinase kinase kinase (MKKK or MEKK). The MKKK once activated by extracellular stimuli,

goes on to phosphorylate and activate a MAP2K which phosphorylates and activates the MAPK. The MAPK then activates a variety of downstream substrates to regulate the various downstream effects of the cellular response.

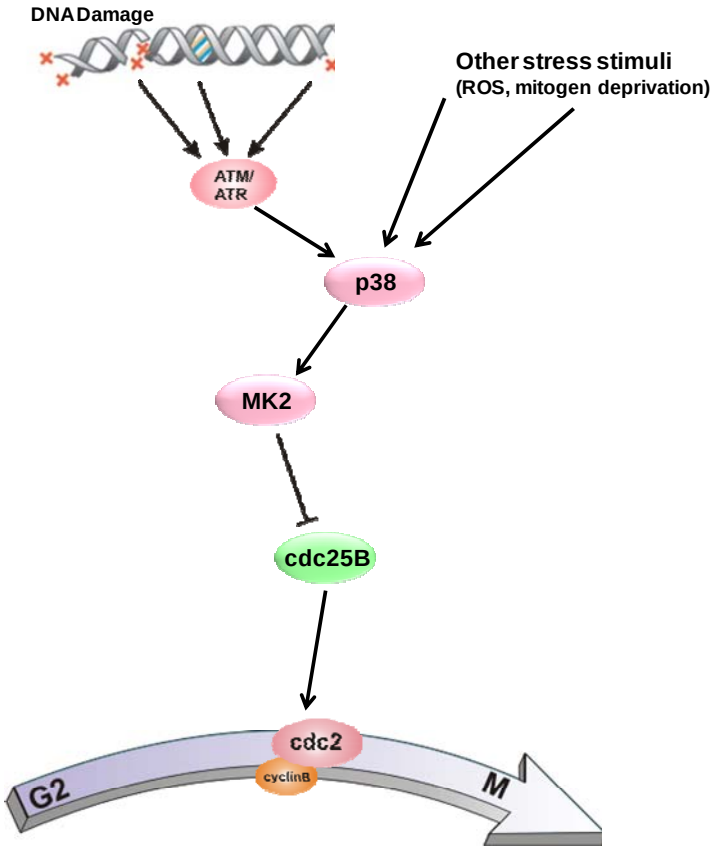


Figure 1.2: Pathway for p38 mediated control of G_2/M cell cycle arrest

p38 MAPK is activated in response to various stress stimuli and cytokines by its upstream MKKK/MKK cascade. The p38 MAPK pathway plays a role in a diverse range of cellular functions including cellular stress response, inflammation, cell cycle

regulation, DNA-damage response, oncogene-induced senescence and contact-inhibition [119]. Because of its role in cell differentiation, growth inhibition and apoptosis, p38 has been defined as a tumor suppressor. This definition is supported by experimental evidence. In animal models p38 deficiency enhances proliferation of cells of multiple origins and promotes chemically-induced tumorogenesis [120, 121]. Conversely, persistent activation of p38 by constitutive activation of MKK6, the upstream kinase of p38, inhibits tumorogenesis in mice [122, 123]. Modulation of p38 activity may thus be an important mechanism of chemoprotection. malignancies.

1.9 Summary

Renal cell carcinoma (RCC) is a highly lethal cancer that is characterized by late diagnosis due to lack of early symptoms, and resistance to conventional radiation and chemotherapy. Our study identifies a natural product, phenethyl isothiocyanate (PEITC) that can target RCC at two different levels: by reducing the risk of RCC (chemoprevention) and by targeting the tumor and killing cancer cells (chemotherapy).

Cruciferous vegetables are a group of vegetables whose consumption has been linked to reduced risk of a variety of human cancers including kidney cancer. They contain a unique class of sulfur-containing phytochemicals called isothiocyanates (ITCs). Different ITCs have been shown to have a variety of anti-cancer activities both *in vitro* and *in vitro*. PEITC is one of the most well studied ITCs, and it has been shown to have a variety of anti-cancer properties *in vitro* and *in vitro* in different cancers. However, its effect on renal carcinoma has not been studied before. Two of the important anti-cancer properties of PEITC that control proliferation and survival of cancer cells include

induction of cell cycle arrest and induction of apoptosis. However, the mechanism by which PEITC induces cell cycle arrest and apoptosis is not fully understood. In this study we look at the effect of PEITC on the proliferation and survival of renal carcinoma cells. We show that PEITC induces cell cycle arrest and apoptosis in human RCC cells and we study the mechanism by which it does so. Understanding the mechanism by which PEITC inhibits proliferation and survival of RCC cells may help develop PEITC as a potential agent of chemoprevention and/or chemotherapy against kidney cancer.

Chapter 2: Phenethyl Isothiocyanate-induced G2/M arrest in renal carcinoma cells is mediated by activation of p38 and MK2

2.1 Abstract

Epidemiological data suggest reduced risk or renal cell carcinoma (RCC) with increased consumption of isothiocyanate-rich (ITC) cruciferous vegetables. Phenethyl Isothiocyanate (PEITC) is one such ITC found naturally in watercress. We investigated the anti-proliferative effect of PEITC on the human RCC cell Caki-1. Treatment with 10 μM PEITC significantly reduced proliferation of Caki-1 cells over a 4-day period as measured by Calcein AM. This inhibition of proliferation was associated with an induction of G¬2/M arrest: treatment with 10 μM PEITC for 24 hrs increased percentage of cells in G2/M phase from 22% to 46%. PEITC induced activation of p38 and increased phosphorylation of its downstream kinase MK2 (Thr334). Use of p38 inhibitor SB203580 reversed the G2 arrest induced by PEITC. We also showed that, in contrast to a few other studies, PEITC does not cause direct DNA damage in cells; the apparent DNA damage observed is a later effect of apoptosis induced by PEITC.

2.2 Introduction

Renal cell carcinoma (RCC) is a highly lethal cancer with overall five-year survival rate less than 50% [4]. The incidence of renal cancer has been rising steadily over the last few decades with 2010 estimates at 57,760 new cases and 12,980 deaths from the disease in the US alone [124]. Risk factors for RCC include cigarette smoking and obesity which have repeatedly been shown to have strong associations. Other risk factors are family history, hypertension and diet [1]. Many dietary factors have been studied in relation to RCC including increased risk with meat consumption and a reduced risk with intake of fruits and vegetables. Studies that looked at sub-groups within fruits and vegetables have found one sub-group: cruciferous vegetables to have a consistent negative association with RCC risk [16, 17]. Cruciferous vegetables contain a unique class of phytochemicals called isothiocyanates (ITCs) that have been shown to have variety of anti-cancer properties both *in vitro* and *in vitro* [19]. Phenethyl Isothiocyanate (PEITC) is one such ITC found naturally and abundantly in water-cress. PEITC has been shown to be a powerful inhibitor of tumorogenesis in animal models of lung, esophageal, colon and prostate cancer [68, 125-132]. *In vitro* anti-cancer effects of PEITC include inhibition of Phase I enzymes that activate pro-carcinogens [133, 134], induction of Phase II enzymes that remove pro-carcinogens from cells [75, 135], induction of cell-cycle arrest [83, 86] and induction of apoptosis [56, 88]. PEITC has been shown to induce G_2 / M arrest in colon, prostate and bone cancer cells [83, 86, 136]. The mechanism by which PEITC induces cell cycle arrest in cancer cells is not well understood. Among proposed mechanisms that have been studied are generation of reactive oxygen species [136], induction of DNA damage [86], upregulation of p21 [62, 86], inhibition of STAT3 [137] and tubulin degradation [83]. The effect of PEITC on the

proliferation of RCC cells has not been studied before. In this study we look at the p38 MAPK pathway as a potential mechanism by which PEITC induces cell cycle arrest in RCC cells.

Mitogen activated protein kinases (MAPKs) are protein kinases that respond to extracellular stimuli such as growth factors, cytokines, mitogen, osmotic stress, heat shock, UV radiation, etc. and regulate various cellular processes such as gene expression, differentiation, proliferation, cell survival and apoptosis [138, 139]. One of three known MAPK sub-families, p38 MAPK pathway is activated in response to a variety of stress stimuli [140, 141] including DNA damage, reactive oxygen species (ROS), UV radiation, etc. p38 has been shown to play an important role at both the G_1/S checkpoint [142] as well as the G_2/M checkpoint [143]. In conditions of stress such as DNA damage, p38 can induce a G_2/M arrest through activation of its important downstream target MAPKAP Kinase-2 (MK2) which goes on to inhibit cdc25B through selective degradation [144]. In this study we show that PEITC induces a G_2/M arrest in Caki-1 cells, and this arrest is associated with increased p38 phosphorylation and phosphorylation of MK2 at Thr334. The p38 inhibitor SB203580 prevents the induction of G_2/M arrest, suggesting that the cell cycle arrest induction of PEITC is dependent on p38. Understanding the mechanism by which PEITC induces cell cycle arrest in RCC cells may help us better understand and utilize the chemoprotective properties of PEITC.

2.3 Materials and Methods

2.3.1 Materials

PEITC was purchased from LKT Laboratories (St. Paul, MN). Cell culture reagents, fetal bovine serum (FBS), cell extraction buffer (catalog # FN0011) and Calcein AM were purchased from Invitrogen (Carlsbad, CA). Propidium Iodide and RNAse A was from Sigma-Aldrich (St. Louis, MO), BCA reagent was from Thermo Fisher (Rockford, IL), SB203580 from Tocris Bioscience (Ellisville, MO). Antibodies against p-p38, total p38, p21, p-MK2 (222), p-MK2 (334), total MK2 and actin were purchased from Cell Signaling (catalog # 4631, 9212, 2947, 3316, 3007, 3042 and 4907 respectively); HRP-conjugated secondary antibody and ECL reagent were purchased from GE Healthcare Biosciences (Pittsburgh, PA); H2A.X phosphorylation assay kit (catalog # 17-344) was purchased from Millipore (Billerica, MA). Caki-1 cells were purchased from ATCC (Manassas, VA).

2.3.2 Cell culture and proliferation assay

Caki-1 cells were grown in RPMI 1640 medium containing 10% FBS and supplemented with 2 mM L-glutamine. The cells were cultured in 75 cm^2 flasks in a humidified incubator with 5% CO_2 / 95% air at 37 °C. Stock solution of PEITC was prepared in DMSO, stored at -20 °C and diluted in medium during treatment so that the final concentration of DMSO was 0.1% in the medium. For proliferation assay 1000 cells / well was plated in 96-well plate, and the different concentrations of PEITC were added to the confluent cells (DMSO was added as control). After the indicated time periods the wells were gently washed with PBS and 1 uM Calcein AM in PBS added to

each well and incubated at 37 °C for 1 hr before reading fluorescence at 570nm ex / 585nm em on a Biotek Synergy2 plate reader.

2.3.3 Cell cycle analysis

Adherent cells growing in 6-well plates were treated with varying concentrations of PEITC (or DMSO only for control) for 24 hrs. For SB203580 treatment, cells were first incubated with the inhibitor for 1 hr followed by incubation with inhibitor and PEITC for 24 hrs. Treated cells were trypsinized and harvested, washed with PBS and fixed with 70% ethanol at -20 °C overnight. Fixed cells were resuspended in PBS containing 0.1 mg/mL RNAse A and 5 μg/mL PI and incubated at 37 °C for 40 min. The cells were then washed with PBS and run on an Accuri C6 Flow Cytometer and final data analysis performed using FlowJo software. Events were gated for FL2 peak height and area to exclude subcellular debris and aggregates. A minimum of 20,000 events were recorded for each sample. A frequency histogram of FL2 peak area was plotted and cell cycle analysis performed on FlowJo software using the Dean-Jett-Fox model.

2.3.4 DNA damage assay

Adherent cells growing in 6-well plates were treated with varying concentrations of PEITC (or DMSO only for control) for varying time periods. For SB203580 treatment, cells were first incubated with the inhibitor for 1 hr followed by incubation with inhibitor and PEITC for 24 hrs. Cells were then harvested, fixed and stained for γ-H2AX phosphorylation using Millipore H2A.X phosphorylation assay kit following manufacturer's protocol. Cells were then analyzed on an Accuri C6 Flow Cytometer.

2.3.5 Immunoblotting

Cells growing in 75 cm^2 flasks were exposed to the different concentrations of PEITC as described above for the indicated time period. Cells were then trypsinized and washed once with cold PBS and lysed on ice using cell extraction buffer (Invitrogen) according to manufacturer's instructions. The protein concentration of the clear lystate was measured using BCA reagent. Lysates containing 20 – 50 μg of protein were run on 10% SDS-PAGE gel and the protein transferred to PVDF membrane. After blocking with 5% non-fat dry milk for 1 hr the membrane was incubated with the indicated primary antibody (1:1000 dilution) at +4 °C overnight. The membrane was then washed and probed with appropriate secondary antibody (1:5000 dilution) for 1 hr, washed again and the immunoreactive bands visualized using enhanced chemiluminescence reagent according to manufacturer's instructions. The membranes were then stripped and reprobed for actin as a loading control.

2.3.6 Statistical Analysis

All statistical analyses were performed using SPSS software. Data were analyzed using one-way analysis of variance followed by the Tukey test for comparison of more than two treatments or a two-tailed Student *t* test for comparison between two treatments to determine statistical differences. Differences were considered statistically significant at $P < 0.05$.

2.4 Results

2.4.1 PEITC inhibits proliferation of Caki-1 cells

Treatment with 10 μM PEITC significantly reduced the proliferation of Caki-1 cells as followed over a 4 day period [Fig. 2.1]. At 20 μM PEITC concentration the cells did not proliferate at all, but we have shown previously that 20 μM concentration PEITC induces significant apoptosis in the Caki-1 cells and thus there is very little viability of the cells at the high concentration. No significant apoptosis was observed in the Caki-1 cells at 10 μM concentration (data previously shown), thus the observed inhibition of proliferation is not a result of cell death.

2.4.2 PEITC induces G2/M arrest

Treatment with increasing concentration of PEITC for a 24 hr period resulted in a dose-dependent increase in the sub-population of cells in the G_2/M phase with the highest arrest observed at the 10 μM concentration [Fig. 2.2A]. With the 10 μM PEITC treatment ~46% of the cells were in G_2/M phase compared to ~22% in G_2/M phase in the DMSO control treated cells. There was a corresponding decrease in the G_0/G_1 sub-population with the PEITC treatment, but no significant change in the S-phase sub-population. In the subsequent experiment, cells were pre-treated with 24 hr serum starvation to synchronize the cells in G_0/G_1 phase, and then released from serum starvation in the presence or absence of PEITC. Serum starvation synchronized the cells in the G_0/G_1 phase as expected (data not shown), and upon release from serum starvation by the addition of 10% FBS media, in the presence or absence of PEITC, a similar G_2/M arrest was observed again, but this time there was an even greater sub-population of cells in the G_2/M phase as compared to our previous experiment where cells weren't

synchronized [Fig. 2.2B]. However even the DMSO treated cells showed increased G_2/M population but this can be explained by the increased proliferation of the cells once they were released from the serum starvation. When serum-starved synchronized cells were treated with PEITC in the complete absence of FBS, the G_2/M arrest was observed at the 5 μM concentration, but at 10 μM concentration there was high cell death (data not shown).

2.4.3 PEITC treatment downregulates p21

One of the mechanisms by which cell can undergo a G_2 arrest is through p21. We wanted to see if the G_2 arrest we observed in Caki-1 cells treated with PEITC was mediated by p21. Contrary to what was expected, Western blot analysis showed a significant reduction in protein levels p21 [Fig. 2.3]. The downregulation of p21 was also confirmed by flow cytometric analysis of cells stained with fluorescent tagged p21 antibody (data not shown). This suggested that the observed G_2 arrest in Caki-1 cells is not mediated by p21 upregulation.

2.4.4 PEITC treatment increased phosphorylation of p38 and MK2

An alternate mechanism for G_2 arrest in cells involves the p38 MAPK pathway. We wanted to see if p38 was involved in the G_2 arrest mediated by PEITC. Western blot analysis revealed increased phosphorylation of p38 after 8 hr treatment with 10 μM PEITC and the phospho p38 level stayed high at the 12 hr timepoints as well [Fig. 2.4]. The total p38 protein level was not affected by the treatment. We then looked at the status of MAPKAP kinase 2 (MK2), a downstream target of p38 known to mediate the G_2/M arrest effect of p38. There was a significant increase in levels of Thr334

phosphorylated MK2 as well as a slight increase in the Thr222 phosphorylated MK2 after the 8 and 12 hr treatments that corresponded to the p38 phosphorylation. The phosphorylation status of MK2 is also apparent from the total MK2 immunoblot: the band position shifted up slightly which is what is expected with MK2 phosphorylation (49 kDa vs 47 kDa).

2.4.5 SB203580 attenuates PEITC-induced G2 arrest

In order to determine if p38 activation is required for the G_2/M arrest induced by PEITC, we pretreated the cells with the selective p38 inhibitor SB203580 (25 μM) for 1 hr prior to the 24 hr PEITC (10 μM) treatment. Cell cycle analysis revealed that pretreatment with the inhibitor attenuated the G_2 arrest inducing effect of PEITC [Fig. 2.5]. Western blot analysis showed that SB203580 did not affect the phosphorylation of p38 caused by PEITC but inhibited phosphorylation of the downstream target MK2. With the use of the inhibitor we were thus able to determine that p38 activity is required for the G_2/M arrest induced by PEITC.

2.4.6 PEITC does not cause direct DNA damage

P38 activation and G_2/M arrest is often a response to cellular DNA damage, so we wanted to see if the p38 activation and G_2 arrest that we observe in Caki-1 cells in response to PEITC treatment is due to DNA damage caused by PEITC. Using fluorescent labeled antibody we looked at γ-H2A.X phosphorylation as a measure of DNA double strand breaks (DSB). 10 μM PEITC treatment for up to 24 hrs showed only a very small increase in γ-H2A.X phosphorylation, however 15 and 20 μM PEITC treatment for 24 hrs resulted in 5% and 20% of cells staining positive for phospho γ-

H2A.X suggesting increased DSB [Fig. 2.6]. However when the cells were co-treated with the caspase inhibitor z-vad-fmk, this observed DNA damage was almost completely reversed suggesting that the observed DNA damage is actually a late effect of the apoptosis induced by PEITC (that we have previously shown), and not a result of direct DNA damage caused by PEITC.

2.5 Discussion

Phenethyl Isothiocyanate (PEITC) has been shown to have a variety of anti-cancer properties in different *in vitro* and *in vitro* models of cancer, but its effect on renal carcinoma has not been studied before. In this study we look at the anti-proliferative activity of PEITC on the human RCC cell line. We show that PEITC induces a G_2/M arrest and identify p38 as a key pathway involved in the cell cycle arrest. PEITC has been previously shown to induce G_2 arrest in other cancer cell lines through p53 and p21 upregulation, but this is the first time we show PEITC mediated activation of p38 as being responsible for the induction of G_2/M arrest.

Cell cycle arrest is an important way in which phytochemicals can exert their chemoprotective as well as chemotherapeutic effects. Most cancer cells maintain uncontrolled proliferation by overcoming some of the key cell cycle checkpoints. The G_2/M checkpoint controls passage of cell into mitosis, and in response to cellular stress such as DNA damage or mitogen deprivation cells can be arrested at this checkpoint [145]. The key regulator at the G_2/M checkpoint is the Cyclin B1 – cdc2 complex. During G_2 this complex is inactive and accumulated in the cytoplasm. When cells are ready to enter mitosis, cdc2 and cyclin B1 are activated by a series of phosphorylation and dephosphorylation and the complex translocates to the nucleus where it activates a series of substrates leading to the early events of mitosis. Cdc25B is an important regulator in the activation of cyclinB1-cdc2 complex. It activates the complex by dephosphorylation of key residues on cdc2 [146]. During conditions of G_2/M arrest, cdc25B is inactivated by upstream kinases thus preventing cdc25B mediated activation of cdc2. G_2/M arrest can also be p53 – mediated: activated p53 upregulates p21 which can interact with the cyclinB1-cdc2 complex preventing its activation [147].

One of the important upstream pathways that can lead to cdc25B suppression is the p38 MAPK pathway. The p38 MAPK pathway is one of three known MAPK pathways. It has a diverse range of functions including cellular stress response, inflammation, cell cycle regulation, DNA-damage response, oncogene-induced senescence and contact-inhibition [119]. In animal models p38 deficiency enhances proliferation of cells of multiple origins and promotes chemically-induced tumorogenesis [120, 121]. Conversely, persistent activation of p38 by constitutive activation of MKK6, the upstream kinase of p38, inhibits tumorogenesis in mice [122, 123]. These findings allow p38 to be considered a tumor suppressor. For our study we are interested in the role of p38 in cell cycle regulation, specifically its ability to arrest cells at the G_2/M checkpoint. In response to stress signals p38 is phosphorylated by its upstream kinases, and once activated it can induce a G_2/M arrest by phosphorylation and inactivation of cdc25B, the key phosphatase that activates the cyclinB1-cdc2 checkpoint. P38 may directly phosphorylate cdc25B or it phosphorylates and activates its downstream kinase MK2 which goes on to phosphorylate cdc25B. Bulavian *et al.* first reported phosphorylation of cdc25B by p38 in response to UV radiation [143] however later studies by Manke *et al.* showed that p38 was not directly phosphorylating cdc25B instead it was through MK2 phosphorylation [144]. More recent studies suggest that cdc25B has multiple phosphorylation sites, and both p38 and MK2 can phosphorylate cdc25B at the different sites. Once phosphorylated, cdc25B is sequestered in the cytoplasm by 14-3-3 and this prevents activation of the cyclinB1-cdc2 complex and progression into mitosis.

In our study we have shown that PEITC induces a G_2/M arrest in Caki-1 cells, which is associated with increased phosphorylation of p38 and MK2, and use of the p38 inhibitor SB203580 attenuates the cell cycle arrest. PEITC has been shown to induce

G_2/M arrest in other cancer cell lines through p53 activation and upregulation of p21, but our findings suggest that in Caki-1 cells, PEITC induced G_2/M arrest is not mediated by p21. In fact we observe a downregulation of p21 in response to PEITC treatment. What role, if any, this downregulation of p21 may play in the survival and proliferation of Caki-1 cells, is hard to predict. How PEITC is downregulating p21 is also a question that needs to be answered, and these are potential areas of future studies.

One of the common cellular stresses that can lead to p38 MAPK activation and G-$_2$/M arrest is DNA damage. There are several studies that suggest that the effects of PEITC and other ITCs on cancer cells are through the induction of DNA damage [86, 148, 149]. We also wanted to determine if PEITC caused any DNA damage in Caki-1 cells that may be responsible for the G_2/M arrest. Using γ-H2AX phosphorylation as a measure of DSB, we observed a slight increase in γ-H2AX phosphorylation at 10 μM PEITC concentration and a significant increase at 15 and 20 μM PEITC, but only after 24 hr treatment. No increase in γ-H2AX phosphorylation was observed at early timepoints (data not shown). This led us to believe that the observed DNA damage may actually be a later effect of the DNA fragmentation caused by apoptosis and not a direct effect of PEITC on the DNA. We repeated the experiment, this time including a general caspase inhibitor z-VAD-fmk to inhibit apoptosis. Use of the apoptosis inhibitor almost completely reversed the observed γ-H2AX phosphorylation caused by PEITC at all treatment concentrations. We were thus able to verify that the observed DNA damage is actually a result of the apoptosis induction effect of PEITC and not direct DNA damage. In the three previous studies mentioned above where the authors reported DNA damage caused by ITCs, DNA damage was observed at concentrations and timepoints that are enough to induce apoptosis. Zhang *et al.* assayed for DNA damage by looking at γ-

H2AX phosphorylation, the other two studies used comet assay to look at DNA damage. DNA fragmentation resulting from apoptosis can manifest as false positive in both γ-H2AX phosphorylation and comet assay [150, 151], something the authors failed to address in all three studies. Our finding that the apoptosis inhibitor z-VAD-fmk reverses the observed DNA damage, and that no DSB is observed at early timepoints suggest that PEITC does not cause direct DNA damage. This is an important finding because DSB is a procarcinogenic effect and induction of DSB by PEITC would be inconsistent with epidemiological data that show reduction of cancer risk with increased consumption of ITC-rich cruciferous vegetables. A recent study by Mi *et al.* further corroborates our finding. The authors showed that PEITC did not form DNA adduct or cause any oxidative DNA damage in cells [94].

Cell cycle checkpoints are important in preventing the uncontrolled proliferation of cells which is cancer. PEITC induces G_2/M arrest in renal carcinoma cells and this may be an important way in which PEITC exerts its chemoprotective effects against renal cancer. PEITC is found naturally in dietary cruciferous vegetables, and has been shown to have high bioavailability in humans. In a human study plasma concentration of PEITC after a 100 g watercress diet peaked at an average 929 nM within 2.6 hrs [52]. Although this dose is less than the dose we use for our study, the local bioavailability within the kidneys may be different from the plasma bioavailability. In one study rats gavaged with PEITC showed highest distribution of PEITC within the liver and the kidneys [53]. PEITC being a natural component of diet also has the added advantage of low toxicity. Studies in mice show no toxicity at doses up to 20 μM [55] and a Phase I human trial is currently in progress to determine maximum tolerable dose of oral PEITC administration. These findings make PEITC a potential candidate for chemoprevention against renal

cancer. Larger human trials in the future may help us determine if PEITC supplementation can be effective in reducing risk of renal cancer.

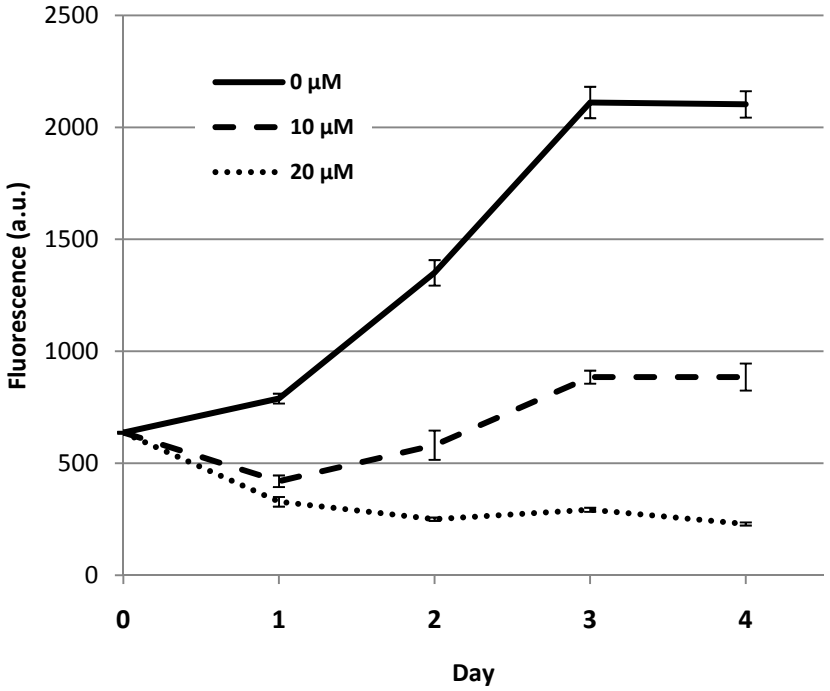
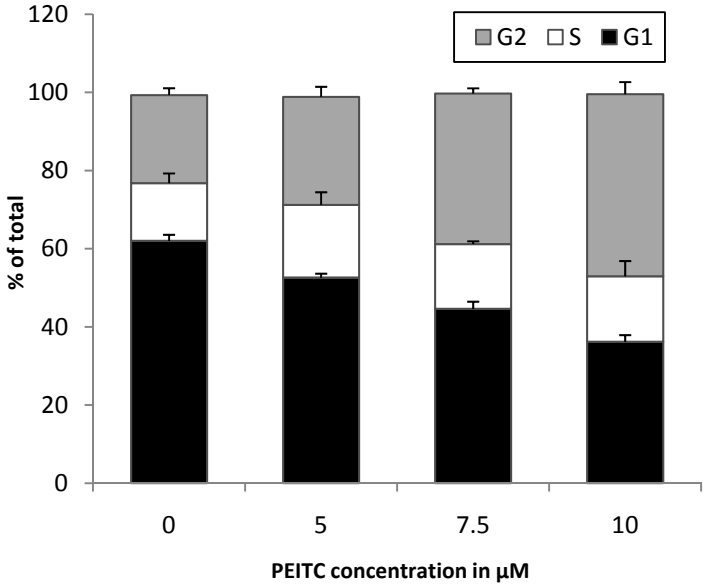
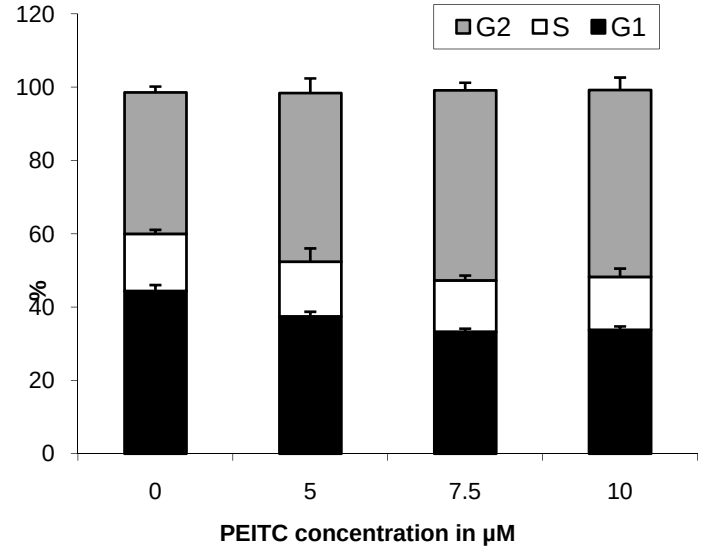


Figure 2.1: PEITC inhibits proliferation of Caki-1 cells. Sub-confluent cells growing in 96-well plate were cultured with 0, 10 or 20 µM PEITC and after each day the cell proliferation measured using Calcein AM assay (n = 4 ± S.E.). 10 µM PEITC significantly reduced proliferation and 20 µM PEITC resulted in complete loss of cell proliferation that could be due to complete loss of cell viability.



(A)



(B)

Figure 2.2: PEITC induced G_2/M arrest in Caki-1 cells. Cells growing in normal conditions (A) or that have been synchronized by serum starvation for 24 hr (B) were treated with various concentrations of PEITC for 24 hr after which they were harvested, fixed and stained with propidium iodide. Stained cells were analyzed by flow cytometry to determine the percentage of cells in each phase of the cell cycle (n = 3 ± s.d.)

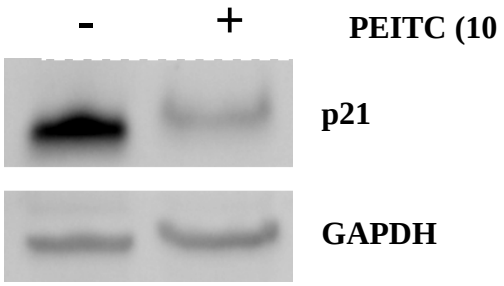


Figure 2.3 Effect of PEITC on p21 protein levels. Cells were treated with DMSO (control) or 10 μM PEITC for 24 hrs and the protein levels of p21 analyzed by western blot. GAPDH level was used as loading control.

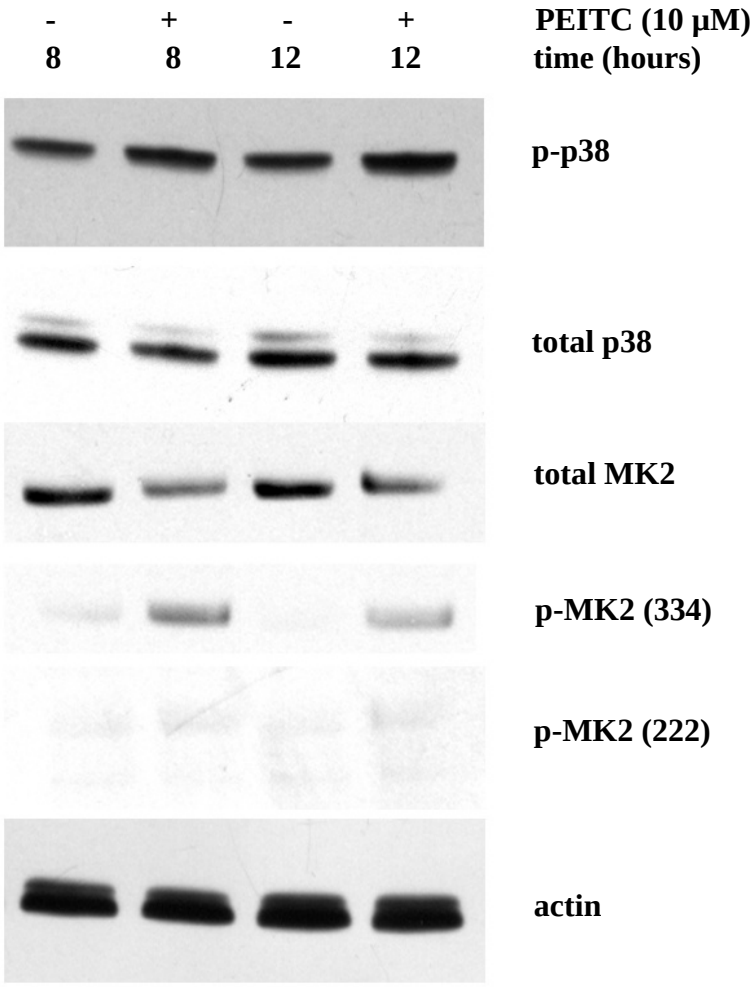


Figure 2.4: PEITC induces p38 and MK2 phosphorylation. Cells were treated with DMSO (control) or 10 µM PEITC for 8 and 12 hrs and the expression and phosphorylation of p38 and MK2 analyzed by western blot. Actin levels was used as loading control.

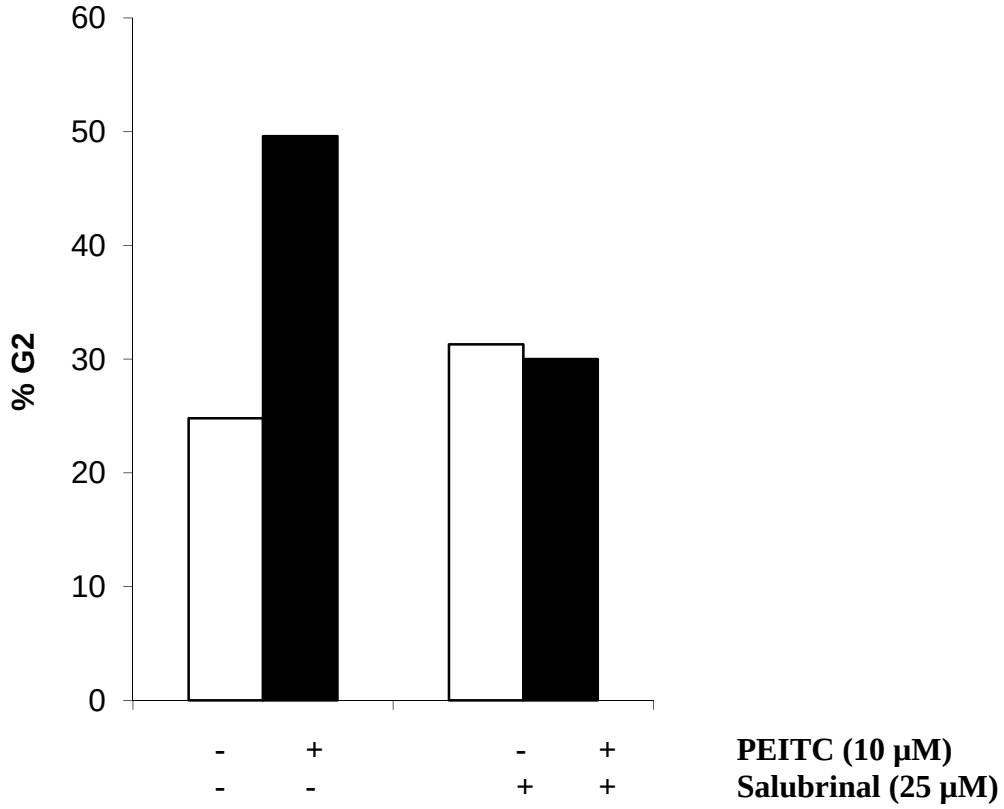


Figure 2.5: Salubrinal attenuates the G_2 arrest induced by PEITC. Adherent cells growing in 6-well plates were treated with 0 or 10 µM PEITC in the presence of absence of 25 µM salubrinal for 24 hrs. Cells were then harvested, fixed and stained with PI and analyzed by flow cytometry to determine the percentage of cells in each phase of the cell cycle.

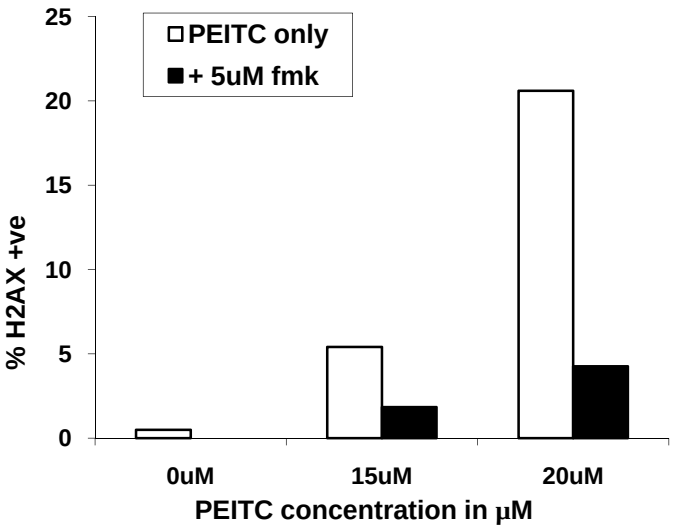


Figure 2.6: Induction of DSB by PEITC is inhibited by co-treatment with caspase inhibitor. Adherent cells in 6-well plates were treated with 0, 15 and 20 μM PEITC in the presence or absence of the general caspase inhibitor z-VAD-fmk (5 μM) for 24 hr. Cells were then harvested, fixed and stained with FITC labeled phospho γ-H2AX antibody, and the cells analyzed by flow cytometry.

Chapter 3: Phenethyl Isothiocyanate-induced apoptosis in renal carcinoma cells is associated with upregulation of death receptor 5 and Fas

3.1 Abstract

Phenethyl isothiocyanate (PEITC) has been shown to be active against various types of cancer but its efficacy against renal cell carcinoma (RCC) has not been shown before. In this study we looked at the effect of PEITC treatment on two different human RCC cell lines, Caki-1 and Caki-2. PEITC reduced viability of both cell lines with an IC_{50} of 7.5 μM in Caki-1 and 14.5 μM in Caki-2 cells. Using Annexin V staining and caspase-3 activity measurement we showed that PEITC induced apoptosis in both cell lines within 18 hours of treatment. The induction of apoptosis was associated with cleavage and activation of caspase-8, BID and caspase-9. There was also an upregulation of death receptor 5 and Fas death receptor in cells treated with PEITC. The induction of apoptosis as determined by Annexin V staining was completely blocked when cells were treated with the general caspase inhibitor z-VAD-fmk. Taken together our results suggest that PEITC induced apoptosis in RCC cells by activation of both the extrinsic and intrinsic apoptotic pathways. PEITC may therefore have potential as a chemotherapeutic agent against RCC, a disease that is highly resistance to conventional chemotherapeutic drugs available today.

3.2 Introduction

The incidence of renal cancer has been rising steadily over the last few decades with 2010 estimates of 57,760 new cases and 12,980 deaths in the US [124]. Renal cell carcinoma (RCC) is the most common type of kidney cancer, accounting for 80-85% of all malignant tumors of the kidney [1]. RCC is a highly lethal cancer with overall five-year survival rate less than 50% [4]. This is because it is a highly metastatic disease: 50% of RCC patients have metastatic disease at diagnosis or develop metastasis after initial treatment [5]. Metatstatic RCC (mRCC) is generally unresponsive to conventional radiation and chemotherapy and carries a dismal prognosis with 5-year survival less than 10% [4]. Immunotherapies such as interleukin-2 and interferon-alpha have been effective only in a small proportion of patients and often with severe side effects [6, 7]. In recent years there has been considerable improvement in treatment options for mRCC with the development of biologically active drugs that target pathways such as VEGF and mTOR, but these modalities have increased median survival by only 4-8 months. The identification and development of new chemotherapeutic drugs that can overcome the resistant nature of mRCC is of vital importance.[94]

Phenethyl Isothiocyanate (PEITC) belongs to the class of phytochemicals called isothiocyanates that are isolated from cruciferous vegetables. Found abundantly in water-cress, PEITC has been shown to have a variety of anti-cancer effects both *in vitro* and *in vitro*. PEITC has been shown to be a potent inhibitor of tumorogenesis in animal models of lung, esophageal, colon and prostate cancer [68, 125-132]. *In vitro* anti-cancer effects of PEITC include inhibition of Phase I enzymes that activate pro-carcinogens [133, 134], induction of Phase II enzymes that remove pro-carcinogens from cells [75, 135], induction of cell-cycle arrest at the G2/M checkpoint [83, 86] and induction of apoptosis

[56, 88]. PEITC and related isothiocyanates have been shown to induce apoptosis in a variety of cancer cell lines including prostate [56], lung [94], pancreatic [152], breast [88] and ovarian cancer [153]. The mechanism of apoptosis induction by PEITC is not fully understood. Known cellular responses to PEITC, and other related isothiocyanates, that may be involved in the apoptosis induction include generation of reactive oxygen species (ROS) [55], activation of MAPK pathways such as ERK, JNK and p38 [154-156], inhibition of STAT3 [137, 152], and selective degradation of tubulin [95]. To our knowledge, the anti-cancer properties and apoptosis induction of PEITC has not been studied in renal carcinoma cells.

In this study we examined the effect of PEITC on two different RCC cell lines. We demonstrate that PEITC induced apoptosis by upregulation of the death receptors DR5 and FAS and is associated with cleavage and activation of caspase-8, BID and caspase-9. This is the first demonstration, to the best of our knowledge, of these effects of PEITC.

3.3 Materials and Methods

3.3.1 Materials

PEITC was purchased from LKT Laboratories (St. Paul, MN). Cell culture reagents, fetal bovine serum (FBS), caspase-3 activity kit (catalog # E13184), cell extraction buffer (catalog # FN0011) and Calcein AM were purchased from Invitrogen (Carlsbad, CA). Annexin V-FITC kit was from Biovision (Mountain View, CA), BCA reagent was from Thermo Fisher (Rockford, IL), z-VAD-fmk and DeadEnd Fluorometric TUNEL kit were from Promega (Madison, WI). Antibodies against caspase-8, caspase-9, BID, DR5 and FAS were purchased from Cell Signaling (catalog # 9746, 9502, 2002, 3696 and 4233 respectively). Caki-1 and Caki-2 cell lines were purchased from ATCC (Manassas, VA).

3.3.2 Cell culture and viability assay

Caki-1 and Caki-2 cell lines were grown in RPMI 1640 medium containing 10% FBS and supplemented with 2 mM L-glutamine. The cells were cultured in 75 cm^2 flasks in a humidified incubator with 5% CO_2 / 95% air at 37 °C. Stock solution of PEITC was prepared in DMSO, stored at -20 °C and diluted in medium during treatment so that the final concentration of DMSO was 0.1% in the medium. For viability assay 5000 cells / well was plated in 96-well plate, and the different concentrations of PEITC were added to the confluent cells (DMSO was added as control) and treated for 24 hours after which the wells were gently washed with PBS and 1 uM Calcein AM in PBS added to each well and incubated at 37 °C for 1 hr before reading fluorescence at 570nm ex / 585nm em on a Biotek Synergy2 plate reader.

3.3.3 Apoptosis detection

Apoptosis induction in control and PEITC treated cells was determined using two methods: Annexin V / PI staining and measurement of caspase-3 activity. Annexin V staining was performed as follows: Adherent cells growing in 6-well plates were treated with varying concentrations of PEITC (or DMSO only for control) for 18 and 24 hrs after which cells were trypsinized and harvested, washed with PBS and resuspended in Annexin V binding buffer containing PI and Annexin V – FITC according to the manufacturer's protocol. The cells were then analyzed using an Accuri C6 Flow Cytometer and final data analysis performed using FlowJo software. Cell debris was first gated out using FSC/SSC plot; Annexin V – FITC and PI was then plotted as FL1 vs FL2. Cells that stained positive for Annexin V but negative for PI (lower right quadrant) were considered to be the early apoptotic population, cells that stained positive for both (upper right quadrant) were considered to be late apoptotic / necrotic population.

3.3.4 Caspase-3 activity

For the caspase-3 activity assay, the EnzChek Caspase-3 kit was used. Adherent cells were treated in 6-well plates; cells were then trypsinized, harvested, lysed and the caspase-3 activity of the lysate measured according to the manufacturer's protocol. The caspase-3 activity of the lysate was standardized against the protein concentration of the lysate as measured using BCA reagent, to account for differences in cell count in the different treatments.

3.3.5 Immunoblotting

Cells growing in 75 cm^2 flasks were exposed to the different concentrations of PEITC as described above for the indicated time period. Cells were then trypsinized and washed once with cold PBS and lysed on ice using cell extraction buffer (Invitrogen) according to manufacturer's instructions. The protein concentration of the clear lystate was measured using BCA reagent. Lysates containing 20 – 35 μg of protein were run on 10% SDS-PAGE gel and the protein transferred to nitrocellulose membrane. After blocking with 5% non-fat dry milk for 1 hr the membrane was incubated with the indicated primary antibody (1:1000 dilution) at +4 °C overnight. The membrane was then washed and probed with appropriate secondary antibody (1:5000 dilution) for 1 hr, washed and the immunoreactive bands visualized using enhanced chemiluminescence reagent according to manufacturer's instructions. The membranes were then stripped and reprobed for actin as a loading control.

3.3.6 Statistical Analysis

All statistical analyses were performed using SPSS software. Data were analyzed using one-way analysis of variance followed by the Tukey test for comparison of more than two treatments or a two-tailed Student *t* test for comparison between two treatments to determine statistical differences. Differences were considered statistically significant at $P < 0.05$.

3.4 Results

3.4.1 PEITC reduced the viability of RCC cells

Treatment with increasing concentrations of PEITC for 24 h resulted in reduced survival of both Caki-1 and Caki-2 cells, however, the Caki-1 cells were more sensitive to its effects (Fig. 3.1). At the highest concentration tested PEITC reduced the viability of both cell lines by almost 80%. The IC_{50} of PEITC was determined to be 7.5μM for Caki-1 cells and 14.5 μM for Caki-2 cells.

3.4.2 PEITC induced apoptosis

The effect of PEITC on apoptosis in the two RCC cell lines was examined by annexin V / PI staining. As shown in Fig. 3.2, PEITC caused a profound increase in both early and late apoptotic cell population in both cell lines.

3.4.3 PEITC induced caspase-3 activity

To further investigate the induction of apoptosis by PEITC we measured the activation of caspase-3 in the two cell types using a fluorescent substrate assay. Both Caki-1 and Caki-2 cells showed more than 3-fold increase in caspase-3 activity after 18 hr treatment with 15 μM PEITC (Fig. 3.3). With 20 μM PEITC treatment there was more than 5-fold increase in caspase-3 activity in Caki-1 cells and more than 6-fold increase in Caki-2 cells. 10 μM treatment did not show any significant difference from control. z-VAD-fmk, a general caspase inhibitor used as a negative control in this assay, brought the caspase-3 activity in both cell lines treated with the maximum dose of PEITC down to basal levels. We went back to our Annexin V assay and determined that addition of the

caspase inhibitor z-VAD-fmk almost completely inhibited the induction of apoptosis by PEITC (Fig 3.2) thus showing that the apoptosis induction by PEITC as determined by Annexin V staining is dependent on caspase-3 activation.

3.4.4 PEITC treatment led to caspase-8, caspase-9 and BID cleavage

In order to determine the mechanism of apoptosis induction by PEITC, we examined the pathway upstream of the effector caspases (caspase-3/7). Fig 3.4 demonstrates that Caki-1 cells treated with increasing concentration of PEITC have increased BID, caspase-9 and caspase-8 cleavage. This was observed after 8 h and more prominently after 12 h when treated with PEITC, at the same effective dose that induced apoptosis.

3.4.5 PEITC upregulated DR5 and FAS

We examined possible upstream mediators of caspase-8. Caspase-8 can be activated by cell surface death receptors such as DR4/5 and FAS or by downregulation of FLIP, an inhibitor of caspase-8. We looked at DR5, FAS and FLIP expression in Caki-1 cells treated with PEITC. Western blot analysis showed upregulation of DR5 and FAS protein levels in response to 15 uM treatment with PEITC for 8, 12 and 16 h. There was no change in the protein levels of FLIP (Fig. 3.5).

3.5 Discussion

PEITC has been shown in many *in vitro* and *in vitro* studies to exhibit cytotoxic activities towards various tumor types. Phase 1 clinical trials are now being carried out to determine the maximal tolerated dose in patients with lymphoproliferative disorders as a first step in determining whether PEITC will prove to be an effective drug for lymphoproliferative disorders. A naturally occurring dietary component, PEITC has excellent bioavailability in rodents and humans following dietary administration [49, 157] and exhibits very low toxicity in rodents [57]. Furthermore, PEITC preferentially distributes to the kidneys [53], making it particularly promising in the treatment of RCC. RCC is highly resistant to standard chemotherapeutic drugs, in part because renal tubule epithelial cells, and hence the tumor cells derived from them, express high levels P-glycoprotein, which allows the cells to excrete drugs [158, 159]. It was recently shown that PEITC is not a substrate for P-glycoprotein [160] and thus evades one of the key mechanisms conferring resistance to RCC. Because PEITC's effects on RCC have not been studied, we examined the ability of PEITC to cause cytotoxicity in human RCC cells *in vitro*. The cell lines chosen for this study, Caki-1 and Caki-2, are human RCC cell lines derived from patient tumors. Caki-1 was derived from a skin metastatic site, while Caki-2 was derived from primary renal tumor. Both cell lines are wild-type for p53, Caki-1 is also wild-type for Von Hippel Lindau (VHL) gene, a tumor suppressor gene mutated in a majority of kidney tumors, while Caki-2 has mutated VHL. Both cell lines have been extensively used as *in vitro* models of RCC [161, 162]. In rodents, plasma concentrations similar to the concentrations used in the current study were readily achieved [53].

Our study demonstrates that PEITC reduces the viability of renal carcinoma cells by inducing apoptosis. The apoptotic response to PEITC is rapid and and occurs at

physiologically achievable concentrations. In the Caki-1 cells, with 20 μM PEITC treatment a strong apoptotic response is observed within 18 hrs by Annexin V / PI staining: 12% of the cells are in the early apoptotic state and another 35% in the late apoptotic state; a similar response is observed in the Caki-2 cells. These results were confirmed by a second measure of apoptosis: the TUNEL assay. We then investigated the mechanism of apoptosis induction. PEITC caused the cleavage of caspace-8. Caspase-8 is an early-response caspase that is responsible for activating the effector caspaces, such as caspace 3. Consistant with this data, caspase-3 was also activated in the presence of PEITC. Thus, PEITC triggers the intrinsic apoptotic pathway in RCC cells, similar to what has been previously demonstrated in other tumor types [88, 153].

Caspase-8 can either be activated by membrane bound death receptors, or it can also be activated by downregulation of c-FLIP, a pro-survival molecule that inhibits activation of caspase-8. We saw no change in FLIP protein level in response to PEITC. We therefore examined the extrinsic death receptor pathways and if they are involved in the activation of caspase-8. Our results indicate that both DR5 and FAS are upregulated in response to PEITC treatment, thus suggesting that this upregulation by PEITC is what activates the apoptotic response in the cells.

This is the first demonstration that PEITC upregulates DR5 and triggers the extrinisic apoptotic pathway. Only two other previous studies have shown upregulation of a member of the death receptor family by an isothiocyanate. Matsui *et al*. showed induction of DR5 expression in osteosarcoma cells in response to treatment with sulforaphane (SFN), another important isothiocyanate [163]. However in their study, SFN alone wasn't able to induce apoptosis, it only sensitized the cells to TRAIL induced apoptosis. Basu *et al*. showed upregulation of DR4 in pancreatic cancer cells by benzyl

isothiocyanate [164]. DR4 and DR5, also known as TRAIL receptors 1 and 2, are the only two active members of the death receptor family that bind to TRAIL to mediate apoptosis. TRAIL mediated apoptosis has emerged as a promising pathway in cancer therapeutics because of the selectivity of TRAIL to induce apoptosis in cancer cells while leaving normal cells unaffected [165-167].

Fas, another member of the death receptor family, also triggers apoptosis by caspase-8 activation. Fas is activated by the binding of its ligand, FasL, to its extracellular domain. Fas plays a key role in the induction of apoptosis and there is evidence indicating that decreased expression of FAS facilitates development and progression of tumors by reducing the tumor cell apoptosis [168, 169]. Fas polymorphism was found to be associated with a significantly increased risk for RCC [170]. Unlike TRAIL, which has been shown to selectively kill cancer cells *in vitro*, FasL induces severe liver toxicity in mice due to high expression of Fas in hepatocytes. As such systemic administration of FasL as a candidate for anti-tumor therapy cannot be considered. However, there is accumulating evidence that suggest that Fas can oligomerize and initiate an apoptotic response independent of FasL binding [112, 171, 172]. Overexpression or upregulation of Fas has been shown to induce apoptosis independent of FasL [172, 173]. Our study shows that PEITC treatment upregulates Fas in RCC cells, and this may be important in the induction of apoptosis by PEITC.

In summary, our study is the first to demonstrate that PEITC has activity against RCC, through the novel mechanism of upregulating DR5 expression, as well as upregulating Fas expression and the subsequent activation of early and late caspases.

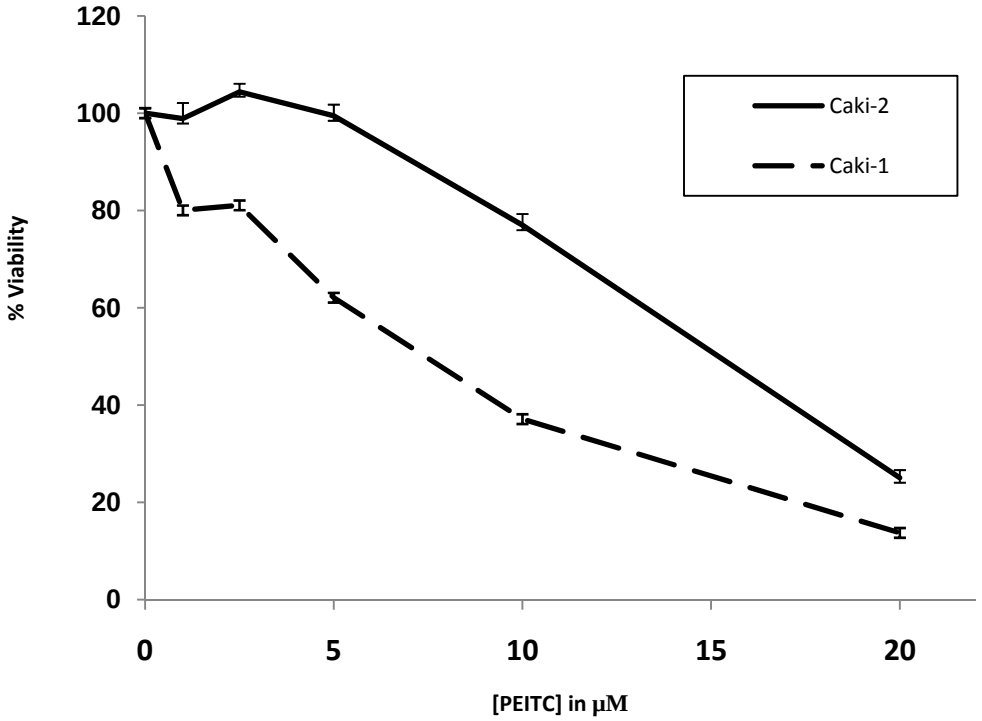


Figure 3.1: Effect of PEITC on the viability of Caki-1 and Caki-2 cells. Cells were treated with different concentrations of PEITC for 24 hrs and the cell viability measured using Calcein AM assay. Values are means (n = 4) ± SEM.

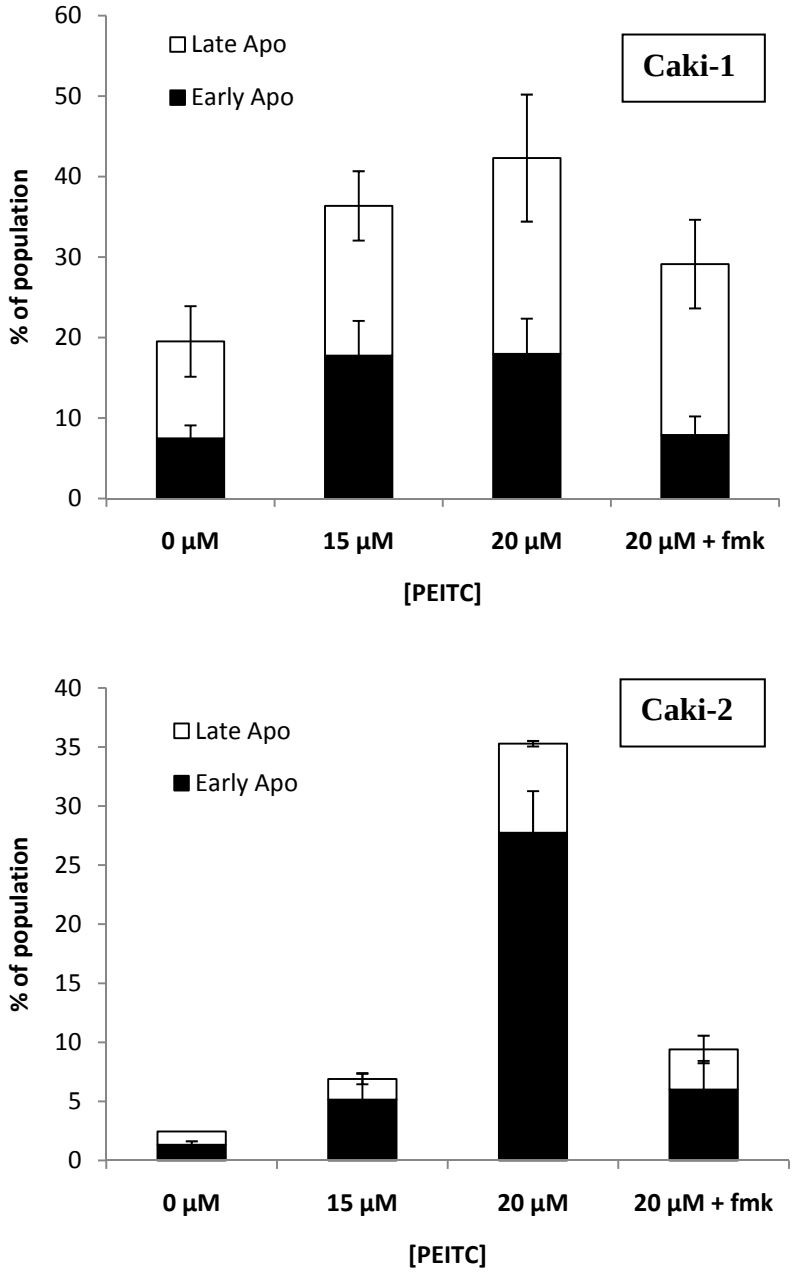


Figure 3.2: PEITC induces apoptosis in RCC cells. Caki-1 and Caki-2 cells were treated with 0, 15 and 20 µM PEITC, in the presence or absence of the general caspase inhibitor z-VAD-fmk (5 µM) as indicated, for 24 hours. Cells were then harvested and stained with Annexin V-FITC and PI, and analyzed by flow cytometry. Cells that stained positive for Annexin but not PI were considered to be early apoptotic, those that stained positive for both Annexin and PI were considered to be late apoptotic/necrotic. Values are means of three independent experiments ± S.D.

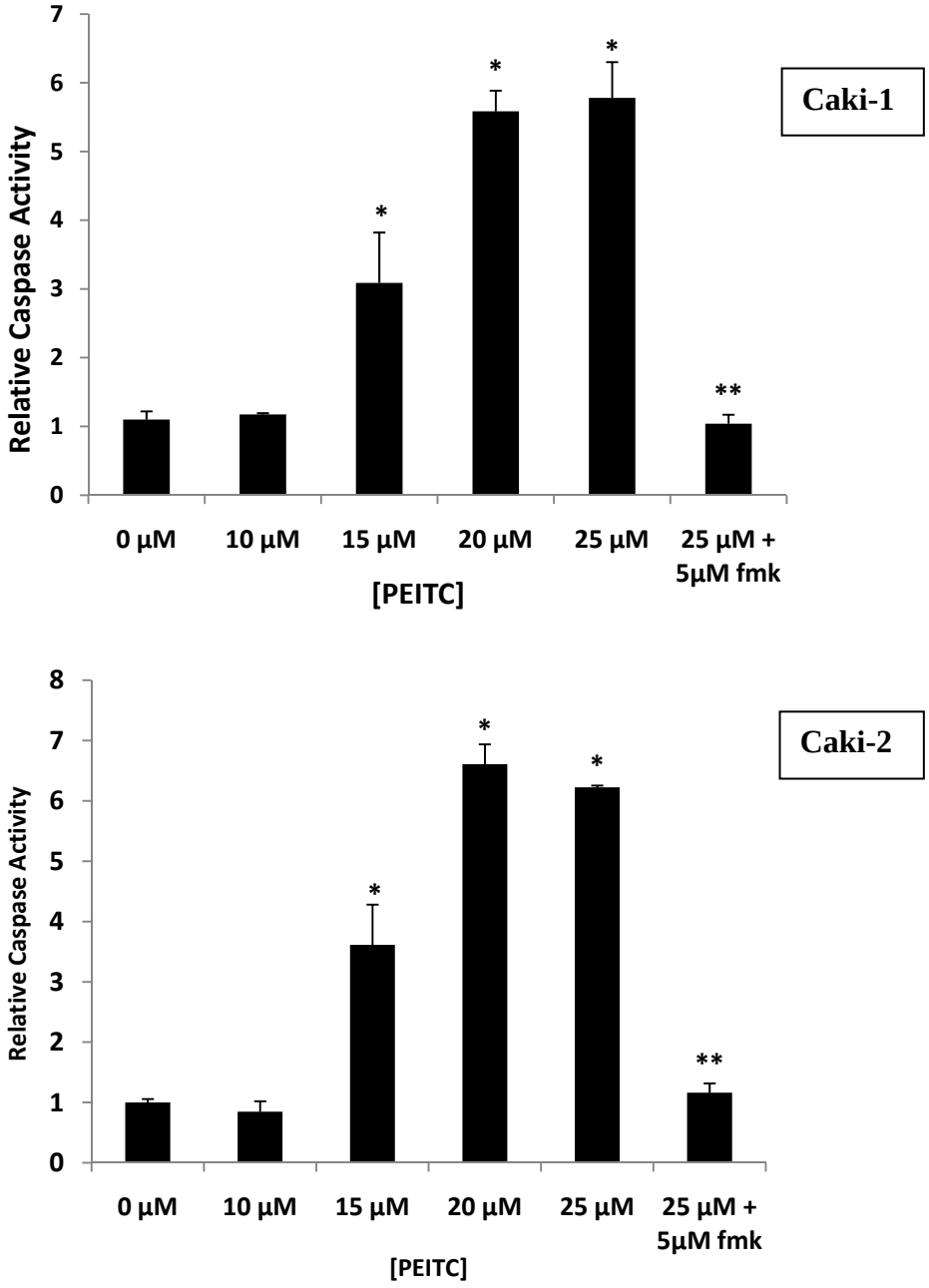


Figure 3.3: PEITC induces caspase-3 activity. Caki-1 and Caki-2 cells were treated with different concentrations of PEITC in the presence or absence of the general caspase inhibitor z-VAD-fmk as indicated for 18 hrs. Caspase-3 activity of cell lysate was measured using fluorescent substrate assay. 15 µM and higher concentrations of PEITC induced increased caspase-3 activity in both cells. The caspasae inhibitor z-VAD-fmk inhibited the induced activity, showing that the measured fluorescence is due to the caspase activity. (n = 3 + S.D.)

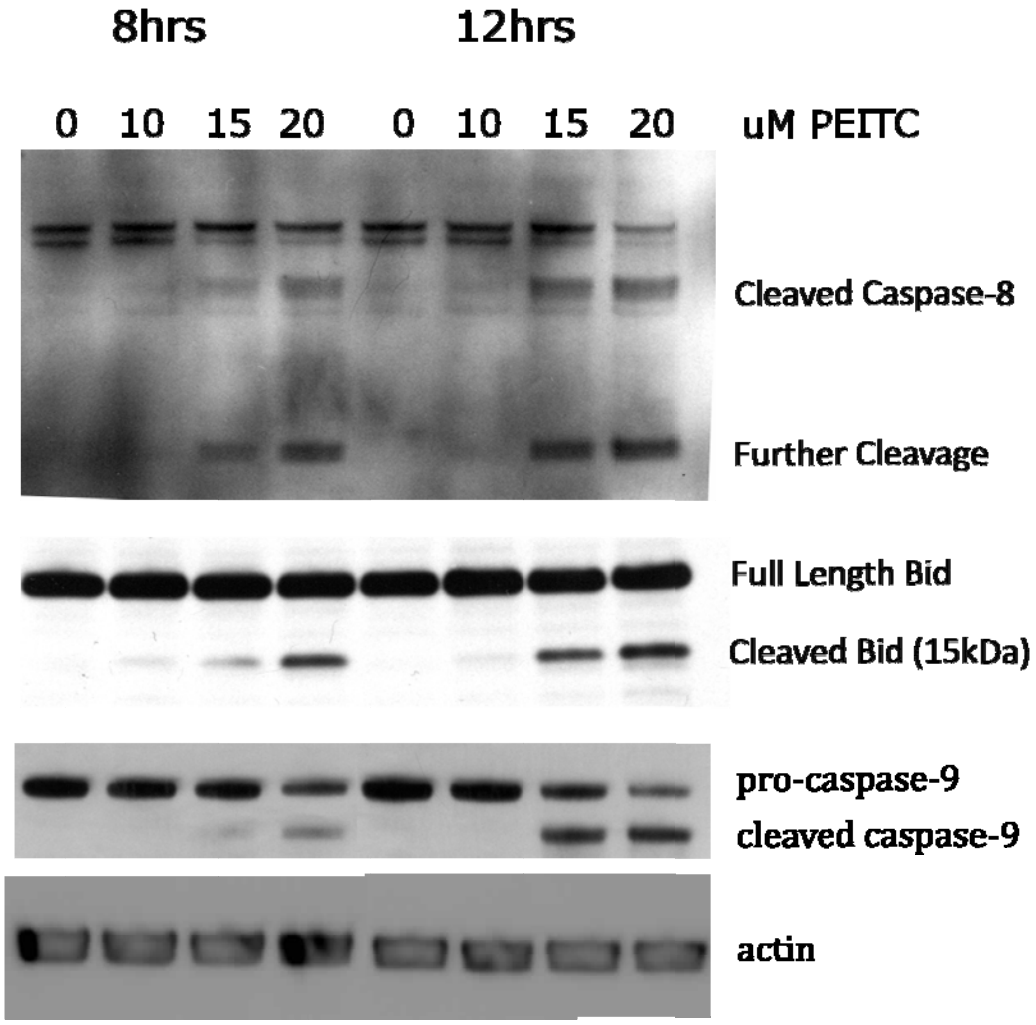


Figure 3.4: Effect of PEITC on proteins involved in the apoptosis cascade. Caki-1 cells were treated with increasing concentrations of PEITC for 8 and 12 hrs and the expression and cleavage of caspase-8, Bid and caspase-9 analyzed by western blot. PEITC 15 and 20 μM PEITC induced cleavage of caspase-8, Bid and caspase-9. Actin was used as loading control. Data is representative of three independent experiments.

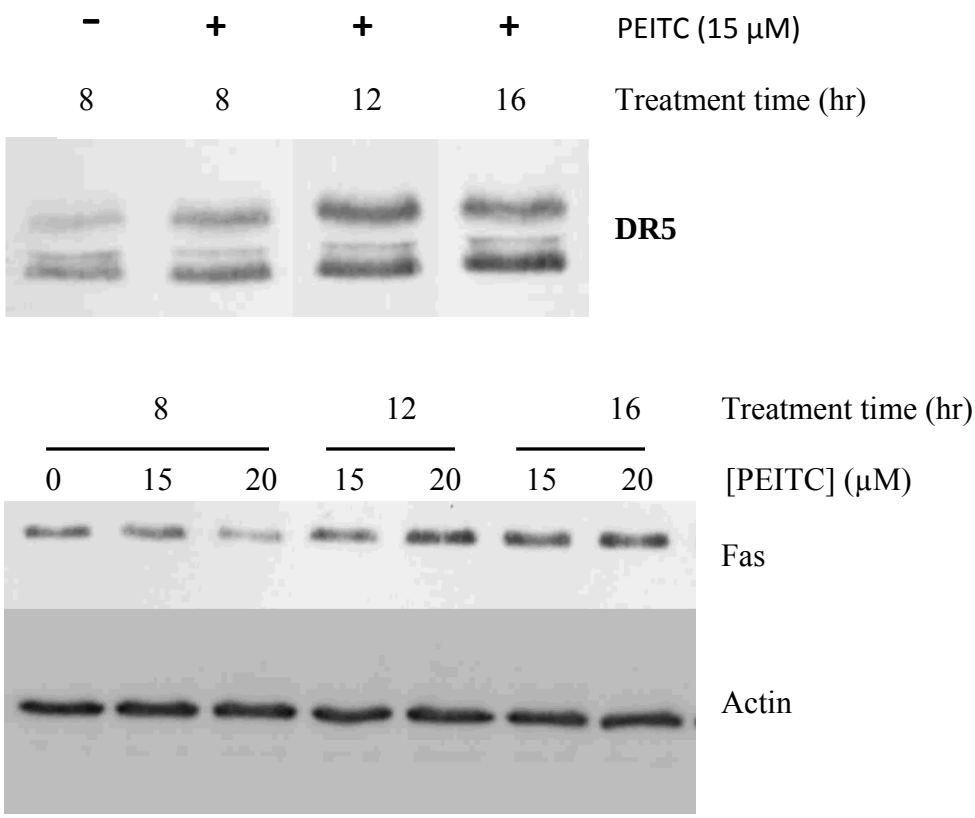


Figure 3.5: Effect of PEITC on DR5 and Fas protein levels. Caki-1 cells were treated with different concentrations of PEITC for 8, 12 and 16 hrs and the expression of DR5 and Fas analyzed by western blot.

Chapter 4: Summary and Future Direction

4.1 Summary

In this dissertation we explore the anti-cancer properties of PEITC, a naturally occurring phytochemical, on renal cell carcinoma (RCC). We determine the mechanism behind two of PEITC's anti-cancer properties against RCC cells: induction of cell cycle arrest and induction of apoptosis. The unique findings in this study are as follows: 1) PEITC induces G_2/M arrest through p38 and MK2 activation. PEITC has been shown to induce p38 MAPK activity in various cancer cell lines before, but this is the first time we show that the p38 MAPK activation is required for the G_2/M arrest induced by PEITC. 2) PEITC does not cause direct DNA damage; the observed DNA damage is a result of the later apoptosis inducing effect of PEITC. A few studies have suggested that PEITC causes DNA double-strand breaks (DSB) which is responsible for the cell cycle arrest and apoptosis induced by PEITC [86, 148, 149]. We show that this observed DSB is not due to direct DNA damage by PEITC; PEITC induced apoptosis results in DNA fragmentation that can manifest as false positive in the different assays used for measuring DSB such as γ-H2A.X phosphorylation and Comet assay. Use of an apoptosis inhibitor reverses this observed DSB. 3) PEITC induces apoptosis by upregulation of the two death receptors DR5 and Fas. PEITC has been shown to induce apoptosis in various cancer cell lines, but this is the first time we show that the apoptosis induction is associated with upregulation of DR5 and Fas.

The number of cases of kidney cancer has risen steadily over the last several decades. RCC which makes up 85% of kidney cancer cases [1] is a highly lethal cancer

mainly because of two reasons. 1) Due to lack of early symptoms, it is detected late and many patients have progressed into metastatic disease by the time of diagnosis. 2) RCC is highly resistant to conventional radiation and chemotherapy. As such it is important to find dietary components or supplements that may have chemopreventive role in the population. It is also important to find new drugs that can have chemotherapeutic role in the clinic. PEITC, a naturally occurring dietary component has been shown to have potent anti-cancer properties in various *in vitro* and *in vitro* cancer models, but its effect on RCC has not been studied before. We show that PEITC inhibits RCC cell proliferation by inducing a G_2/M arrest. At higher concentrations PEITC is a potent inducer of apoptosis in the RCC cells. Understanding the mechanism by which PEITC has these effects is important for designing prevention or therapeutic strategies. Our study shows that PEITC induces the cell cycle arrest by activating p38 MAPK, an important tumor suppressor gene. p38 MAPK controls a variety of cellular functions that are dysregulated in cancer, including cell cycle regulation, cellular differentiation and apoptosis [119]. It plays an important role at the G_2/M checkpoint. In response to various stress stimuli, p38 gets phosphorylated and it activates its downstream target MK2 which goes on to phosphorylate cdc25B [143, 144]. Once phosphorylated, cdc25B is sequestered in the cytoplasm, thus preventing its activation of cdc2/cyclinB1, the important checkpoint regulator that allows the cell to progress from G_2 to M (mitosis). Our study shows that PEITC induces a G_2/M arrest in Caki-1 cells, and this arrest is associated with increased p38 phosphorylation and phosphorylation of MK2 (Thr334). Inhibition of p38 by the specific inhibitor SB203580 attenuates the cell cycle arrest, thus demonstrating that p38 is required for the induction of G_2/M arrest by PEITC. At higher doses, PEITC induces apoptosis in Caki-1 and Caki-2 human RCC cell lines. The apoptosis induction is associated with increased caspase-3 activity, cleavage and activation of caspase-9, Bid

and caspase-8 as well as upregulation of DR5 and Fas. Treatment with the general caspase inhibitor z-VAD-fmk inhibited the apoptosis induction by PEITC showing that the caspase cascade is required for the apoptosis effect. Caspase-8 is an initiator caspase that is upstream of Bid, caspase-9 and caspase-3. It can be activated either by the various death receptors including DR5 and Fas [97], or by downregulation of FLIP [98], a regulator that inhibits activation of caspase-8. Our study shows that PEITC has no effect on FLIP levels, but it upregulates DR5 and Fas. This upregulation of DR5 and Fas may be the mechanism by which PEITC initiates caspase-8 activation which leads to the apoptotic cascade. Both DR5 and Fas are important death receptors that are relevant in cancer biology. DR5 is activated by binding of its ligand, TRAIL which initiates a potent apoptotic response. TRAIL has important therapeutic potential in cancer because of its selectivity in inducing apoptosis in cancer cells while leaving normal cells unharmed. However many cancer cells are resistant to TRAIL mediated apoptosis, and downregulation or low DR5 expression may be responsible [115, 116]. PEITC by upregulating DR5 may make RCC cells more susceptible to TRAIL induced apoptosis. Fas, the other death receptor modulated by PEITC binds to its ligand FasL which leads to a strong apoptotic response through the caspase-8 initiated cascade. Unlike TRAIL however, FasL cannot be administered to patients due to its non-selectivity: it induces apoptosis in normal cells as well, and has shown severe liver toxicity in animals [111]. However, there is emerging evidence that suggests that Fas can be activated to initiate the apoptotic response independent of FasL. Overexpression of Fas has been shown to be enough to induce apoptosis *in vitro* independent of FasL [112]. Thus upregulation of Fas by PEITC may be an important mechanism for the apoptosis inducing effect of PEITC.

4.2 Future Directions

Our study shows that the G_2/M cell cycle arrest induced by PEITC in Caki-1 cells is mediated by p38. However, how PEITC leads to p38 activation is not known. One of the most common cellular stresses that can lead to p38 mediated G_2/M arrest is DNA damage. However our study shows that PEITC does not induce direct DNA damage. Another possible effect of PEITC that can lead to p38 activation is oxidative stress. It will be interesting to see if PEITC generates reactive oxygen species (ROS) in Caki-1 cells, and if this ROS generation is responsible for the p38 mediated cell cycle arrest. PEITC may also be creating cellular stress by affecting microtubule dynamics. One study suggests that PEITC causes proteasome mediated selective degradation of α- and β-tubulin, while another study shows that PEITC disrupts microtubule polymerization. For our future studies we would like to know what role, if any, PEITC's effect on microtubule dynamics plays in the p38 mediated G_2/M arrest induced by PEITC.

We show that PEITC mediated apoptosis in Caki-1 and Caki-2 cells is associated with upregulation of the death receptors DR5 and Fas. How PEITC may upregulate these death receptors still remains to be explored. One possibility is through endoplasmic reticulum (ER) stress. ER stress is caused by an accumulation of unfolded or misfolded proteins in the ER. This triggers a cellular stress response called unfolded protein response (UPR) the function of which is to temporarily halt protein translation and increase synthesis of molecular chaperones involved in protein folding, in an effort to restore normality. If the protein misfolding is excessive or prolonged and the cell is unable to restore normal function, UPR leads to apoptosis of the cell. One of the mechanisms by which ER stress promotes apoptosis is by upregulation of death

receptors. We have preliminary data that shows that PEITC treatment leads to upregulation of C/EBP homologous protein (CHOP). In fact CHOP is believed to be the component of UPR that mediates the upregulation of DR5 in response to ER stress. Future studies can look at what role ER stress plays in the apoptosis induction by PEITC. By using siRNA to knock down CHOP, we can determine whether activation of CHOP is required for the upregulation of DR5 by PEITC. To date, there has been no study looking at ER stress induction as a possible mechanism of action of PEITC, or for that matter, any other related isothiocyanates. ER stress mediated apoptosis can play an important role in killing cancer cells. PEITC may be inducing ER stress through generation of ROS, or by directly binding to proteins. Understanding the mechanism of apoptosis induction by PEITC will help us advance the potential of PEITC as a chemotherapeutic drug against RCC.

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