AN ABSTRACT OF THE DISSERTATION OF

Christine A. Larsen for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on January 4, 2010.

Title: Suppression of Met Signaling by the Green Tea Polyphenol (-)-Epigallocatechin-3-Gallate (EGCG)

Abstract approved:

_____

Roderick H. Dashwood

Met is a prognostic indicator of colorectal cancer patient survival. Therapies that target Met may therefore have beneficial outcomes in the clinic. Recently, EGCG was reported to suppress Met activation, although the mechanisms were not elucidated. HCT116 and HT29 human colon cancer cells were used to examine the relationships between Met activation, EGCG treatment, and H_2O_2 generation. At concentrations of 0.5, 1 and 5 μM, EGCG suppressed the activation of Met induced by its ligand, hepatocyte growth factor (HGF). Concentrations of 10 μM EGCG and below generated low amounts of H_2O_2 (<1.5 μM), whereas higher H_2O_2 concentrations (>5 μM) were required to directly increase the phosphorylation of Met. Moreover, suppression of Met activation by EGCG occurred in the presence or absence of catalase, suggesting that such

effects were not an ‘artifact’ of H_2O_2 generated from EGCG in cell culture media. Molecular docking and enzyme kinetic analyses suggested that EGCG is a competitive inhibitor, binding to the kinase domain of Met with a K_i of 3.3 μM EGCG. The downstream effect of EGCG mediated suppression of the Met receptor included decreased signaling to members of the MAPK and PI3KK signaling pathways. Cell proliferation and migration was also significantly inhibited by EGCG. Overall, the data presented in this dissertation support that EGCG is able to suppress HGF-induced Met signaling. These findings demonstrate that EGCG might be a beneficial therapeutic agent in the colon, inhibiting Met signaling and helping to attenuate tumor metastasis.

Suppression of Met Signaling by the Green Tea Polyphenol (-)Epigallocatechin-3-Gallate (EGCG)

by

Christine A. Larsen

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented January 4, 2010

Commencement June 2010

Doctor of Philosophy dissertation of Christine A. Larsen presented on January 4, 2010.

APPROVED:

_____

Major Professor, representing Molecular and Cellular Biology

_____

Director of the Molecular and Cellular Biology

_____

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

_____

Christine A. Larsen, Author

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and appreciation to Dr. Roderick Dashwood for the mentorship and support throughout my graduate study. I would also like to show my appreciation for my committee members, Dr. Emily Ho, Dr. Robert Tanguay, Dr. David Williams and Dr. Claudia Maier. Thank you for all of your advice and suggestions. You played an instrumental role in my studies and in my development as a scientist.

I would like to thank the members of the Dashwood Lab for their assistance, advice and companionship. Special recognition belongs to Mohaiza Dashwood for maintaining a smooth running lab, ordering supplies and training me on many of the techniques used in these studies.

I would like to acknowledge the Molecular and Cellular Biology Program and the Linus Pauling Institute for providing an excellent learning environment with many opportunities to present my research. I would also like to thank the people who make up these institutions for all their help and encouragement.

Finally, I would like to thank my family and friends for their love and support. Without them, I do not know where I would be. Words cannot express the gratitude that I feel for their presence in my life. Thank you for your patience and belief in me. You gave me strength when I wanted to give up.

CONTRIBUTION OF AUTHORS

CHAPTER 2: Dr. William H. Bisson performed molecular docking and constructed the figures of ATP, triazolopyridazine inhibitor and EGCG docked with Met.

CHAPTER 3: Dr. Roderick Dashwood provided critical review and assisted with the writing.

CHAPTER 4: Dr. William H. Bisson performed molecular docking and constructed the figures of tea catechins docked with Met. Dr. Roderick Dashwood provided critical review and assisted with the writing.

CHAPTER 5: Hui Nian performed statistical analysis of the cell proliferation experiment and Dr. Praveen Rajendran assisted with the calculations of IC_{50} values for EGCG and SU11274 with the Met receptor. Dr. Roderick Dashwood provided critical review and assisted with the writing

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

Chapter 4

LIST OF FIGURES (Continued)

Figure Page

Chapter 5

LIST OF TABLES

Table Page

Chapter 2

Chapter 1: General Introduction

Suppression of Met signaling by the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG)

Introduction

Cancer development can be divided into four steps: initiation, tumor promotion, malignant conversion and tumor progression. In the initiation stage, mutations accumulate in cells. These mutations inactivate tumor suppressor genes (such as p53 or adenomatosis polyposis coli (APC)) or activate oncogenes (such as Ras or PI3K) [1, 2]. Tumor initiation is also associated with epigenetic change in the pre-cancerous cell. Tumor suppressor genes have been found to be transcriptionally silenced by DNA methylation of promoter regions in preneoplastic human lung and colon tissues [3]. These genetic and epigenetic changes allow the initiated cells to be free from normal proliferative control; thus allowing clonal expansion, which is a hallmark of the tumor promotion stage. Further genetic and epigenetic changes in the promotion change allow cells to express the malignant phenotype in the process of malignant conversion. The propensity for genomic instability and uncontrolled growth is a prominent characteristic of the malignant phenotype [4].

Tumor progression is the stage at which malignant cells acquire more aggressive characteristics. Further functional loss of tumor suppressor genes and activation of oncogenes is observed. These changes allow cells to have the capacity for regional invasion and ultimately distant metastatic spread. The major cause of death from cancer is metastasis that is resistant to conventional therapies [5]. The ability of neoplastic cells to invade adjacent

tissues, survive in foreign compartments and proliferate at distant sites is defined as “invasive growth” [5].

Invasive growth occurs under normal conditions during embryonic development and in adult life during wound healing. This is a dysregulated process that contributes to tumorigenesis and metastasis in cancer cells. Early invasive growth requires a process known as epithelial-mesenchymal transition (EMT). In this process cells release cell-cell contacts, undergo cytoskeletal rearrangement to change polarity, and attain the ability to move through the extracellular environment. In EMT, cells lose their epithelial phenotype and acquire a mesenchymal one.

Expression of the Met receptor tyrosine kinase has been shown to lead to invasive growth in animal models [6-8]. In humans, aberrant Met expression has been found in many solid tumors, in particular it is found in liver metastasis of colon cancers [9]. The expression of Met and its ligand, hepatocyte growth factor (HGF), is associated with poor prognosis in colon cancer patients [10].

HGF is a large protein that consists of six domains (Fig. 1), including an amino terminal domain (N), four kringle domains (K1-K4) and a serine proteinase homology (SPH) domain. The kringle domain consists of ~80 amino acids and is characterized by having limited secondary structure and defined by having three conserved disulfide bonds. As a result of mutations in essential residues, the SPH domain lacks enzymatic activity [11]. HGF is

synthesized as an inactive precursor (pro-HGF) that must be proteolytically converted into its active, disulfide linked α- and β-chain heterodimer. Many serine proteinases are responsible for conversion of pro-HGF to the active form, including urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA).

Met is also a disulfide-linked heterodimer that must be cleaved to produce the mature protein. It consists of an extracellular α chain and a longer β chain that encompasses the extracellular, transmembrane and cytoplasmic portions of the cell (Fig 2). The Sema domain of Met is homologous to semaphorin axon-guidance proteins and is responsible for ligand binding [12]. The remainder of the extracellular portion of Met consists of a small cysteine-rich sequence, followed by four repeating immunoglobulin domains that hold the protein in correct orientation for proper ligand binding [13]. The juxtamembrane domain has the ability of regulating catalytic function. Serine 985 is located in this domain; when this serine is phosphorylated Met activity is suppressed [14]. The tyrosine kinase domain has the ability to catalyze the transfer of the γ-phosphate of ATP to tyrosine residues on protein substrates. The combination of the juxtamembrane domain, kinase domain and carboxy-terminal tail is essential for downstream signaling [13].

Met has been proposed to be an optimal pharmacological target for cancer therapy because it plays a central role in cancer development and metastatic spread [5]. Since invasion and metastasis are the main cause of

death for cancer patients, drugs that target these pathways are attractive options. Many strategies have been employed to reduce Met signaling in cancer cells. These include ligand antagonists, kinase inhibitors and expression inhibitors.

NK4 is a ligand antagonist. It is a molecule that contains the N-terminal region and four kringle domains of HGF. It binds the Met receptor but fails to activate it. In a mouse model, it effectively impaired tumor invasion, angiogenesis and metastasis [15].

Neutralizing anti-HGF antibodies are also ligand antagonists. These antibodies bind HGF at subnanomolar concentrations and inhibit the ability of HGF to bind Met. In a tumor xenograft study, these antibodies impaired HGF-dependant tumor growth and caused significant regression of the xenografts [16].

Another approach to block Met activation is with the use of receptor competitors. These are molecules that can bind to the receptor's extracellular domain to reduce receptor activation by inhibiting receptor dimerization. Examples of these are monoclonal antibodies to Met, decoy Met, and anti-Sema antibodies that target the Sema domain on Met.

Alternatively, peptides that compete with the receptor's docking sites to reduce cellular signaling could be employed. Also, down-regulation of receptor expression can be achieved by using shRNA vector construct carried by an adenovirus.

Finally, small molecule kinase inhibitors can be used to block the Met receptor. These inhibitors are usually low molecular weight molecules that compete for the ATP binding site of the receptor. Examples of these are K252a, PHA-665752, PF-2341066 and SU11274. K252a is a broad spectrum inhibitor of many receptor tyrosine kinases [17]. SU11274 is an inhibitor that has been shown to be more selective for the Met receptor [18]. In xenograft studies, both PHA-665752 [19] and PF-2341066 [20] decreased angiogenesis and tumor size.

Most recently, EGCG has been identified as a Met inhibitor [21-25]. In this dissertation, the ability of EGCG to act as a Met inhibitor was examined and potential mechanisms of action were investigated in human colon cancer cells. The cellular effects of Met inhibition were studied, alongside the selective Met kinase inhibitor, SU11274.

Figure Legends

Figure 1: The domain structure of HGF. Abbreviations are as follows: amino terminal domain (N), kringle domains (K1-K4), serine proteinase homology domain (SPH).

Figure 2: The domain structure of Met. Abbreviations are as follows: sema domain (S), cysteine-rich domain (C), immunoglobulin domains (Ig1-4), transmembrane domain (TM), juxtamembrane domain (JM) and tyrosine kinase domain (TK).

Figure 1.

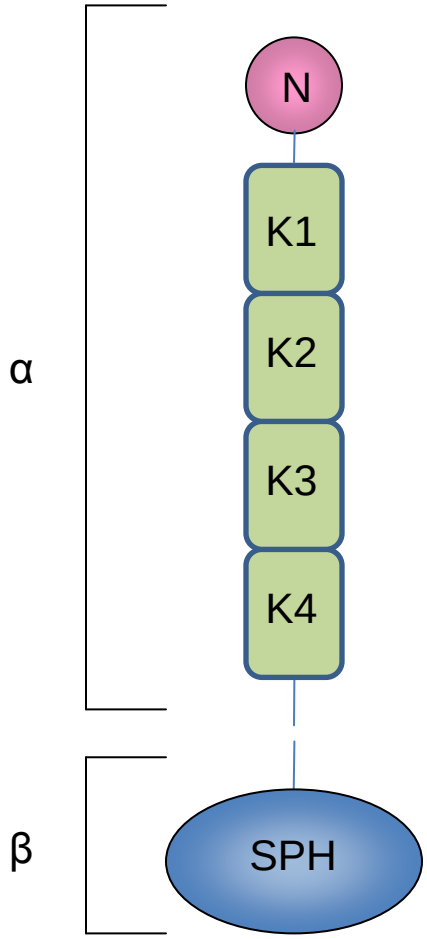
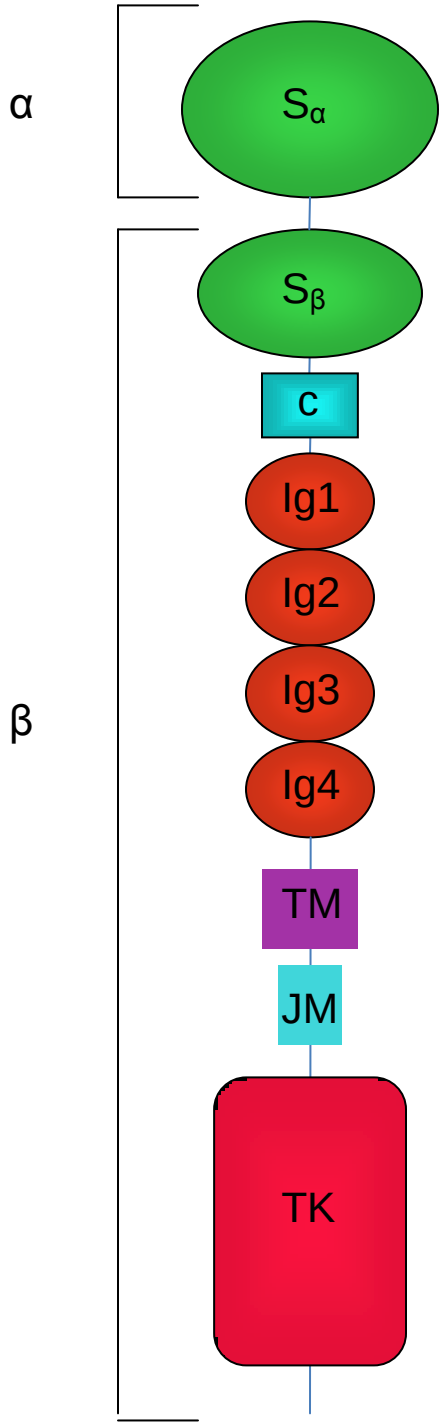


Figure 2.



Chapter 2

Tea Catechins as Inhibitors of Receptor Tyrosine Kinases

Christine A. Larsen[1,2], William H. Bisson[2] and

Roderick H. Dashwood[2,3]

[1] Molecular and Cellular Biology Program, [2] Linus Pauling Institute, [3] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR

Formatted for submission

Abstract

Receptor tyrosine kinases (RTKs) play important roles in the control of fundamental cellular processes such as maintenance of the balance between cell proliferation and death. RTKs have emerged as molecular targets for the treatment of various cancer types. Green tea and its polyphenolic compounds, the catechins, have been shown to have chemopreventive and chemotherapeutic properties in many cancer types in animal models. Epidemiological studies are somewhat less convincing, but some positive correlations have been observed. The tea catechins, including (-)-epigallocatechin-3-gallate (EGCG), have pleiotropic effects on cellular proteins and signaling pathways. This review focuses on the ability of the tea catechins to suppress RTK signaling and summarizes the possible mechanisms by which the catechins exert their protective effects.

Introduction

Tea is made from the leaves of the *Camellia sinensis* plant. The cultivation of the tea plant dates back to more than 5000 years, and originally its leaves were used medicinally. Today it is a popular beverage that is consumed by two-thirds of the world's population. In recent years, green tea has received attention for its beneficial health effects, in particular the prevention of cancer. In 2009, Yang et al. reviewed the possible targets that could account for the chemopreventive effects of EGCG [26]. The diverse mechanisms included inhibition of matrix metalloproteinases (MMPs), cyclin dependant kinases (CDKs), proteosomes, DNA methyltransferase (DMNT), vitmentin, BCL-2, MAPKs and RTKs, increased p53 expression, and induction of apoptosis.

This review focuses specifically on the effects of tea and its constituents on RTKs. First, the biochemical properties and bioavailability of the tea catechins will be discussed. Then, the ability of tea to inhibit tumorigenesis in animal models and human epidemiological data will be presented. RTKs and their downstream signaling pathways will be described. Finally, the interaction of tea catechins on these pathways and potential mechanisms of action will be discussed.

Green Tea and Cancer Chemoprevention

Aside from water, tea is the most widely consumed beverage worldwide [27]. This popular beverage has gained much attention for its purported health benefits, in particular for its possible role in preventing and treating cancer [28]. The chemistry of green tea, compared to other teas, is quite well characterized [29]. Green tea is produced by steaming or pan-frying the leaves of the *Camellia sinensis* plant. This process prevents the oxidation of the tea constituents. Among these constituents is a class of polyphenolic compounds known as the catechins (Fig. 1). Green tea catechins (GTCs) include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)epigallocatechin-3-gallate (EGCG).

A typical cup of brewed green tea has been defined as 2 g of tea leaves in 200 mL of hot water. The catechins make up 30-40% (by dry weight) of the water extractable material [30]. EGCG is the most well studied and most abundant of the catechins, accounting for 50-80% of the total catechin content. This represents 200-300 mg per cup of brewed green tea [31].

Tea catechins undergo methylation, glucuronidation, sulfation and ring fission metabolism [32-34]. The biotransformation of tea catechins was extensively reviewed by Lambert et al. [35]. Catechin pharmacokinetics has been studied by several groups. In one study, rats and mice were given 0.6% GTCs as drinking fluid [36]. EGCG accounted for 78% of the catechins

present, but plasma concentrations were much lower for EGCG than EGC and EC. High levels of EGCG were found in the feces whereas high levels of EGC and EC were found in urine. In another study, examination of tissues showed that EGCG was distributed widely in the colon, small intestine, liver, lung, and other organs [33]. After intravenous administration of green tea to rats, EC was found mainly in the intestine, bladder and kidney, EGC was found in the intestine, bladder, kidney and lung, and EGCG was found mostly in the colon and liver [36, 37].

In humans, high concentrations of individual catechins were administered orally. The plasma concentration for each catechin was observed to be as high as 1.53 μM for a dose of 1050 mg EC [38], 3.1 μM for a dose of 644 mg ECG [39], 5 μM for a dose of 459 mg EGC [39], and 6.35 μM for a dose of 1600 mg EGCG [40].

Tea extracts and tea constituents have gained much attention for their abilities to inhibit tumor formation in different animal models (reviewed in [31, 41-44]). The inhibition of small intestine, colon, prostate, bladder, breast, stomach, liver, pancreas, esophagus, oral cavity, lung and skin cancers have been reported in animal models (Table 1). Although mostly positive (i.e. chemopreventive) results were reported, some equivocal results in which tea had no chemopreventive effect were observed. Many variables could explain inconsistencies in these studies. Differences in diets used, protocol for tumor initiation, the type and dose of tea polyphenols or extracts used may explain

the variability in some of the animal study results. Another key issue may be the stability of tea polyphenols in solution and in the diet.

A positive association between human cancer risk and tea consumption may be more difficult to establish from epidemiological studies. There are many reviews on this topic [45-50]. Some studies show a negative association, others report no association, and a positive association has been observed in other investigations. Most recent reviews conclude that the protective effects of tea depend on the various etiological factors involved in different cancer types and even for the same cancer types in different geographical areas. Furthermore, the consumption of green and black tea differs significantly among studies. One might expect that due to these large differences between epidemiological studies, it is perhaps naïve to expect a simple conclusion concerning tea and cancer prevention in human populations.

Case control studies and cohort studies have observed an association between green tea consumption and lowered risk for stomach cancer in Japan (reviewed in [50, 51]). One of the most promising intervention studies was performed by Bettuzzi et al. [52]. Two hundred individuals with high-grade prostate intraepithelial neoplasia (PIN) received either 600 mg GTC or placebo for 12 months. In the GTC group only 3% developed prostate cancer whereas 30% developed prostate cancer in the placebo group. The latter findings strongly demonstrate the importance of carefully controlled

intervention trials with tea and tea compounds, and similarly conducted studies are warranted.

Membrane Associated RTKs and Their Downstream Signaling Pathways

Receptor tyrosine kinases (RTKs) are key regulators of cell signaling pathways involved in cell proliferation, differentiation, migration and death. The activation of RTKs by specific ligands (growth factors and cytokines) and downstream effects is summarized in Fig. 2, which provides a simplified overview, but is nonetheless useful in describing general principles.

RTKs function in cell signaling by catalyzing the transfer of the γ-phosphate of ATP to tyrosine residues on protein substrates. This phosphorylation modulates enzymatic activity and creates binding sites for the recruitment of downstream signaling proteins. It is in this way that a signal cascade is established.

RTKs are activated by binding of a ligand to the extracellular domain. This induces dimerization of the RTK and cross-phosphorylation of tyrosine residues. This phosphorylation results in the stabilization of the dimer conformation, allows opening of the kinase domain for ATP binding, and access to docking sites for adapter proteins.

The MAPK pathway is activated by recruitment and activation of the adapter proteins (Grb2, Shc, and Sos) that in turn activate Ras. The serine/threonine kinase Raf-1 is activated by Ras which then phosphorylates and activates MEK 1/2 on two serine residues. MEK1/2 then activates ERK1 ($p44^{MAPK}$) and ERK2 ($p42^{MAPK}$). The result of MAPK activation results in the

activation of transcription factors, such as AP-1, which lead to changes in expression of genes that are important for cell proliferation and migration [53, 54].

The PI3K pathway is another important signaling pathway that is activated by RTKs. PI3Ks are heterodimeric lipid kinases that are composed of a regulatory (p85) and catalytic (p110) subunit. When PI3K is activated by RTKs it induces the synthesis of a lipid second messenger, PIP_3. PIP_3 is necessary for the phosphorylation of Akt. Akt is involved in cell survival. It phosphorylates and deactivates Bad, a proapoptotic protein [55]. Caspase-9 is also phosphorylated by Akt. This phosphorylation inhibits the catalytic activity of Caspase-9 thus enhancing cell survival [56]. The transcription factor NFκB is also activated by Akt. NFκB regulates genes that promote survival in response to apoptotic stimuli [57].

In the cytoplasm, NFκB is kept in its inactive form through interaction with IκB. Ubiquitination and degradation of IκB is mediated by phosphorylation of IκB kinase (IKK). This releases NFκB such that it is able to translocate to the nucleus. Akt has an effect on NFκB by phosphorylating and activating IKK [57]. NFκB-inducing kinase (NIK) also controls the phosphorylation and activation of IKK [58, 59]. The MAPK pathway has been shown to activate the NIK/IKK/NFκB pathway [60].

The activation of AP-1 and NFκB by ERK and Akt pathways can act independently or coordinately to regulate specific target genes such as cyclin

D1 and Cox-2. The promoters of these two genes contain both AP-1 and NFκB binding sites [61-63]. The dysregulation of AP-1 and NFκB play important roles in the development of many types of cancer [58, 64].

Tea Catechins and RTKs

Insulin Like Growth Factor-1 Receptor (IGF-1R)

IGF-1R is activated by its ligands IGF-1 and IGF-2. It has an essential role in cell growth and development [65, 66]. It is typically expressed at low levels in normal hepatocytes, but is overexpressed in hepatocellular carcinomas (HCC) [67]. It has also been found to be overexpressed in other types of cancer, including prostate [68] and colorectal cancer [69]. Activation of IGF-1R leads to the activation of PI3K and MAPK pathways [70]. The activity and function of the IGF ligands are controlled by IGFBP-3 [67]. IGFBP-3 is found to be decreased in HCC samples compared to non-neoplastic liver tissues [71].

In one study, Shimizu et al. [72] treated HepG2 cells (human HCC cells) and Hc cells (normal hepatocytes) with 20 µg/mL EGCG. A preferential growth inhibition by EGCG in the HepG2 cells was observed. They found decreased phosphorylation of IGF-1R, ERK, Akt, Stat-2 and GSK-3β with EGCG treatment. Decreases in protein and mRNA of IGF-1 and IGF-2 and an increase in IGFBP-3 protein were also observed.

Another study by Li et al. [73] demonstrated that EGCG competitively binds to the ATP binding site of IGF-1R, blocking downstream signaling of this receptor. They calculated the IC_{50} value to be 14 µM EGCG.

Epidermal Growth Factor Receptor (EGFR) Family

The EGFR signaling pathway is important for the regulation of cell growth, proliferation, differentiation and survival. The EGFR family of receptors includes EGFR (ErbB1), HER-2 (HER-2/neu, ErbB2), HER-3 (ErbB3) and HER-4 (ErbB4). Many different ligands have been identified for this family of receptors including epidermal growth factor (EGF), transforming growth factor (TGF)-α, β-cellulin and neoregulins.

Liang et al. [74] reported the inhibition of EGFR phosphorylation in A431, human epithelial carcinoma cells, by EGCG. In ligand binding assays, it was suggested that EGCG blocks the binding of EGF to its receptor. Others reported that not only does EGCG decrease receptor phosphorylation, the tea catechin also inhibits receptor expression by inhibiting the activity of ERK, which regulates the transcription factor Egr-1. Egr-1 controls the expression of EGFR [75]

Another study proposed that the oxidation of EGCG is necessary to reduce EGFR activation [76]. This was demonstrated by adding superoxide dismutase (SOD) to cell culture media to stabilize EGCG. This stabilized EGCG lost its ability to suppress receptor activation. In conflicting studies, Adachi et al. [77, 78] demonstrated that EGCG and polyphenon E (a mixture of GTCs), but not EC, disrupted lipid order and membrane organization. This caused the internalization of EGFR such that EGF could no longer bind. The addition of SOD had no effect on this process.

Vascular Endothelial Growth Factor Receptor (VEGF-R)

The VEGF-R family is involved in proliferation, migration and tube formation in endothelial cells. VEGF-R family members include VEGF-R1 (Flt-1), VEGF-R2b (KDR, Flk-1), and VEGF-R3 (Flt-4). Their ligands are vascular endothelial growth factor (VEGF), placental growth factor (PIGF) and VEGF-B, -C, -D. VEGF-R expression can be induced by many cytokines and growth factors including IL-1, IL-6, PDGF-BB, TGF and HGF [79, 80].

Lee et al. [81] showed that doses of EGCG as low as 7 μM in chronic lymphoid leukemia cells (CLL) could inhibit the phosphorylation of both VEGF-R1 and VEGF-R2 and induce apoptosis by down-regulating anti-apoptotic Bcl-1, Mcl-1 and XIAP proteins. The activation of Caspase-3 and cleavage of PARP was also observed. When Kondo et al. [82] investigated EC, ECG, EGC and EGCG, all four tea catechins were found to inhibit angiogenesis in an *in vitro* model using human umbilical vein endothelial cells (HUVECs) in concentrations ranging from 1.56-100 μM. EGCG was the most effective of the catechins, and was the only catechin that was able to inhibit VEGF binding to its receptor. In the HuH7 hepatoma cell line, EGCG reduced the expression of VEGF mRNA by inhibiting ERK and Akt phosphorylation, which regulates VEGF expression. Growth of the HuH7 cells was also inhibited by EGCG [83].

Platelet Derived Growth Factor Receptor (PDGF-R)

The PDGF-R family is expressed in connective tissue and glial cells. They play an important role in wound healing. The PDGF ligand is a dimeric protein that is composed of A- and B-chains. These chains combine to form three isoforms, PDGF-AA, PDGF-BB and PDGF-AB. The PDGF-Rα receptor can bind all three isoforms whereas the PDGF-Rβ only binds PDGF-BB with high affinity.

EGCG and ECG have been shown to suppress PDGF-BB-induced activation of PDGF-Rβ in rat and human vascular smooth muscle cells and in A172 human glioblastoma cells [84-86]. In these studies, EC had no effect.

In a study by Suzuki et al. [87] PDGF-BB was found to be bound to EGCG that had been immobilized on an agarose gel. Binding studies with [^{125}I]-PDGF-BB was performed on EGCG-treated vascular smooth muscle cells (VSMC) [88]. This group found a 50% reduction of [^{125}I]-PDGF-BB binding, suggesting that ECGC disrupts the ability of PDGF-BB to bind to its receptor.

Met

Met is important during embryonic development and wound healing [89]. It is dysregulated in many cancer types and is associated with poor prognosis [89]. Activation of Met by its ligand, hepatocyte growth factor (HGF), results in increased proliferation, survival, angiogenesis, tissue regeneration, scattering, motility, and morphogenesis [90].

In the breast cell line MCF-10A, EGCG, ECG, EGC and EC were studied. The gallated catechins, EGCG and ECG, inhibited the activation of Met, Akt and ERK at 0.6 µM. Non-gallated, EGC did not inhibit Met activation but repressed Akt and ERK signaling at concentrations less than 2.5 µM. EC did not have an effect on Met, Akt or ERK phosphorylation [21]. This suggested that the presence of a trihydroxyphenyl on the B ring and a gallate group are important structural features for inhibition of Met and its downstream signaling pathways.

We reported similar findings with the Met receptor in HCT116 colon cancer cells. Using 5 µM of each catechin, we found that EC was unable to suppress Met activation. EGC marginally suppressed Met activity and ECG and EGCG had the greatest effect on suppressing receptor phosphorylation [22]. In an *in vitro* kinase assay, however, the inhibitory effect of EGC was not detected. Molecular docking and enzymatic kinetic analysis suggested a competitive mechanism of inhibition, involving GTCs containing a gallate group associating with the ATP binding domain of the Met receptor [22].

Mechanisms of RTK Inhibition by Tea Catechins

Most studies with respect to inhibition of RTK activity by tea constituents have been performed with EGCG. Among the various GTCs examined to date, it has been found that EC has little or no effect in the suppression of EGFR [77], VEGFR [82], PDGF-R [85, 86] or Met [21, 22]. All receptors were inhibited most effectively by EGCG, followed by ECG and EGC. This suggests that the presence of a gallate and the trihydroxyphenyl on the B ring are important structural features for RTK inhibition.

Many possible mechanisms have been proposed and tested to account for the effect of EGCG on RTKs. These mechanisms include effects on receptor transcription/translation, hydrogen peroxide generation, disruption of lipid rafts, receptor internalization, and competitive inhibition.

It has been demonstrated that EGCG can decrease the levels of IGF-1 and IGF-2 mRNA and protein. IGFBP-3 is also increased by EGCG treatment [72]. These changes in protein expression could account for the inhibitory effects of EGCG on IGF-R activity.

EGCG can interact with subcellular kinase activities of ERK and Akt directly [91]. Decreased activity of ERK leads to inhibition of Egr-1, a key transcription factor that regulates the production of EGF. Decreased ERK and Akt activities have been observed to attenuate VEGF expression [83].

Another factor contributing to EGCG-mediated inhibition of RTK activity is the stability of the catechin under various conditions. EGCG is known to be unstable in cell culture media [92]. When SOD is added to cell culture media it stabilizes EGCG. The co-treatment of SOD and EGCG decreased the effectiveness of EGCG to decrease EGFR activation [76]. This suggested that in some circumstances the effects on EGFR are due to the generation of reactive oxygen species, and not that of parent EGCG. However, Adachi et al. saw no difference on EGFR activity when EGCG was incubated in the presence or absence of SOD [78].

Hydrogen peroxide (H_2O_2) is one of the oxidation products of EGCG in cell culture media. High levels of H_2O_2 (≥ 500 μM) have the ability to suppress Met receptor activation by inducing phosphorylation of serine 985 (a negative regulatory site) of Met [14]. We reported that physiologically relevant concentrations of EGCG produce much less H_2O_2 in cell culture media, and that the suppressive effects on Met were maintained in the presence or absence of catalase [23].

Lipid rafts are areas of the plasma membrane that contain a high level of cholesterol and sphingolipids [93, 94]. EGFR [95] and IGF-R [96] are associated with lipid rafts. Ligand binding and tyrosine kinase activity of erbB family receptors is influenced by the lipid environment [97]. Adachi et al. demonstrated that EGCG disrupted the ordered lipid domains of the plasma membrane, and this was associated with inhibitory effects of EGCG on EGFR signaling [77].

Lipid organization is also thought to play a role in receptor internalization [98]. When cells are treated with EGF, its receptor is internalized into early endosomes [99]. Adachi et al. also showed that treatment of EGCG induced internalization of EGFR into endosomes [78]. The EGF-stimulated cells were still able to produce a cascade of phosphorylation. In contrast, EGCG-treated cells do not allow the signaling of EGFR by sequestering the receptor inside the cell, thereby making it inaccessible to EGF ligand.

EGCG has also been shown to bind directly to EGF [74], VEGF [82], and PDGF [87] ligands. This prevents the growth factors from interacting with their corresponding receptors and activating downstream signaling cascades.

There is evidence that EGCG can compete with the ATP binding site of some proteins and act as an ATP mimetic of the adenine moiety [100]. Recently, Hsp90 was shown to have a C-terminal nucleotide binding site that has the ability to bind both ATP and EGCG. We have shown by kinetic analysis and molecular docking that EGCG interacts with the ATP binding site of the Met receptor [22]. The γ-phosphate of ATP is transferred to tyrosine residues on protein substrates to initiate a cascade of signaling from the Met receptor (Fig. 3A). A triazolopyridazine inhibitor was previously found to compete with the ATP binding site [101]. Comparative molecular docking simulations of the triazolopyridazine inhibitor and EGCG (Fig. 3B and 3C) revealed that both molecules interacted favorably with the ATP binding site of Met [22].

Discussion

Tea and its catechin constituents have gained much attention for their anticancer properties. There are many animal models of carcinogenesis in which tea compounds were shown to have a chemoprotective effect. However, human epidemiological data are somewhat less convincing. Possible reasons for this discrepancy include human genetic variability, lifestyle, amount of tea and type consumed, and various cancer etiologies. It is therefore important to establish carefully conducted case control studies to verify the possible beneficial effects of tea in a human population, for cancer and other chronic diseases.

Bioavailability studies have shown that tea catechins can widely distribute to different tissues, but mostly accumulate in the oral cavity and digestive tract [102]. Thus, catechins may be more effective at targeting cancers of oral and gastrointestinal tissues.

Tea catechins have many different effects on proteins (reviewed in [26]). Here, we focused on the effects of tea catechins on RTKs. Many RTKs are found to be mutated, dysregulated or overexpressed in a variety of cancer types. Activation of RTKs results in the activation of downstream signaling pathways that control proliferation, differentiation, migration and survival. It is for these reasons that targeting RTKs is an attractive cancer therapeutic approach. Indeed, RTK inhibitors are used at present in the clinical setting.

One example is trastuzumad (Herceptin®), a humanized monoclonal antibody against HER-2, used in the treatment of metastatic breast cancer [103].

Many possible mechanisms of RTK inhibition by tea catechins have been proposed. It is possible to explain, at least in part, the multitude of different mechanisms by which tea compounds act because of the complex circuitry involved in RTK signaling [104]. One might anticipate that EGCG interacts with these pathways in many different ways. However, among RTKs the tyrosine kinase domain is highly conserved [105]. It is plausible, therefore, that EGCG acts as a competitive inhibitor for most of the RTKs, although further studies are warranted on this topic.

Finally, most of the studies focusing on tea catechins and RTK signaling have been performed in cell culture. Tea compounds and their metabolites need to be evaluated more extensively *in vivo*, and also in human clinical trials, to ascertain the true relevance of RTK inhibitory mechanisms in human cancer prevention and/or therapy. In general, EGCG and other tea catechins modulate multiple pathways that are defective or dysregulated in cancer cells, and continue to hold promise as anticancer agents. More studies are needed to support the application of ECGC in the management of cancer.

Figure and Table Legends

Table 1: Summary of animal data in which tea or tea compounds were used as chemopreventive agents.

Table 2: Summary of the suppressive effects of EGCG on different RTKs.

Figure 1: Structure of tea catechins.

Figure 2: RTK signaling map.

Figure 3: EGCG competes for the ATP binding site of the Met receptor. (A) ATP docked in the Met kinase domain. (B) Triazolopyridazine inhibitor (TPI) [101] docked in the Met kinase domain. (C) EGCG docked in the Met kinase domain.

Table 1.

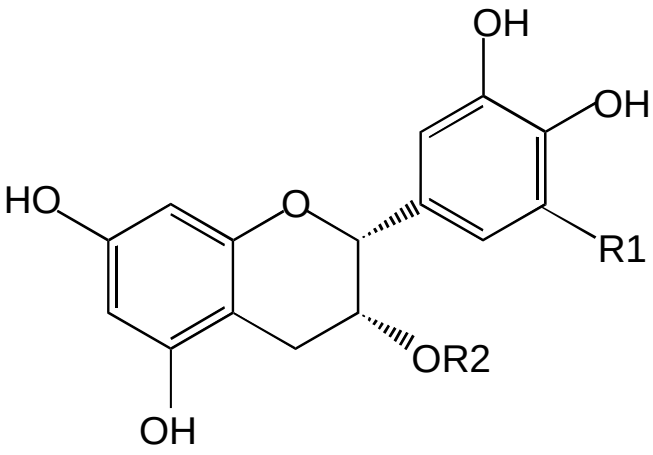
Tea and inhibition of cancer in animal models		
	Positive Results	Negative Results
Lung	23 (1)	2
Bladder	3 (1)	0
Oral Cavity	6	0
Esophagus	5	0
Stomach	9	0
Small Intestine	9	1
Colon	11 (3)	6
Liver	8	1
Pancreas	2 (2)	0
Prostrate	6 (5)	0
Breast	10 (8)	0
Thyroid	1	0
Skin	29 (1)	0

*Updated from [26] to include data from 2009. The number of xenograft studies is shown in parentheses.

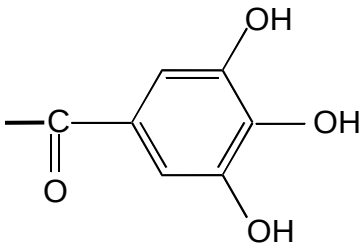
Table 2.

RTK	Effective [EGCG]	Cell line	Ref
EGFR	2.2 µM	SW480	[78]
	10 µM	A431	[106]
	20 µM	KYSE150	[76]
	21.8 µM	YCH-H891, YCU-N861	[107]
	25 µM	HSC	[75]
	30 µM	ECE16-1	[91]
	32.7 µM	A431	[106]
	43.6 µM	HT-29	[77]
Her-2/neu	87.3 µM	NF639	[108]
	87.3 µM	NF639, SMF	[109]
IGF-R1	14 µM	MCF-7, HeLa, NIH3T3	[73]
	43.6 µM	HEPG2	[72]
Met	1 µM	FaDu	[25]
	5 µM	MDA-MB-231	[21]
	5 µM	HCT116	[22]
	10 µM	H2122, H358, H460	[24]
PDGF-R	5 µM	LI90	[110]
	10 µM	hVSMC	[111]
	10 µM	NIH3T3	[106]
	12.5 µM	Fibroblasts	[87]
	20 µM	VSMC	[86]
	32.7 µM	NIH3T3	[74]
	50 µM	A172	[85]
	50 µM	VSMC	[88]
	50 µM	HSC	[112]
VEGF-R1	1.56 uM	HUVEC	[82]
	6.8 uM	CLL B	[81]
	21.8 uM	HuH7	[83]

Figure 1.



Generic Tea Catechin Structure



Gallate group

Tea Catechin	**Abbreviation**	**R1**	**R2**
Epicatechin	EC	H	H
Epigallocatechin	EGC	OH	H
Epicatechin gallate	ECG	H	Gallate
Epigallocatechin gallate	EGCG	OH	Gallate

Figure 2.

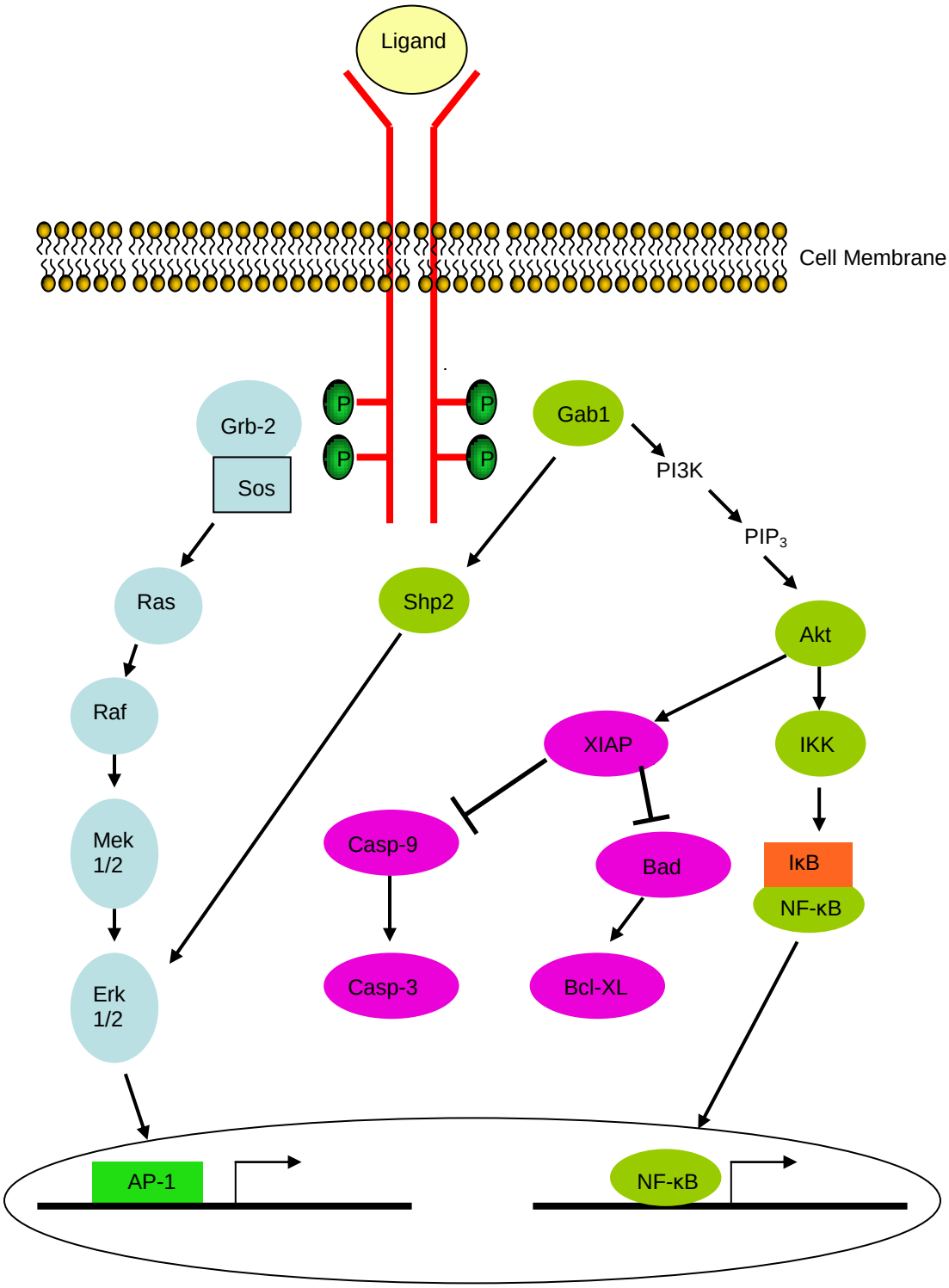
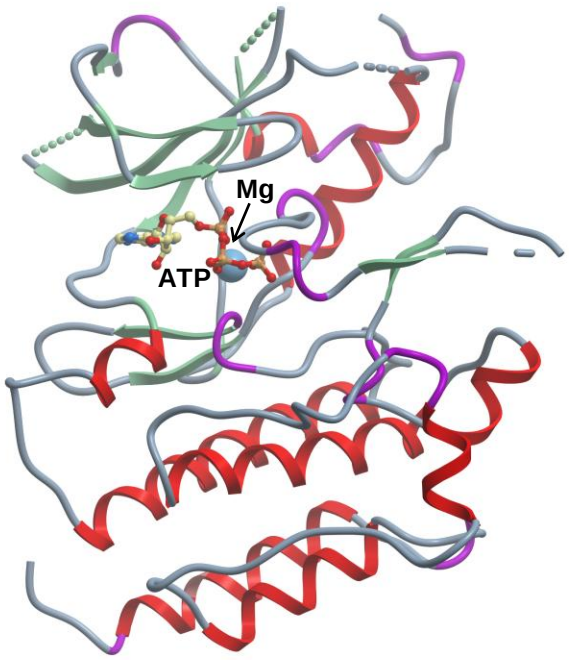
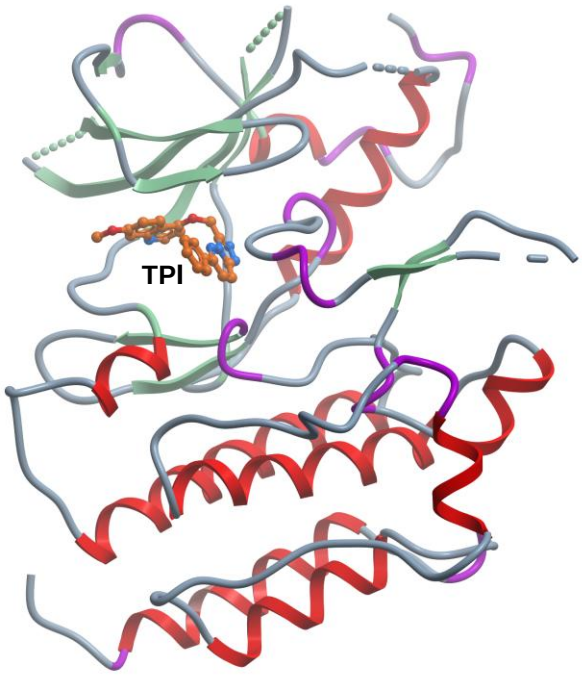

Ligand
Cell Membrane
P
P
P
P
Grb-2
Sos
Gab1
PI3K
PIP3
Ras
Shp2
Akt
Raf
XIAP
IKK
Mek
1/2
Casp-9
Bad
IκB
NF-κB
Erk
1/2
Casp-3
Bcl-XL
AP-1
NF-κB

Figure 3.

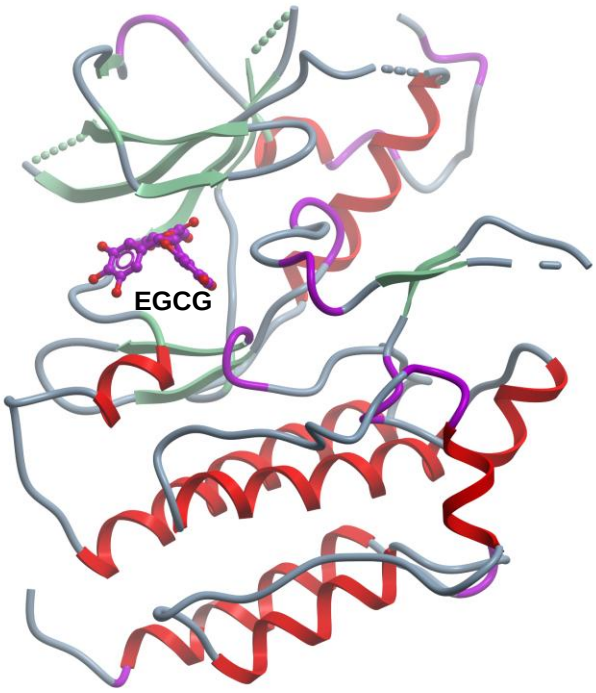
A.



B.



C.



Chapter 3

Suppression of Met activation in human colon cancer cells treated with (-)-epigallocatechin-3-gallate: minor role of hydrogen peroxide

Christine A. Larsen[a] and Roderick H. Dashwood[a,b]

[a] Linus Pauling Institute, [b] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR

Biochemical and Biophysical Research Communications

Elsevier Science, 655 Avenue of the Americas, New York, NY 10010

2009, **389**, 527-530

Abstract

Colorectal cancer is the second leading cause of cancer-related deaths in the U.S. Met, the receptor for hepatocyte growth factor (HGF), is over-expressed in colon tumors and is associated with poor prognosis. Recently, the green tea polyphenol (-)-epigallocatechin gallate (EGCG) was reported to suppress Met activation in breast cancer cells. However, the possible confounding effect of hydrogen peroxide (H_2O_2), produced when EGCG is added to cell culture media, was not assessed. In the present study, the human colon cancer cell lines HCT116 and HT29 were used to examine the relationships between Met activation, EGCG treatment, and H_2O_2 generation. At concentrations of 0.5, 1 and 5 μM, EGCG suppressed markedly the activation of Met in the presence of HGF. Concentrations of 10 μM EGCG and below generated low amounts of H_2O_2 (<1.5 μM), whereas higher H_2O_2 concentrations (>5 μM) were required to directly increase the phosphorylation of Met. Moreover, suppression of Met activation by EGCG occurred in the presence or absence of catalase, suggesting that such effects were not an 'artifact' of H_2O_2 generated from EGCG in cell culture media. We conclude that EGCG might be a beneficial therapeutic agent in the colon, inhibiting Met signaling and helping to attenuate tumor spread/metastasis, independent of H_2O_2-related mechanisms.

Introduction

According to the American Cancer Society, colorectal cancer is the second most common cause of cancer-related deaths in the US. Disease survival is related to tumor stage. For example, Stage I colorectal cancer, in which the carcinoma remains localized in the sub-mucosa of the colon epithelium, has an overall 5-year survival rate of over 90%. In marked contrast, the 5-year survival rate for metastatic disease (Stage IV) drops to less than 10%. Since metastasis accounts for >90% of colon cancer deaths [113], therapies that target this process and block disease progression are of major interest. The receptor tyrosine kinase, Met, has been identified as an important protein that is essential for metastasis in colorectal carcinogenesis [114-118]. When Met is activated by its ligand, hepatocyte growth factor (HGF), it induces proliferation, motility, and invasion; all processes that are required for metastasis to occur.

Green tea and its associated polyphenols have been shown to reduce intestinal tumor formation in the *Apc*$^{Min/+}$ mouse [119, 120]. EGCG, which is the most abundant polyphenol in green tea, has been shown to attenuate Met receptor-mediated signaling in breast cells. The ability of EGCG to suppress Met signaling in colon cancer cells remains to be studied. After oral ingestion, most EGCG accumulates in the intestine [50]. This makes the use of EGCG an attractive therapeutic approach for the possible prevention of colon tumor metastasis.

EGCG has been shown to produce large amounts of hydrogen peroxide (H_2O_2) when added to cell culture media [92]. This has been proposed to represent a possible 'artifact' when working with tea polyphenols *in vitro*. Therefore, empirical evidence is needed whenever cell culture studies use EGCG, to verify that effects are due to EGCG rather than H_2O_2 generated in the experiment. The present study provides evidence that EGCG suppresses Met signaling in human colon cancer cells, and that this effect is independent of H_2O_2 generated under the condition used here.

Materials and Methods

Cell culture.

HCT116 and HT29 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained in McCoy's 5A media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37°C with 5% CO_2.

EGCG/HGF cell treatment.

Cells (0.8×10^6) were plated in 60 mm dishes and grown in serum-containing media for 48 h. Cells were then incubated in serum-free media for 4 h. After serum starvation, cells were pretreated for 30 min with various concentrations of EGCG (Sigma-Aldrich, St. Louis, MO) followed by treatment with 30 ng/ml HGF (Calbiochem, San Diego, CA). In some experiments, 30 U/ml catalase (Roche Applied Science, Indianapolis, IN) were added to the media 10 min prior to addition of EGCG.

H_2O_2 cell treatment.

Cells (0.8×10^6) were plated in 60 mm dishes and grown in serum-containing media for 48 h. Cells were then serum-starved for 4 h. Various concentrations of H_2O_2 (Sigma-Aldrich, MO) were added and cells were incubated for an additional 30 min prior to immunoblot analyses.

Western blotting.

Cells were placed in IP lysis buffer, vortexed, and then centrifuged at 10,000 rpm for 5 min. The supernatant was collected and protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Proteins (10-20 µg) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Novex, San Diego, CA) and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Equal protein loading was confirmed with Amido Black staining and β-actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by overnight incubation with primary antibody at 4°C, and finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: Phospho-Met (Tyr1234/1235), 1:1000 (Cell Signaling Technology, Beverly, MA), Total Met, 1:1000, (Cell Signaling Technology), and β-actin,1:5000 (Sigma). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system.

H_2O_2 measurement.

To measure H_2O_2 production, serum-starved cells were incubated with various concentrations of EGCG in phenol red-free McCoy's 5A media (Invitrogen) for 30 min. Aliquots of media were analyzed using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR), as previously reported [121].

Enzyme-linked immunosorbent assay (ELISA).

Cell culture media was pretreated in the presence or absence of 30 U/ml catalase (Roche). EGCG (5 µM) was then added to the media and cells were incubated for 30 min prior to the addition of 30 ng/ml HGF (Calbiochem). Cells were incubated for an additional 15 min and lysed in RIPA buffer containing phosphatase inhibitors (Pierce). The ELISA was performed according to the instruction manual for STAR phospho-Met (Tyr1230/Tyr1234/Tyr1235) ELISA kit (Upstate).

Cell viability.

HCT116 cells were incubated with various concentrations of H_2O_2 for 15 min. The cells were then collected and stained with Guava Viacount® Reagent (Guava Technologies, Hayward, CA) for 5 min. The percentage of viable cells was determined by using the Guava ViaCount Assay on a Guava Personal Cytometer.

Results

Phosphorylation of the Met receptor previously was reported to be inhibited by EGCG in immortalized and tumorigenic breast epithelial cells [21]. To determine if this occurred in human colon cancer cells, HCT116 and HT29 cells were pre-incubated with various concentrations of EGCG for 30 min followed by the addition of HGF to the media. As expected, in the absence of EGCG pretreatment, HGF alone strongly increased the levels of p-Met in HCT116 cells (Fig. 1A) and HT29 cells (Fig. 1B). However, activation of the Met receptor was suppressed by EGCG concentrations as low as 0.5 μM. This suppression occurred in both cell lines, and was evident after normalizing p-Met to total Met in the immunoblots (Fig. 1C).

The amount of H_2O_2 produced by EGCG under the current conditions was measured after incubating various concentrations of EGCG in cell culture media for 30 min. With no EGCG present, ~0.1 and 0.25 μM H_2O_2 was detected in media from HCT116 and HT29 cells, respectively (open bars, Fig. 2A). The concentration of H_2O_2 increased with EGCG added to the media. However, only at the highest concentration of 10 μM EGCG was H_2O_2 measured at a level above 1 μM, in each cell line (solid bar, Fig. 2A).

To investigate the direct effects of H_2O_2 on Met activation, HCT116 cells were incubated with various concentrations of H_2O_2 for 30 min, in the absence of HGF, and p- Met was analyzed by immunoblotting. Concentrations at or below 2.5 μM H_2O_2 had no apparent effect on Met phosphorylation, as

indicated by the lack of change in p-Met levels compared with the 0 μM H_2O_2 control (Fig. 2B). When the concentration of H_2O_2 exceeded 2.5 μM, however, there was increased phosphorylation of Met, most notably at the highest levels of 10 and 20 μM H_2O_2 (Fig. 2B).

The direct effects of H_2O_2 were further examined in the presence and absence of HGF (Figs. 2C,D). In the absence of HGF, p-Met/total Met levels were markedly increased by concentrations of H_2O_2 in the range 5-100 μM H_2O_2 (Fig. 2D, open bars). A similar trend was seen in cells treated with HGF plus H_2O_2 (Fig. 2D, gray bars). However, compared to –HGF controls, receptor activation was enhanced at concentrations below 20 μM H_2O_2 (compare, for example, white and gray bars at 5 μM H_2O_2 in Fig. 2D). At concentrations of 20, 50, and 100 μM H_2O_2, phosphorylation of the Met receptor was decreased slightly when HGF was present, as compared with the corresponding –HGF controls. The experiment, by design, also sought to test the upper limit of Met activation in response to H_2O_2; concentrations of 500 and 1000 μM H_2O_2 strongly reduced p-Met and total Met in the immunoblots, as well as expression of the loading control β-actin (Fig. 2C).

The viability of HCT116 cells treated with these concentrations of H_2O_2 was assessed. There was little or no effect on viability when cells were exposed to H_2O_2 at concentrations up to 100 μM (Fig. 2E). However, at the highest concentrations of 500 and 1000 μM H_2O_2 the percentage of viable cells was reduced to less than 45% and 8% viability, respectively (Fig. 2E).

To further assess the role of H_2O_2 and to differentiate its effects from those of EGCG, subsequent experiments included catalase. Immunoblotting revealed that, in the presence or absence of catalase, HGF produced a similar level of Met phosphorylation, and EGCG maintained its inhibitory effects (Fig. 3A). This was confirmed using an ELISA kit with the same cell lysates to detect p-MET, revealing that Met activation was virtually identical in the presence or absence of catalase (Fig. 3B). Thus, inhibition of the Met receptor by EGCG can occur in the absence of H_2O_2.

Discussion

Previous studies have shown that tea polyphenols are capable of suppressing receptor tyrosine kinase activity [31, 122, 123]. In particular, the Met receptor was shown to be inhibited in breast cancer cells treated with EGCG, the major polyphenol in green tea [21]. Here, EGCG effectively suppressed the activation of the Met receptor in human colon cancer cells, and at concentrations as low as 0.5 µM EGCG, which are likely to be within the physiological range [124, 125].

EGCG rapidly produces H_2O_2 when added to cell culture media [92]. At a concentration of 25 µM EGCG, for example, 10-12 µM H_2O_2 was detected in serum-free MEM media [121]. Here, we investigated the H_2O_2 generated following the addition of EGCG to McCoy's 5A media, used in the current study. As suggested by Long *et al.* [92], excess H_2O_2 generated in cell culture media from the oxidation of polyphenolic compounds could represent an artifact in mechanistic studies of tea catechins. Thus, it is important to distinguish the effects of EGCG from those of H_2O_2. Under the present conditions, 10 µM EGCG produced less than 1.5 µM H_2O_2 in both HCT116 and HT29 cell lines. This concentration of H_2O_2 did not alter Met phosphorylation levels following direct addition of H_2O_2 to the experiment. However, the Met receptor was activated by H_2O_2 when concentrations exceeded 2.5 µM EGCG in the assay. This is most likely due to the ability of

H_2O_2 to inhibit protein tyrosine phosphatases [126], although this was not tested in the present study.

Others have reported that exposing A549 human lung cancer cells to 500 μM H_2O_2 in the presence of HGF results in the suppression, rather than the activation, of the Met receptor [14]. In the present study, concentrations of 500 and 1000 μM EGCG clearly had a deleterious effect on the viability of human colon cancer cells; thus, the apparent loss of Met phosphorylation must be viewed in this context, along with the reduced levels of total Met and β-actin. Importantly, catalase had no effect in these experiments, either in blocking the HGF-mediated increase in p-MET levels, or diminishing the effectiveness of EGCG to suppress Met activation.

We conclude that, under the present conditions, EGCG can effectively suppress Met signaling in colon cancer cells, at physiologically relevant concentrations. Thus, EGCG may be useful as a therapeutic agent for prevention of metastatic spread of colon tumors. The concern over H_2O_2 as a possible artifact was ruled out through the use of catalase, and by experiments showing that the concentrations of H_2O_2 generated by 1-10 μM EGCG were too low to influence Met phosphorylation. Further studies are warranted to determine the mechanism by which EGCG suppresses activation of the Met receptor.

Acknowledgements

This work was supported in part by grant CA090890 from the US National Cancer Institute.

Figure Legends

Figure 1: EGCG suppresses HGF-induced Met signaling. Human colon cancer cells were serum-starved for 4 h and then treated with various concentrations of EGCG for 30 min. HGF (30 ng/ml) was added to the cell culture media and incubated for an additional 15 min. Immunoblotting was performed on cell lysates obtained from (A) HCT116 cells or (B) HT29 cells, using primary antibodies to phosphorylated-Met (p-Met), total Met, or β-actin (loading control). (C) Quantification of immunoblots by densitometry, with p-Met normalized to total Met. Cells treated with HGF alone (no EGCG) were assigned an arbitrary value of 1.0. Wedge symbol indicates 0, 0.1, 0.5, 1, 5 and 10 μM EGCG. Data were from a single experiment, and are representative of the findings from three separate experiments.

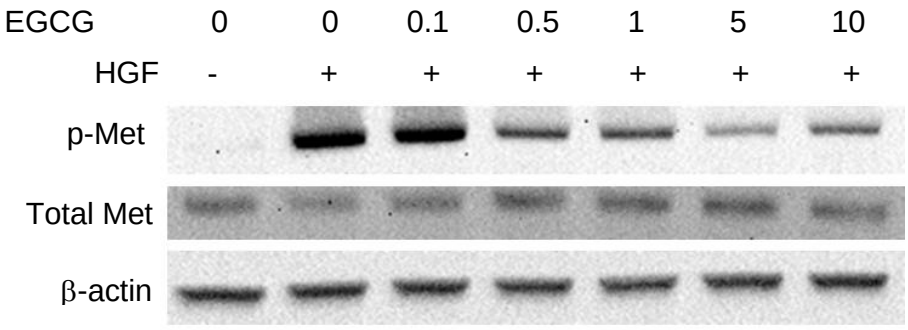
Figure 2: EGCG-generated hydrogen peroxide, and the increased phosphorylation of Met at high H_2O_2 concentrations. (A) HCT116 and HT29 cells were serum-starved for 4 h in phenol red-free McCoy's 5A media. After 30 min of EGCG treatment, aliquots of media were taken and H_2O_2 was measured using the Amplex Red assay. Data (mean±SD, n=3) are representative of the findings from three separate experiments. (B) Western blot analysis of HCT116 cells serum-starved for 4 h then treated with various concentrations of H_2O_2. (C) Immunoblotting of HCT116 cells serum-starved for 4 h, and treated with a broad range of H_2O_2 concentrations, in the presence and absence of HGF. (D) Quantification of immunoblots in (C) by

densitometry, showing p-Met normalized to total Met. (E) Viability of HCT116 cells treated with H_2O_2.

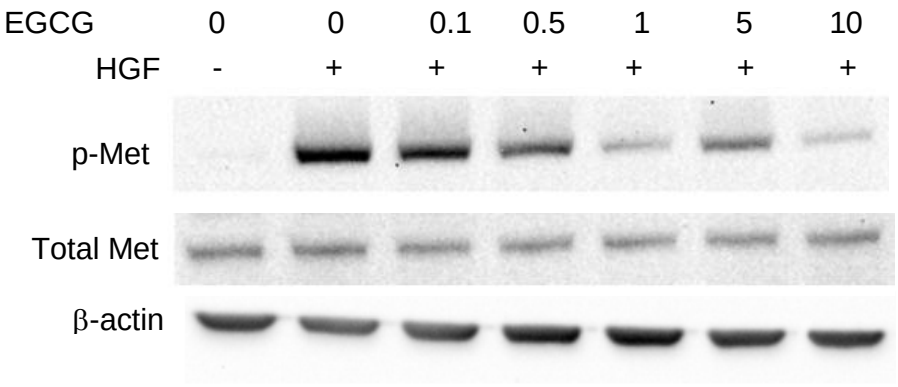
Figure 3: Catalase does not interfere with the inhibitory effects of EGCG on HGF-mediated Met activation. HCT116 cells were serum-starved for 4 h. Catalase (30 U/ml) was added to the cell culture media 10 min prior to the addition of 5 µM EGCG. (A) Western blot analysis of the cell lysates. (B) ELISA using STAR phospho-Met ELISA kit (Upstate), with cell lysates from HCT116 cells treated in (A). Data (mean±SD, n=3) are representative of findings from three separate experiments.

Figure 1.

A.


EGCG 0 0 0.1 0.5 1 5 10
HGF - + + + + + +
p-Met
Total Met
β-actin

B.


EGCG 0 0 0.1 0.5 1 5 10
HGF - + + + + + +
p-Met
Total Met
β-actin

C.

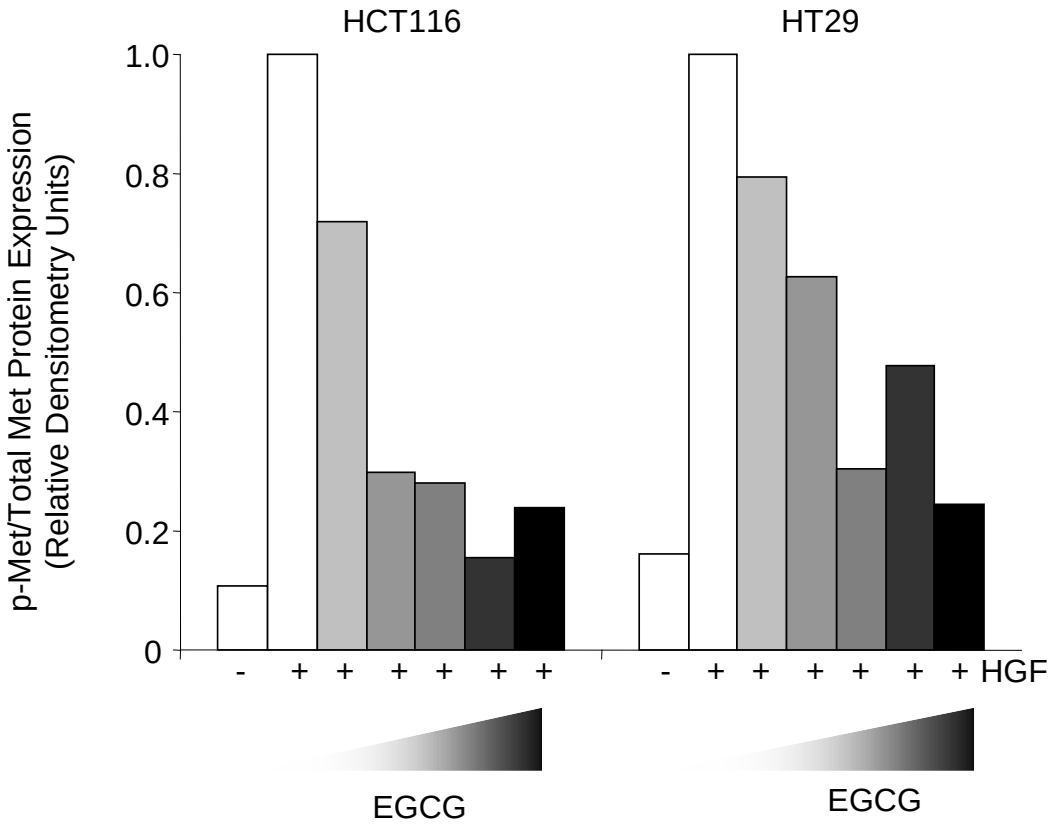
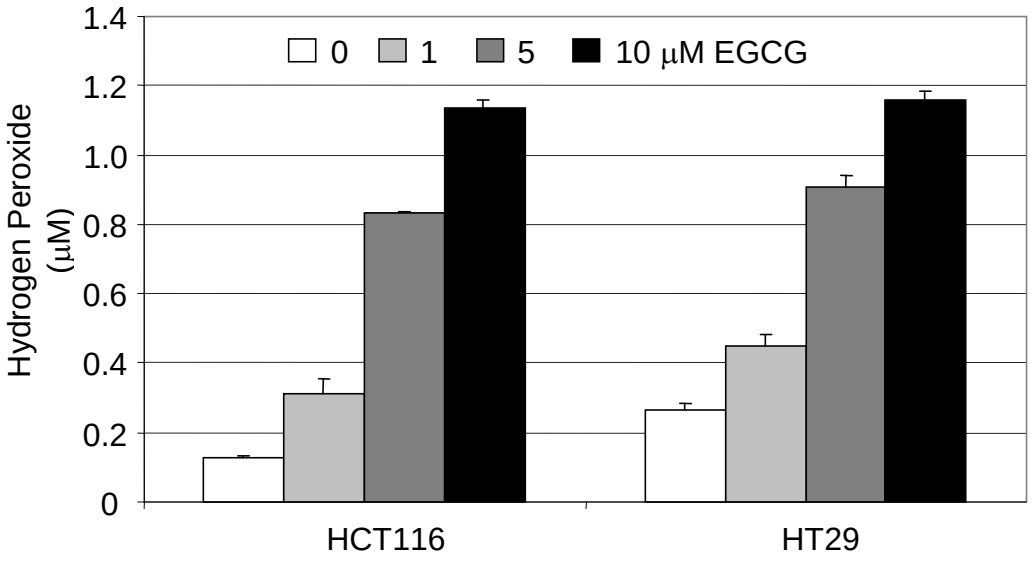
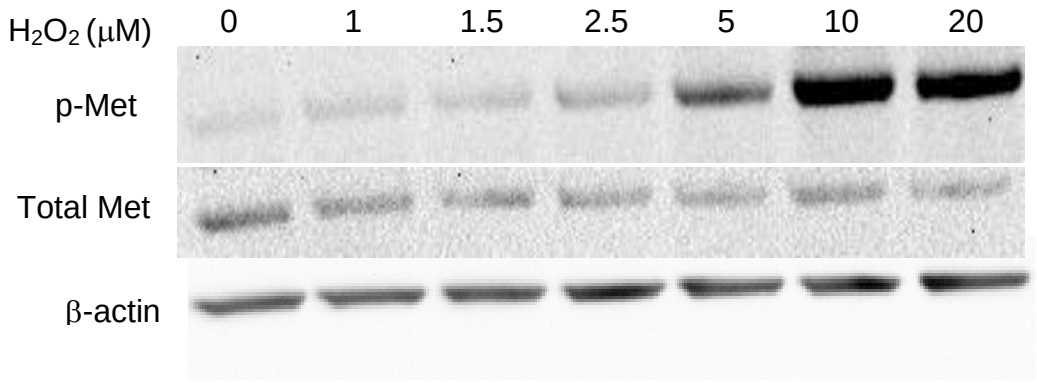

HCT116
HT29
p-Met/Total Met Protein Expression
(Relative Densitometry Units)
1.0
0.8
0.6
0.4
0.2
0
- + + + + + +
- + + + + + + HGF
EGCG
EGCG

Figure 2.

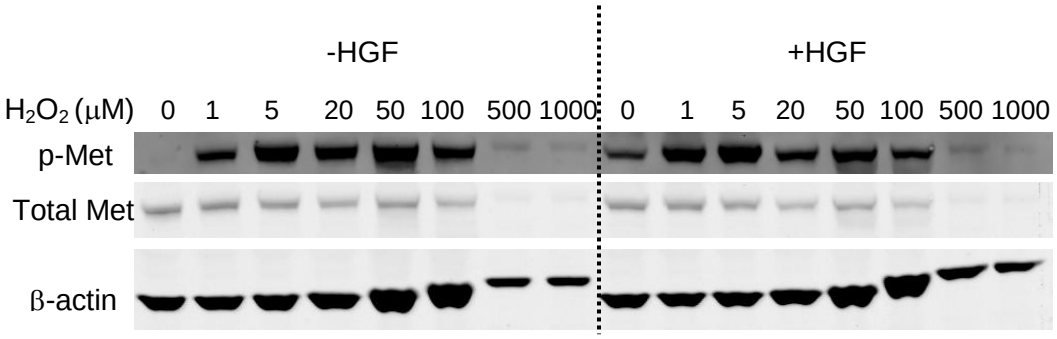
A.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Hydrogen Peroxide (μM)
0
1
5
10 μM EGCG
HCT116
HT29

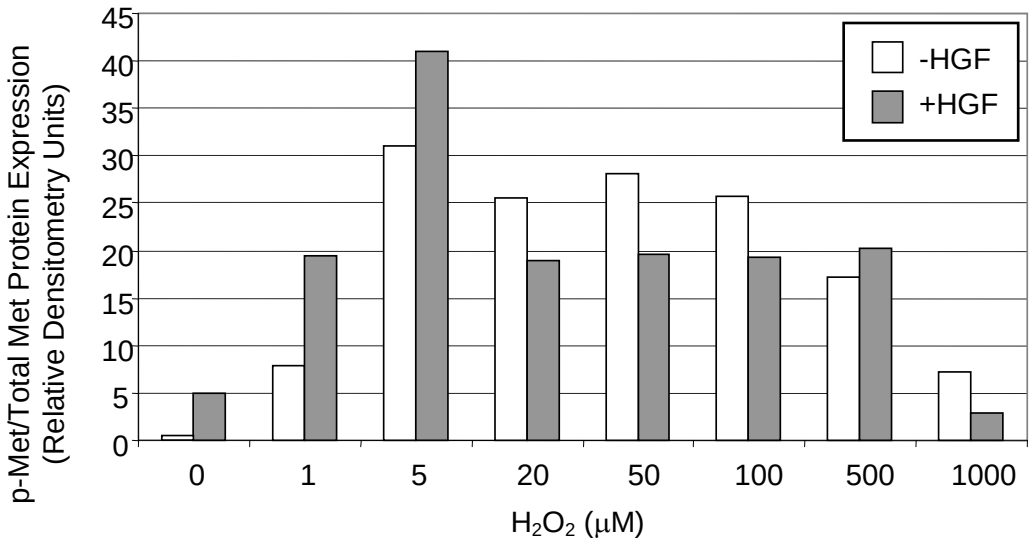
B.


H2O2 (μM)
0
1
1.5
2.5
5
10
20
p-Met
Total Met
β-actin

C.


-HGF
+HGF
H2O2 (μM) 0 1 5 20 50 100 500 1000 0 1 5 20 50 100 500 1000
p-Met
Total Met
β-actin

D.


p-Met/Total Met Protein Expression
(Relative Densitometry Units)
45
40
35
30
25
20
15
10
5
0
-HGF
+HGF
0 1 5 20 50 100 500 1000
H2O2 (μM)

E.

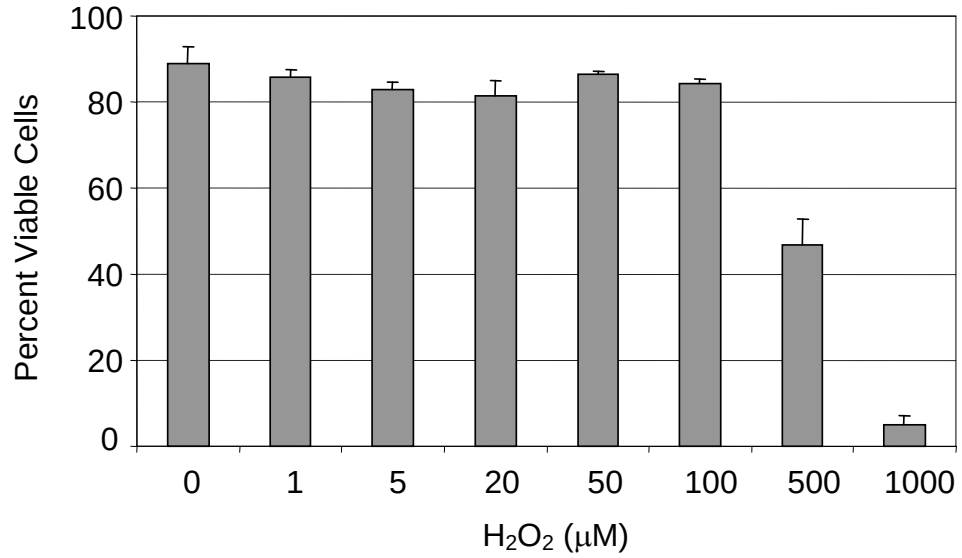
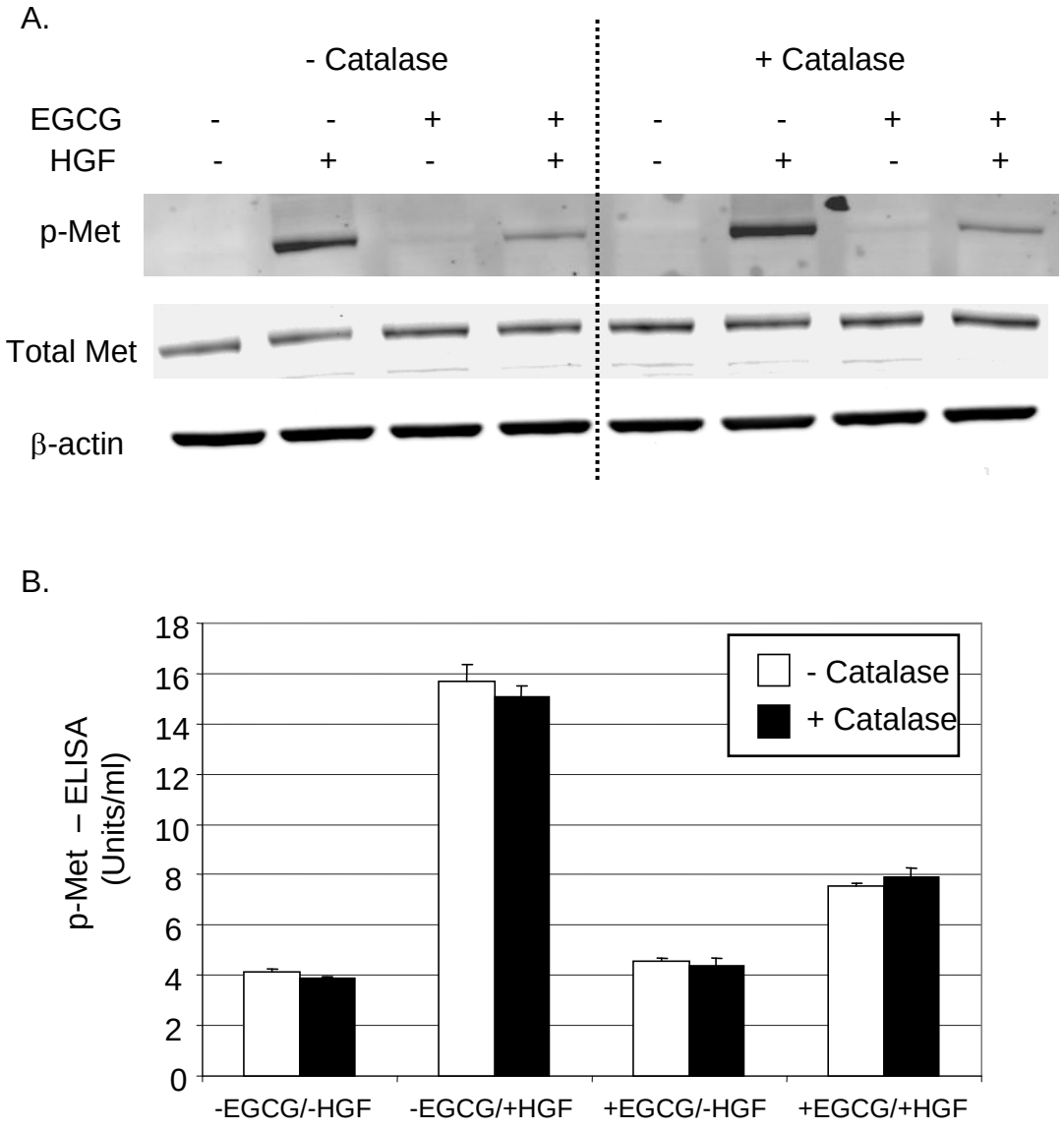

100
80
60
40
20
0
Percent Viable Cells
0
1
5
20
50
100
500
1000
H2O2 (μM)

Figure 3.


A.
- Catalase
+ Catalase
EGCG - - + + - - + +
HGF - + - + - + - +
p-Met
Total Met
β-actin
B.
p-Met – ELISA (Units/ml)
18
16
14
12
10
8
6
4
2
0
- Catalase
+ Catalase
-EGCG/-HGF
-EGCG/+HGF
+EGCG/-HGF
+EGCG/+HGF

Chapter 4

Tea Catechins Inhibit Hepatocyte Growth Factor Receptor (MET Kinase) Activity in Human Colon Cancer Cells: Kinetic and Molecular Docking

Christine A. Larsen[1], William H. Bisson[1] and Roderick H. Dashwood[1,2]

[1] Linus Pauling Institute, [2] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR

Journal of Medicinal Chemistry

1155 Sixteenth Street N.W., Washington, DC 20036

2009, **52**, 6543-6545

Abstract

Most cancer deaths result from spread of the primary tumor to distant sites (metastasis). MET is an important protein for metastasis in multiple tumor types. Here we report on the ability of tea catechins to suppress MET activation in human colon cancer cells and propose a mechanism by which they might compete for the kinase domain of the MET protein.

Supporting Information

Experimental Section

Cell Culture.

HCT116 human colon cancer cells were obtained from ATCC and were maintained in McCoy's 5A media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37°C with 5% CO_2.

Tea catechin/HGF Cell Treatment.

Cells (0.8×10^6) were plated in 60 mm plates and grown in serum containing media for 48 hours. Cells were then incubated in serum free media for 4 h. After serum starvation, cells were pre-treated for 30 minutes with 5 μM of tea catechins (Sigma-Aldrich, MO) followed by treatment with 30 ng/ml HGF (Calbiochem, San Diego, CA).

Western Blot.

Cells were placed in IP lysis buffer and were vortexed and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Proteins (10-20 mg) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Novex, San Diego, CA) and were transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Equal protein loading was confirmed with Amido Black staining and β-actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by overnight incubation with primary antibody

at 4°C, and was finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: Phospho-Met (Tyr1234/1235), 1:1000 (Cell Signaling Technology, Beverly, MA), Total Met, 1:1000, (Cell Signaling Technology) and β-actin,1:5000 (Sigma) Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system.

In vitro Kinase Assay.

The kinase activity assay was performed as described by the HTScan® Met Kinase Assay Kit protocol (Cell Signaling Technology). Briefly, tea catechins were pre-incubated with MET kinase. ATP and substrate were then added to the reaction mix. Phosphorylation of the substrate was detected using a secondary antibody conjugated with HRP.

Molecular Docking.

The receptor is represented by five types of interaction potentials: (i) van der Waals potential for a hydrogen atom probe; (ii) van der Waals potential for a heavy-atom probe (generic carbon of 1.7 Å radius; (iii) optimized electrostatic term; (iv) hydrophobic terms; and (v) loan-pair-based potential, which reflects directional preferences in hydrogen bonding. The energy terms were based on the all-atom vacuum force field ECEPP/3 with appended terms from the Merck Molecular Force Field to account for

solvation free energy and entropic contribution.[R3] Modified inter-molecular terms such as soft van der Waals and hydrogen-bonding as well as a hydrophobic term are added. Conformational sampling was based on the biased probability Monte Carlo (BPMC) procedure, which randomly selects a conformation in the internal coordinate space and then makes a step to a new random position independent of the previous one but according to a predefined continuous probability distribution. It has also been shown that after each random step, full local minimization greatly improves the efficiency of the procedure. In the ICM-VLS (Molsoft ICM v3.5-1p) screening procedure the ligand scoring was optimized to obtain maximal separation between the binders and non-binders. Each compound was assigned a score according to its fit within the receptor, which accounts for continuum and discreet electrostatics, hydrophobicity and entropy parameters [127-129].

Hepatocyte growth factor receptor, also known as MET or c-Met, is important during embryonic development and wound healing [89]. Its ligand is hepatocyte growth factor (HGF), also known as scatter factor. MET is deregulated in a variety of tumor types and is associated with poor prognosis. (*Camellia sinensis*) and its polyphenolic compounds, the catechins, have been studied for their ability to prevent and treat chronic conditions such as cancer, cardiovascular diseases, neurodegenerative disorders, diabetes, and osteoporosis [28]. Green tea catechins include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). (-)-Catechin (CAT) is the epimer of EC, whereas gallocatechin gallate (GCG) is the epimer of EGCG (Figure 1).

Tea catechins recently were reported to inhibit MET signaling in breast [21] and hypopharyngeal carcinoma cells [25]; however, the mechanisms were not examined. In the present investigation, HCT116 human colon cancer cells were treated with the various compounds shown in Figure 1, and the cell lysates were subjected to immunoblotting for phospho-MET. As expected, HGF alone caused an increase in phospho-MET, but this was inhibited markedly by the gallate-containing catechins ECG, EGCG, and GCG (Figure 2).

An *in vitro* MET kinase activity assay next was performed (Figure 3). Consistent with findings from the cell culture experiments, ECG, EGCG, and GCG inhibited MET kinase activity, whereas the other test compounds had

little or no effect. For ECG, EGCG, and GCG, inhibition occurred in a dose-dependent manner, and the concentration for 50% inhibition (IC_{50}) for each compound was ~10 μM.

Because EGCG is the most abundant catechin in green tea, and it was an effective inhibitor in the MET kinase activity assay, EGCG was selected for further kinetic analyses (Figure 4). The Cornish-Bowden plot ([S]/V versus [I]) produced a series of parallel lines, whereas the Dixon plot (1/V versus [I]) had lines that intersected above the x-axis. These findings are consistent with reversible competitive enzyme inhibition [130, 131]. On the basis of linear regression analysis of the Dixon plot (Figure 4B), the inhibitor constant (K_i) was determined to be 3.3 μM EGCG.

Molecular docking [128, 129] was used to investigate how the tea catechins might interact with MET. The MET kinase domain first was constructed using the experimentally resolved crystal structures available in the Protein Data Bank, with access code 3CD8,5 and energetically minimized in the internal coordinate space [132, 133]. To validate the model, a known MET inhibitor [101] was docked within a 4 A° sphere around its 3D position in the crystal structure. The inhibitor docked with a score of -44, having a low all-atoms rmsd compared to the crystallographic orientation. The conformation of the inhibitor after docking was “U-bended” (Figure 5, in orange/blue), with hydrogen bond interactions water-mediated between N1 and the quinoline aromatic nitrogen and the backbone –NH of residues Asp1222 and Met1160, respectively, as described in the literature [101].

By use of the same iterative approach, EGCG was docked into the MET kinase domain. The tea catechin fit favorably into the MET binding site, with a score of -20.08 (Figure 5, in purple). Two of the hydroxyl groups of the gallate moiety interacted with the backbone -NH of Met1160 and Pro1158.

When the MET inhibitor [101] and EGCG alignments were superimposed, the quinoline ring of the former molecule and the gallate phenyl ring of EGCG were orientated in a similar manner in the crystal structure (Figure 5). Thus, the hydrogen bond interaction between the ligand (through-OH groups or the aromatic quinoline nitrogen) and the backbone of Met1160, which was observed in the crystal structure [101] and from docking studies, appears to be an important feature for binding to the kinase domain.

The docking score of EGCG was ~2-fold lower than for the triazolopyridazine inhibitor [101], which predicts a lower affinity of the tea catechin for MET.

Additional tea catechins (Figure 1) were docked into the MET kinase domain. Two docking orientation clusters were identified: GCG, EGCG, ECG (Figure 6) and CAT, EC, EGC (Figure 7), with docking scores of approximately -20 and -10, respectively. Thus, the gallate group was identified as a key structural feature for binding of tea polyphenols to the MET kinase domain. The gallate-containing catechins were observed to allocate the 3-gallate phenyl ring in the same region of the binding pocket. In this position, hydrogen bonding was established between the aromatic hydroxyl

groups of the gallate moiety and the backbone -NH of Met1160 and Pro1158 (with the exception of GCG with no hydrogen bond to Pro1158). In contrast, catechin derivatives lacking the gallate group did not exhibit affinity for Met1160 but interacted with the backbone -NH of Asp1222. This was associated with a lower overall docking score. Thus, from modeling studies we predict the binding affinity toward MET will be in the relative order triazolopyridazine inhibitor [101] > gallate-containing catechins > non-gallate-containing compounds.

It is noteworthy that the computational predictions were in general agreement with the immunoblotting data for phospho- MET (Figure 2) and the MET kinase enzyme activity data in vitro (Figure 3), which revealed little if any inhibition by CAT, EC, and EGC, and intermediate inhibition by ECG, EGCG, and GCG, compared with the strong inhibition reported previously for the triazolopyridazine compound [101]. One apparent limitation of the modeling approach is that a 2-fold difference in docking score can exist in the face of a larger range of IC_{50} values.

In conclusion, gallate-containing tea catechins were effective at suppressing MET activation in human colon cancer cells. Enzyme kinetic studies and computational analyses supported a model in which the gallate moiety was bound to the kinase domain of the MET receptor. Recent work has shown that the 90 kDa heat shock proteins (Hsp90) contain a C-terminal nucleotide binding pocket that binds not only ATP but also cisplatin, novobiocin, paclitaxel, and EGCG [134]. Tea polyphenols and other

flavonoids interact with the ATP-binding site of several kinases, possibly as a mimetic of the adenine moiety of ATP [100]. The ATP binding site of MET also has been used in high-throughput virtual screening for potential new enzyme inhibitors, and some of the candidates identified had IC_{50} values in the micromolar range [135]. The present investigation has revealed that several tea catechins also inhibit MET and are effective in the 1-10 μM range. With pharmacologic oral dosing of EGCG, peak plasma concentrations of 7.5 and 9 μM EGCG were detected in humans and mice, respectively [102]. These concentrations also may be achievable in the gastrointestinal tract following more typical green tea intake, despite extensive methylation, glucuronidation, and sulfation, or conversion to valerolactone breakdown products by the gut microflora [102, 136]. It is noted that despite the promising mechanistic data obtained here and elsewhere using *in vitro* or preclinical models [102], in humans the evidence is less compelling for chemoprevention by tea [28]. In the therapeutic setting there is a clear need for further investigation, including effects of tea on MET and other pathways implicated in metastasis.

Figure Legends

Figure 1: Structures and nomenclature of tea catechins.

Figure 2: Tea catechins inhibit MET activation in human colon cancer cells: (A) immunoblots of whole cell lysates; (B) corresponding densitometry data, with phospho-MET normalized to total MET. Loading control is β-actin. Test compounds, 5 [micro]M.

Figure 3: Tea catechins inhibit MET kinase activity *in vitro*. Data are the mean (SD, n = 3)

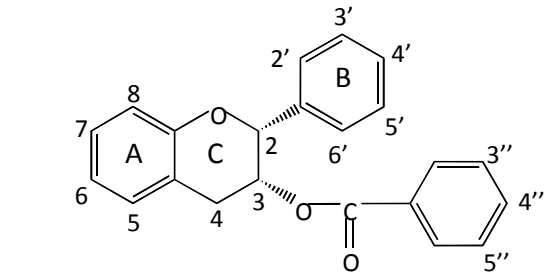
Figure 4: Kinetic analyses of MET kinase inhibition by EGCG: (A) Cornish-Bowden plot; (B) Dixon plot (Ki = 3.3 μM).

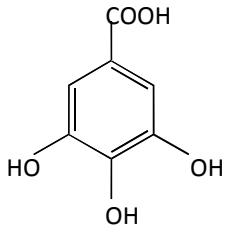
Figure 5: Modeling of the MET kinase active site with bound inhibitors. Triazolopyridazine inhibitor (orange), listed as compound 4 previously [101], and EGCG (purple).

Figure 6: Modeling of the MET kinase active site with bound EGCG (purple), GCG (green), and ECG (blue).

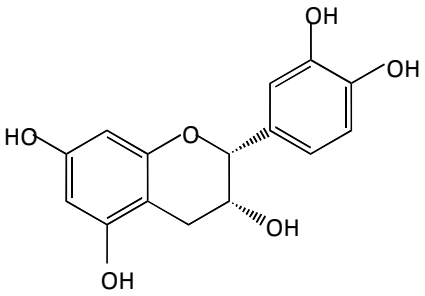
Figure 7: Modeling of the MET kinase active site with bound CAT(purple), EC (green), and EGC (blue).

Figure 1.

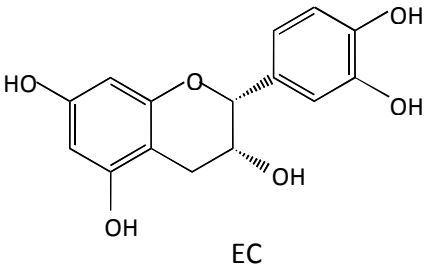




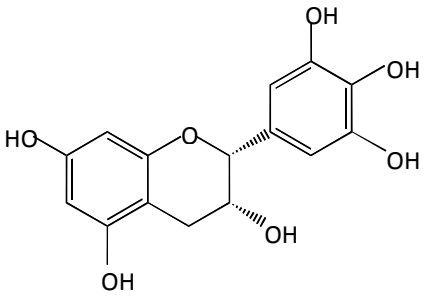
GA



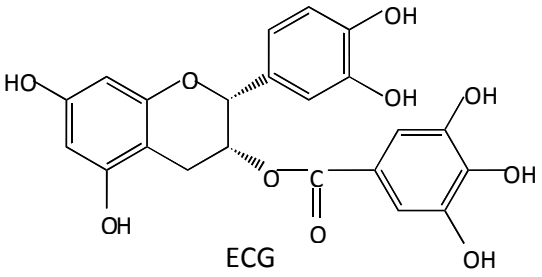
CAT



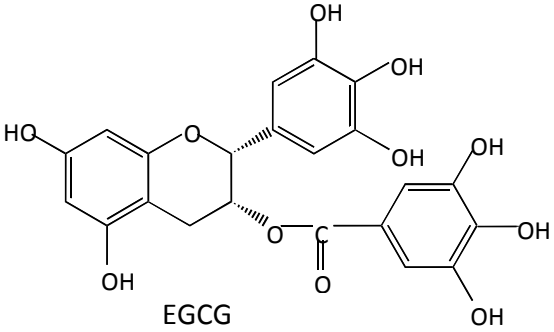
EC



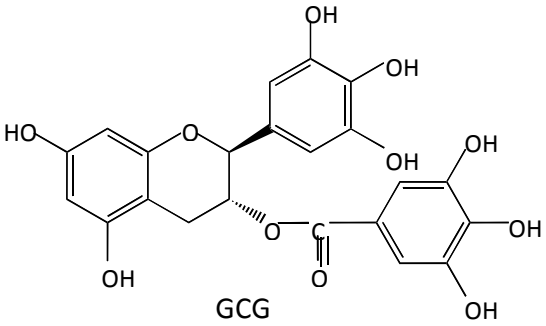
EGC



ECG



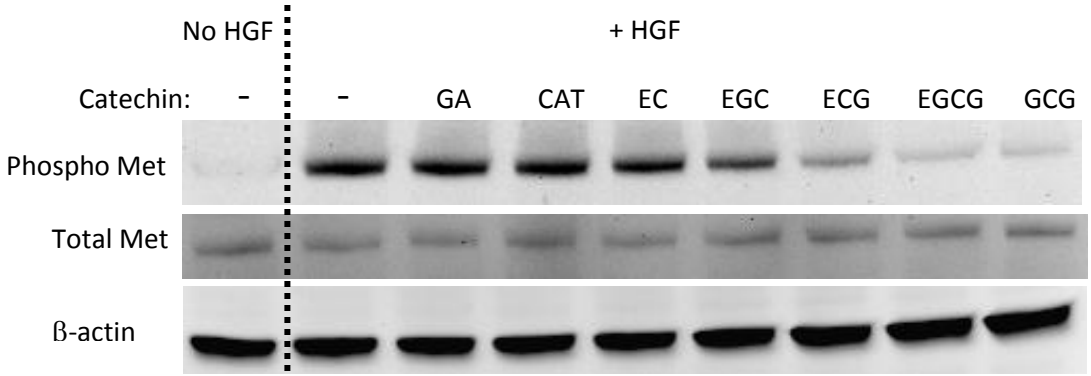
EGCG



GCG

Figure 2.

A.


No HGF
+ HGF
Catechin:
-
-
GA
CAT
EC
EGC
ECG
EGCG
GCG
Phospho Met
Total Met
β-actin

B.

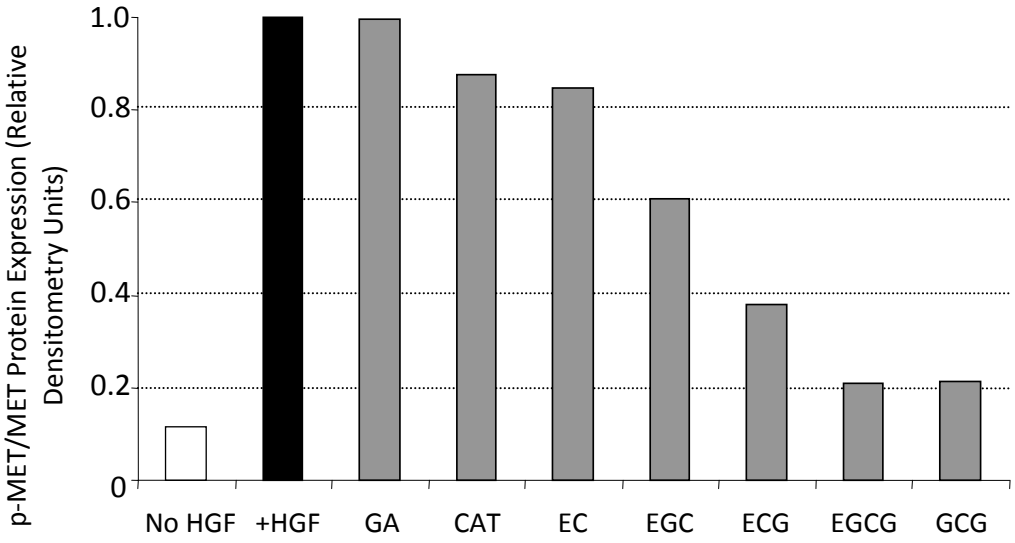

p-MET/MET Protein Expression (Relative Densitometry Units)
1.0
0.8
0.6
0.4
0.2
0
No HGF
+HGF
GA
CAT
EC
EGC
ECG
EGCG
GCG

Figure 3.

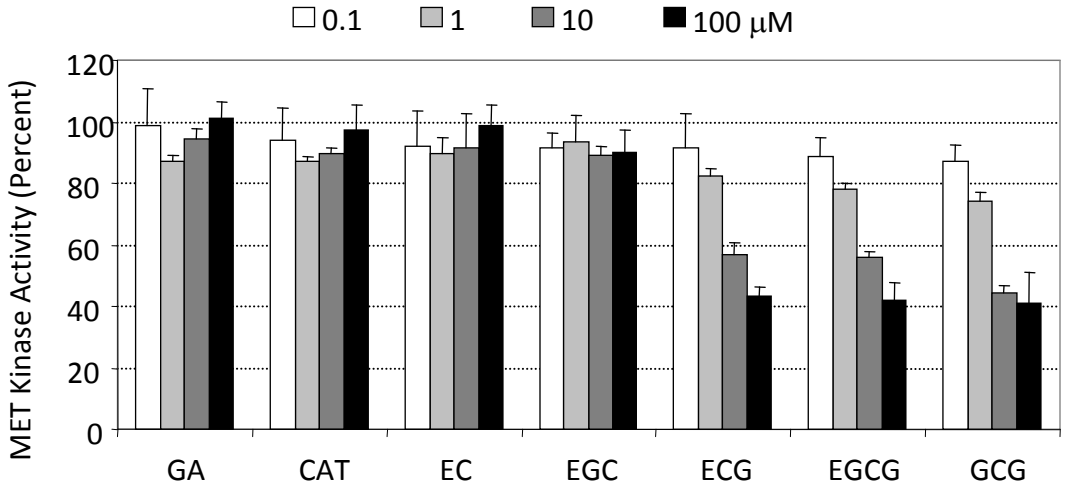
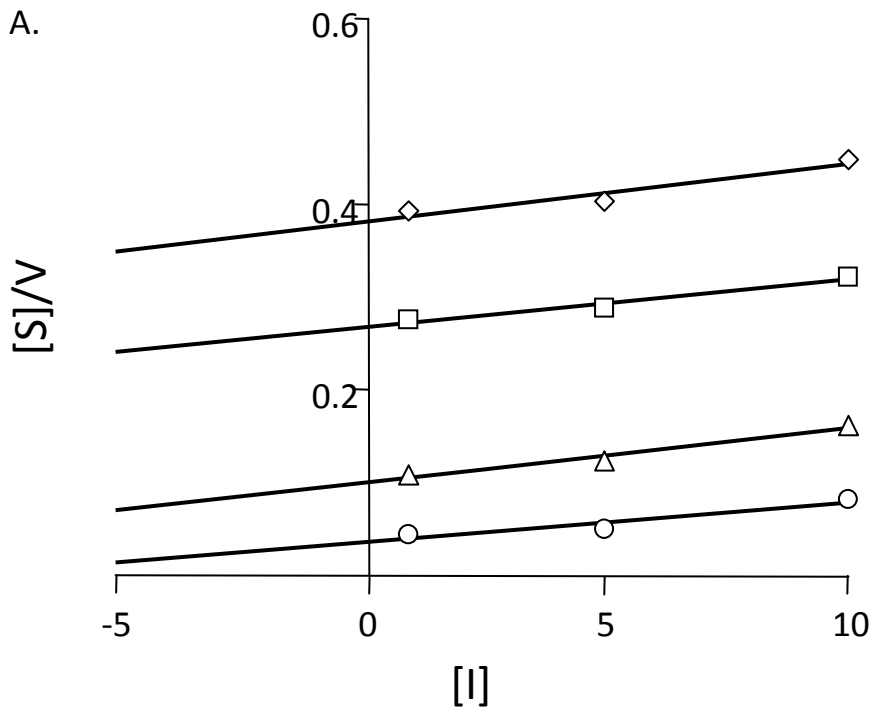

0.1
1
10
100 μM
MET Kinase Activity (Percent)
120
100
80
60
40
20
0
GA
CAT
EC
EGC
ECG
EGCG
GCG

Figure 4.



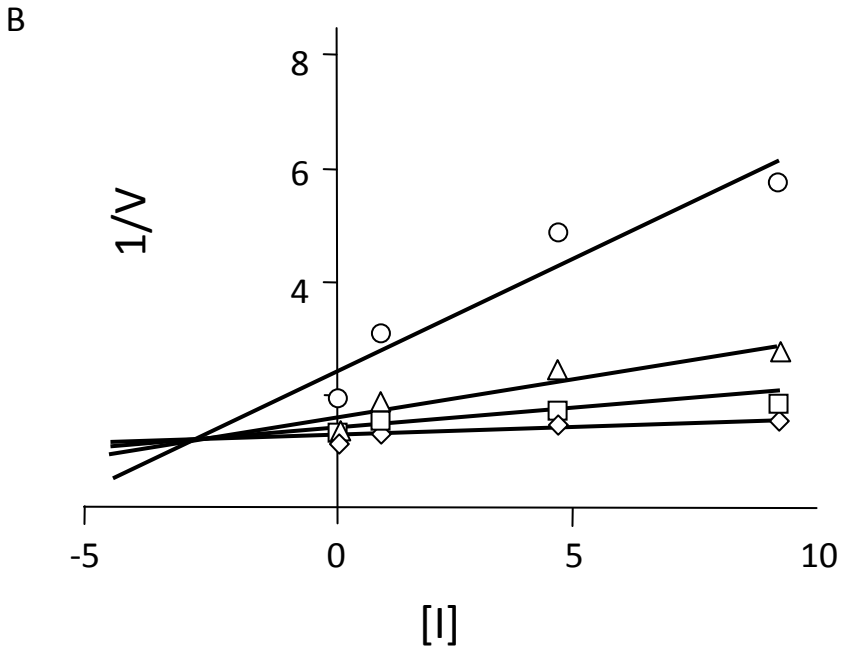


Figure 5.

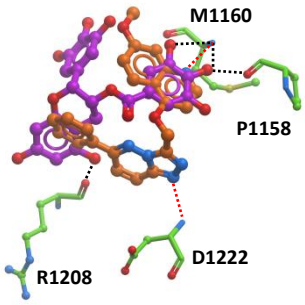


Figure 6.

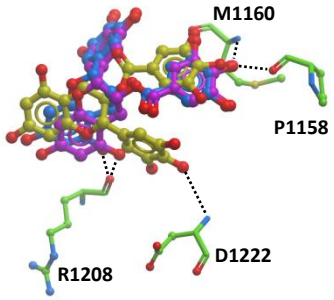
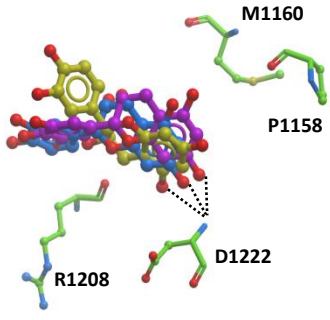


Figure 7.



Chapter 5

(-)-Epigallocatechin-3-gallate inhibits Met signaling, proliferation, and invasiveness in human colon cancer cells

Christine A. Larsen[a] , Roderick H. Dashwood[a,b]

[a]Linus Pauling Institute, [b]Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon, USA

Submitted for publication

Archives of Biochemistry and Biophysics

Elsevier Science, 655 Avenue of the Americas, New York, NY 10010

Abstract

The Met receptor tyrosine kinase is deregulated in a variety of cancers and is correlated with advanced stage and poor prognosis. Thus, Met has been identified as an attractive candidate for targeted therapy. We compared the tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) and a specific Met inhibitor, SU11274, as suppressing agents of Met signaling in HCT116 human colon cancer cells. Treatment with hepatocyte growth factor increased phospho-Met levels, and this was inhibited in a concentration-dependent manner by EGCG and SU11274 (IC_{50} 3.0 *vs* 0.05 μM, respectively). Downstream activation of Erk and Akt signaling pathways also was suppressed. Both compounds at a concentration of 5 μM lowered cell viability and proliferation, with EGCG being more effective than SU11274, and the invasion of colon cancer cells in Matrigel assays was strongly inhibited. These findings are discussed in the context of the pleiotropic effects of tea catechins, their tissue metabolite levels, and the potential to inhibit colon cancer metastasis and invasion.

Abbreviations used: RTK, receptor tyrosine kinase; HGF, hepatocyte growth factor; SU11274, [(3*Z*)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1*H*-pyrrol-2-yl}methylene)-*N*-methyl-2-oxoindoline-5-sulfonamide]; PI3K, phosphoinositide 3-kinase; EGCG, (-)-epigallocatechin-3-gallate; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay.

Introduction

Met is a receptor tyrosine kinase (RTK), activated by the ligand hepatocyte growth factor (HGF). Met and HGF are required for embryonic development, but they can become deregulated in a variety of tumor types [89]. Met is a critical oncogene for tumor metastasis, facilitating cellular proliferation, invasion, and motility [13, 137-140]. In human colorectal cancers, Met and HGF expression predicts tumor phenotype and propensity for metastasis, and is correlated with poor outcome [10]. Thus, Met and HGF are potential therapeutic targets for colorectal cancer.

SU11274 [(3*Z*)-*N*-(3-chlorophenyl)-3-({3,5-dimethyl-4-[(4-methylpiperazin-1-yl)carbonyl]-1*H*-pyrrol-2-yl}methylene)-*N*-methyl-2-oxoindoline-5-sulfonamide] is a Met inhibitor that induces G_1 cell cycle arrest and apoptosis, interfering with phosphoinositide 3-kinase (PI3K) and other signaling pathways [18, 141]. (-)-Epigallocatechin-3-gallate (EGCG) is a tea polyphenol that blocks Met activation in breast [21], hypopharyngeal [25] and colon cancer cells [22, 23]. EGCG also reportedly inhibits signaling via epidermal growth factor, platelet-derived growth factor, insulin-like growth factor 1, and vascular endothelial growth factor receptors [31, 100, 123, 142]. EGCG inhibits the activities of cyclin-dependant kinases 2 and 4, and induces the expression of the Cdk inhibitors p21 and p27, leading to G_1 arrest [143].

Using human HCT116 colon cancer cells, we compared EGCG and SU11274 as broad-spectrum and specific Met kinase inhibitors, and

examined their downstream effects on PI3K and mitogen-activated protein kinase signaling (MAPK), cell growth, and invasion.

Experimental Section

Cell culture.

HCT116 cells were obtained from American Type Tissue Collection (Manassas, VA) and maintained in McCoy's 5A media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37°C with 5% CO_2.

Cell treatments.

Cells were plated at 1.5 x 10^5 cells in 12-well culture dishes and grown in serum containing media for 48 h. Cells were then incubated in serum-free media for 4 h. After serum starvation, cells were pretreated for 30 min with 5 μM EGCG (Sigma-Aldrich, MO) or 5 μM SU11274 (Calbiochem, San Diego, CA) followed by treatment with 30 ng/ml HGF (Calbiochem).

Immunoblotting.

Cells were placed in IP lysis buffer, vortexed, and centrifuged at 10,000 rpm for 5 min. The supernatant was collected and protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins (10-20 μg) were separated by SDS-PAGE on a 4-12% bis-Tris gel (Novex, San Diego, CA) and transferred to nitrocellulose membrane (Invitrogen). Equal protein loading was confirmed by Amido Black staining and β-actin levels. The membrane was blocked for 1 h with Li-Cor Blocking Buffer (Li-Cor Biosciences, Lincoln, Nebraska), followed by

overnight incubation with primary antibody at 4°C, and finally incubated for 1 h with goat anti-mouse secondary antibody conjugated with IRDye800 and goat anti-rabbit antibody conjugated with IRDye680 (Li-Cor Biosciences). Antibody dilutions were as follows: phospho-Met (Tyr1234/1235) 1:1000 (Cell Signaling Technology, Beverly, MA); total Met 1:1000 (Cell Signaling Technology); phospho-Akt 2 μg/ml (Upstate); total Akt 1:1000 (Cell Signaling Technology); phospho-Erk1/2 1:2000 (Cell Signaling Technology); total Erk1/2 1:1000 (Cell Signaling Technology); and β-actin 1:5000 (Sigma). Image acquisition and analysis were performed using the Odyssey® Infrared Imaging System (Li-Cor Biosciences).

Enzyme-linked immunosorbent assay (ELISA).

Cells were pretreated with EGCG or SU11274 for 30 min and then HGF (30 ng/ml) was added. Cells were incubated for an additional 15 min and lysed in RIPA buffer containing phosphatase inhibitors (Pierce). ELISA was performed as described in the instruction manual for STAR phospho-Met (Tyr1230/Tyr1234/Tyr1235) ELISA kit (Upstate). Concentration for 50% inhibition (IC_{50}) was calculated using the sigmoidal dose-response method in GraphPad Prism 4 (GraphPad Software, La Jolla, CA).

Cell viability.

Cells were incubated with 5 μM EGCG or 5 μM SU11274 for 30 min, and HGF (30 ng/ml) was then added to the culture media. Cells were

collected at 0, 24, 48 and 72 h and stained with Guava ViaCount Reagent (Guava Technologies, Hayward, CA) for 5 min. The number of viable cells was determined by using the Guava ViaCount Assay on a Guava Personal Cell Analyzer.

Cell invasion assay.

Matrigel (BD Transduction Laboratories) was diluted to 2 mg/ml in serum-free McCoy's 5A media, and 50 μl were plated on 6.5 mm diameter Costar Transwell Inserts (VWR) and allowed to gel for 1 h at 37°C. Cells were diluted to 2 x 10^4 cells/ml in serum-free media with or without 5 μM EGCG or 5 μM SU11274 and plated in the top of the wells. Serum-free McCoy's 5A with or without 30 ng/ml HGF was plated in the bottom of the wells. Cells were incubated for 24 h at 37°C in a 5% CO_2 incubator. Matrigel and cells remaining on the top surface were removed with a cotton swab. Cells that migrated to the bottom of the insert were fixed in methanol and stained with ProLong Gold DAPI (Invitrogen) and counted using a Zeiss Axiovert 100 fluorescent microscope.

Statistics.

Student's *t*-test was used for pairwise comparisons. Additional analyses used a mixed model procedure with SPSS Statistics software (IBM Corp., Armonk, NY). *P*-values shown in the figures indicate significant differences from multiple comparisons, with Bonferroni correction.

Results

SU11274 and EGCG (Fig. 1A) have both been characterized as Met inhibitors [18], and we performed a side-by-side comparison of their abilities to block Met activation in HCT116 colon cancer cells. Immunoblotting revealed little or no p-Met in the absence of HGF treatment and high levels of p-Met after HGF, with total Met expression remaining constant (Fig. 1B, left lanes). Both test compounds reduced the levels of p-Met in a concentration-dependent manner over the concentration range 0.05-10 μM (Fig. 1B). Quantification of p-Met in the corresponding cell lysates revealed SU11274 to be a more potent Met inhibitor than EGCG, with IC_{50} values of 0.05 μM and 3 μM, respectively (Fig 1C).

We next examined the downstream signaling pathways of Met kinase in time-course experiments. In the absence of EGCG, HGF induced a rapid increase in the phosphorylation of Met, and high p-Met levels were detected between 0.25-2 h, followed thereafter by a return to baseline (Fig. 2A). In the presence of 5 μM ECGC, the increase in p-Met was essentially abolished. Quantification of the immunoblots confirmed that the inhibition by EGCG was significant at all time points between 0.25-6 h (Fig. 2B, upper panel, p-Met normalized to total Met). In the absence of EGCG, HGF increased the levels of p-Akt at 0.25-2 h and p-Erk1/2 at 0.25-1 h (Fig. 2A). There was a trend towards inhibition following EGCG treatment, and this reached significance at

1 h for p-Akt/Akt, at 0.5, 1, and 6 h for p-Erk1/Erk1, and at 1-2 h for p-Erk2/Erk2 (Fig. 2B).

Changes in Met, Erk, and Akt pathways also were compared following EGCG, SU11274, and EGCG plus SU11274 treatment (Fig. 2C). As before, HGF alone increased the levels of p-Met, p-Erk1/2 and p-Akt, and EGCG inhibited p-Met and p-Erk1/2 expression. SU11274 (5 μM) strongly attenuated the levels of p-Met, p-Akt, p-Erk1 and p-Erk2, and there was no additional inhibition when SU11274 was combined with EGCG (compare black and hatched bars in Fig. 2D).

To determine the consequences of Met inhibition by SU11274 and EGCG, cell viability was assessed (Fig. 3A). Both inhibitors reduced cell viability relative to the corresponding control, in the presence and absence of HGF, and significant differences between EGCG and SU11274 treatment groups became apparent with time. For example, in cells treated with HGF, cell viability at 72 h was decreased to 67% by EGCG and to 79% by SU11274 (**P<0.01, compare solid green and solid blue bars, respectively, in Fig. 3A).

Compared to the -HGF control, HGF treatment increased the overall cell number at 24, 48, and 72 h (solid *vs* dashed black lines in Fig. 3D, P<0.001 for all time-points). In the presence or absence of HGF, cell growth was suppressed markedly by SU11274 (blue line *vs* corresponding black line), and it was lowered relative to 0-h controls following EGCG treatment (green lines, Fig. 3B).

A matrigel assay was used to examine cell invasion *in vitro* (Fig 4A). The presence of HGF in the lower compartment increased the invasion of HCT116 cells, as expected (compare gray bars in Fig. 4B, ***P<0.001). Following HGF treatment, EGCG and SU11274 decreased the cell invasion to 32% and 18%, respectively (**P<0.01 for both treatment groups *vs* control), but under these conditions there was no significant difference between EGCG and SU11274 groups (compare green *vs* black bars in Fig. 4B). In the absence of HGF, however, SU11274 was more effective at reducing cell invasion compared to EGCG (*P<0.05, black *vs* green bars).

Discussion

The Met receptor tyrosine kinase is considered an important prognostic factor for metastasis, tumor stage, and reduced survival [10]. We compared the effects of two Met inhibitors in HCT116 human colon cancer cells. SU11274 is a specific Met inhibitor [18], whereas EGCG most likely acts on multiple RTKs [31, 100, 123, 142]. The results were consistent with a potent selective Met inhibitor being more effective than a widely consumed tea catechin in suppressing Met activation. SU11274 had a lower IC_{50} value than EGCG in reducing p-Met levels in HCT116 cells, and SU11274 was more effective than EGCG at inhibiting Akt and Erk pathways. It has been documented that Met activation results in increased MAPK and PI3K signaling, and these pathways have been strongly implicated in HGF-induced cellular invasion [21].

Interestingly, 5 μM EGCG had a greater inhibitory effect on cell viability than the same concentration of SU11274, in both HGF-treated and HGF-untreated cells. EGCG also was more effective than SU11274 at decreasing proliferation of HCT116 cells over a 72-h period. However, in a matrigel invasion assay minus HGF treatment, SU11274 was slightly more effective than EGCG at decreasing the number of invading cells, whereas in the presence of HGF both compounds were equally effective at suppressing invasion. Our interpretation of these findings is that SU11274 acts more effectively on the specific kinase pathways studied, but additional

chemopreventive mechanisms of EGCG [123, 142, 143] likely result in greater overall inhibition of cell viability and proliferation. Both test compounds nonetheless strongly suppressed HGF-mediated invasion *in vitro*.

In this investigation, EGCG had an IC_{50} value of 3 μM for inhibition of Met activation, which is noteworthy given that a peak plasma concentration of 7.5 μM EGCG has been detected in humans after pharmacological oral dosing [102]. These concentrations of EGCG and other tea catechins also might be feasible in the gastrointestinal tract following oral tea intake, despite extensive methylation, glucuronidation, and sulfation, or conversion to valerolactone breakdown products [136, 144]. It remains to be determined whether specific catechin metabolites and breakdown products have improved efficacy towards suppression of Met signaling, cell proliferation, and invasion, and whether this is recapitulated *in vivo*.

Acknowledgments

We thank Dr. Donald Jump of the Department of Nutrition and Exercise Sciences for access to the Li-Cor instrument, Dr. Jeffrey Greenwood and Dr. Hyo Sang Jang of the Cell Imaging & Analysis Core of the Environmental Health Sciences Center for assistance with invasion assays, Dr. Praveen Rajendran for assistance with the calculation of IC_{50} values for EGCG and SU11274 with the Met receptor and Hui Nian for performing statistical analysis of cell proliferation data. This work was supported in part by NIEHS Center grant P30 ES00210 and by Program Project P01 CA090890 from the National Cancer Institute.

Figure Legends

Figure 1: EGCG and SU11274 inhibit Met activation in human colon cancer cells. (A) Chemical structures of SU11274 and EGCG. (B) HCT116 cells were serum-starved for 4 h and then treated with various concentrations of EGCG or SU11274 for 30 min. HGF (30 ng/ml) was added to the cell culture media, followed by incubation for an additional 15 min. Immunoblotting was performed on cell lysates using primary antibodies to phosphorylated-Met (p-Met), total Met, or β-actin (loading control). (C) Results from STAR phospho-Met ELISA kit (Upstate), using cell lysates from (B). Data (mean±SD, n=3) are representative of the findings from three separate experiments.

Figure 2: Inhibitory effects of EGCG on MAPK and PI3K signaling pathways. (A) HCT116 cells were serum-starved for 4 h and treated with or without 5 μM EGCG for 30 min. HGF (30 ng/ml) was added and incubations were continued for the times indicated. Immunoblotting was performed on cell lysates using primary antibodies to phosphorylated-Met (p-Met), total Met, phosphorylated-Akt (p-Akt), total Akt, phosphorylated-Erk (p-Erk), total Erk, or β-actin (loading control). (B) Quantification of immunoblots by densitometry, showing phosphorylated protein normalized to corresponding total protein. Open bars -EGCG, solid bars +EGCG. (C) HCT116 cells were serum-starved for 4 h and then treated with or without 5 μM SU11274 and 5 μM EGCG for 30 min. HGF (30 ng/ml) was added to the culture media and incubated for 1 h. (D) Quantification (relative densitometry) of immunoblot data from three

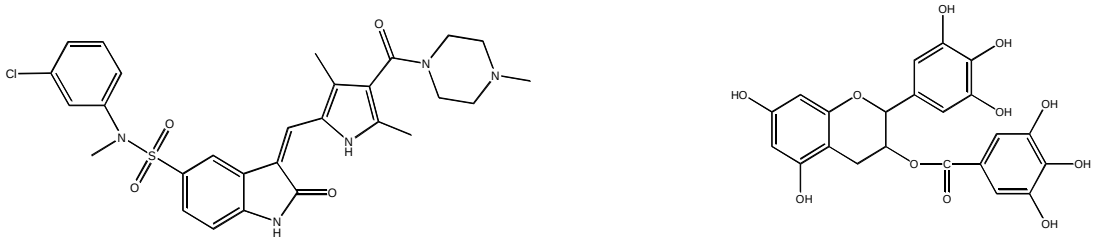
separate experiments. In (B) and (D), data=mean±SD, n=3; *P<0.05; **P<0.01; ***P<0.001; n/s, not significant.

Figure 3: Inhibition of cell viability and proliferation by EGCG and SU11274. (A) Viability of HCT116 cells treated with EGCG, SU11274, or vehicle control in the presence (solid bars) and absence of HGF (hatched bars). (B) Proliferation of HCT116 cells treated with EGCG, SU11274, or vehicle, in the presence and absence of HGF. Data shown in (A) and (B) are representative findings from three separate experiments (mean±SD, n=3).

Figure 4: Suppression of invasion by ECGC and SU11274. (A) Representative images showing invasion of HCT116 cells through matrigel-coated Transwell inserts, under the assays conditions indicated. (B) Quantification of three independent *in vitro* invasion assays (mean±SD, n=3). *P<0.05; **P<0.01; n/s = not significant.

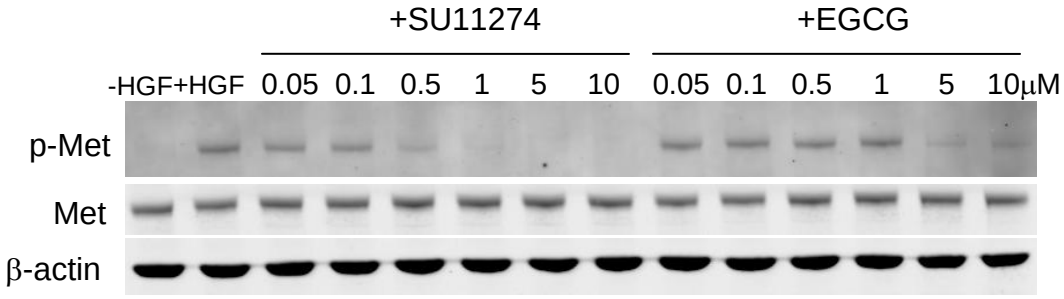
Figure 1.

A



SU11274 EGCG

B



C

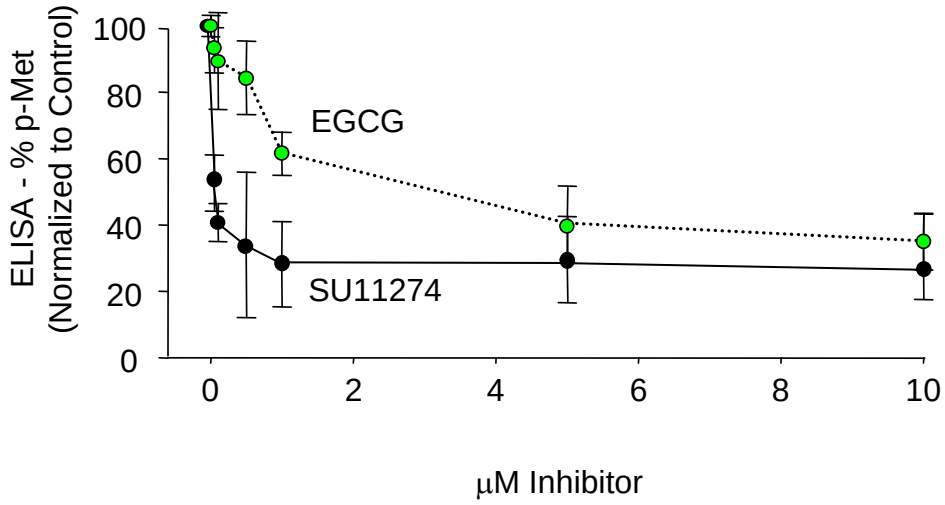
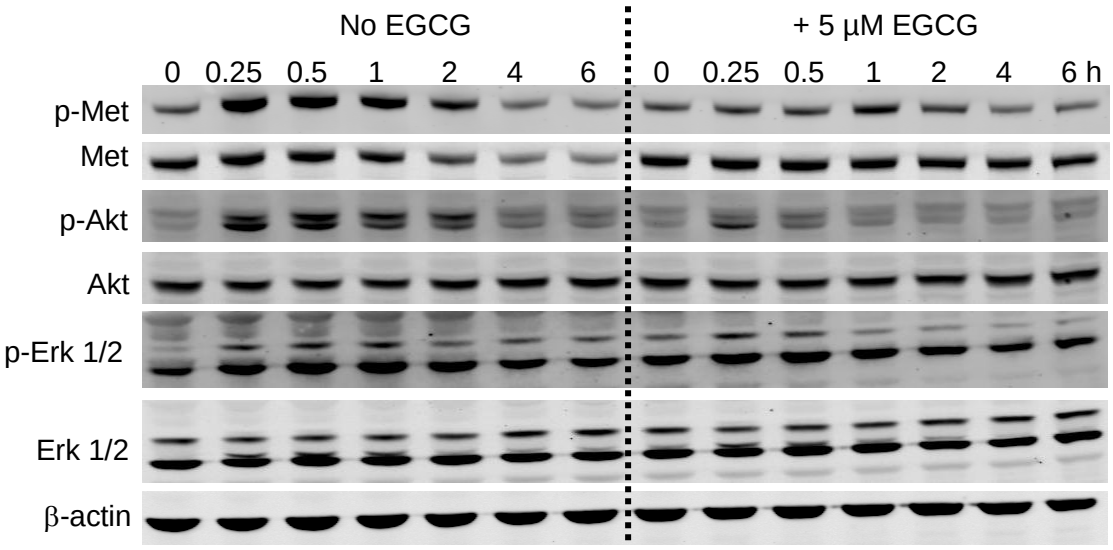
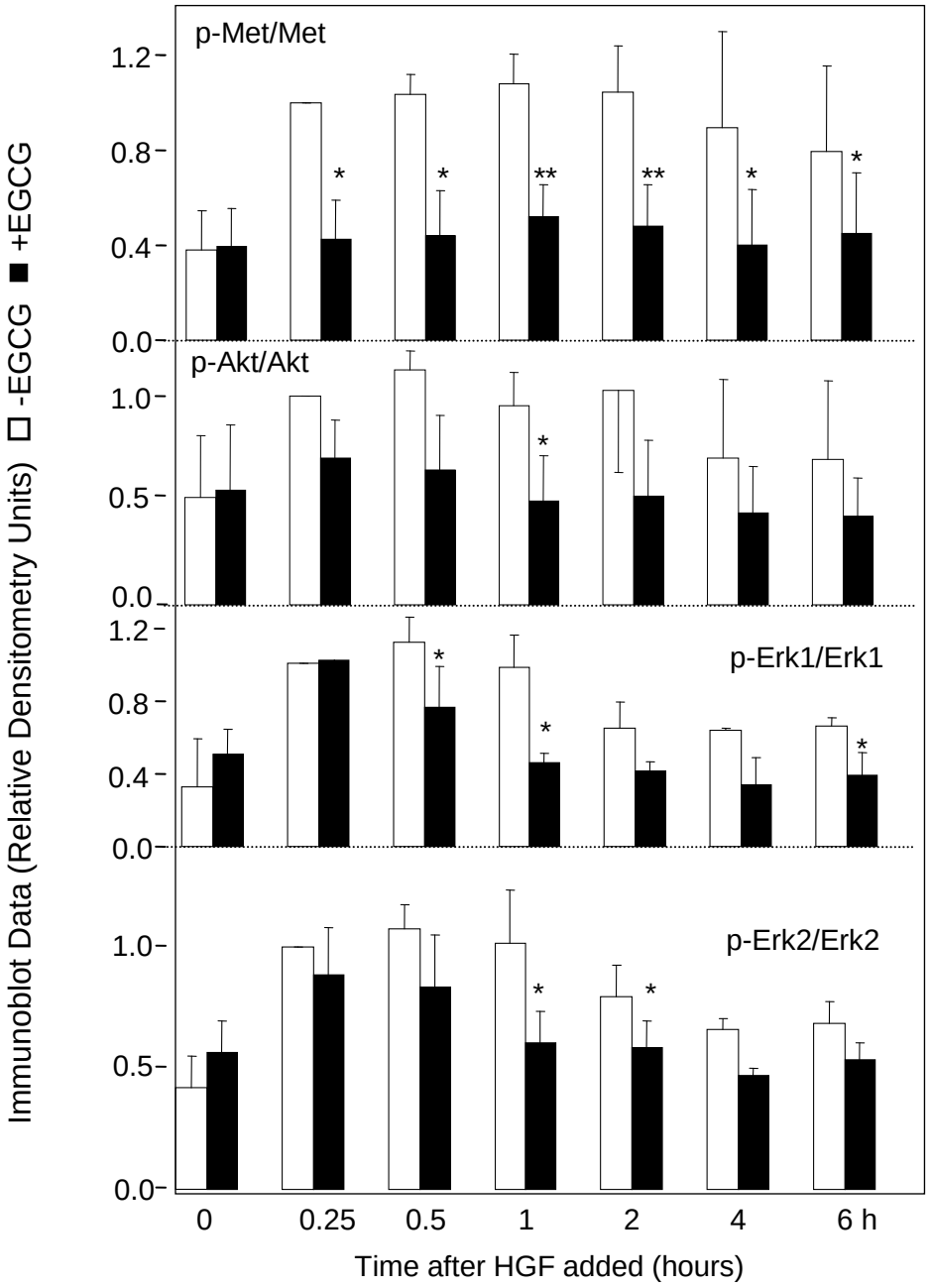


Figure 2.

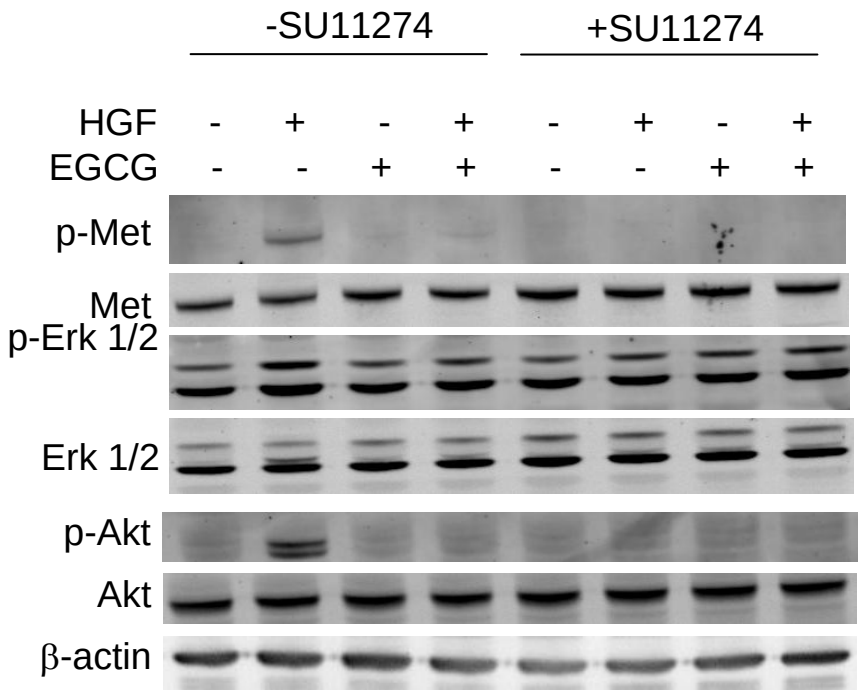
A


No EGCG
+ 5 µM EGCG
0 0.25 0.5 1 2 4 6 0 0.25 0.5 1 2 4 6 h
p-Met
Met
p-Akt
Akt
p-Erk 1/2
Erk 1/2
β-actin

B


p-Met/Met
p-Akt/Akt
p-Erk1/Erk1
p-Erk2/Erk2
Immunoblot Data (Relative Densitometry Units) □ -EGCG ■ +EGCG
Time after HGF added (hours)
0
0.25
0.5
1
2
4
6 h

C


-SU11274
+SU11274
HGF - + - + - + - +
EGCG - - + + - - + +
p-Met
Met
p-Erk 1/2
Erk 1/2
p-Akt
Akt
β-actin

D

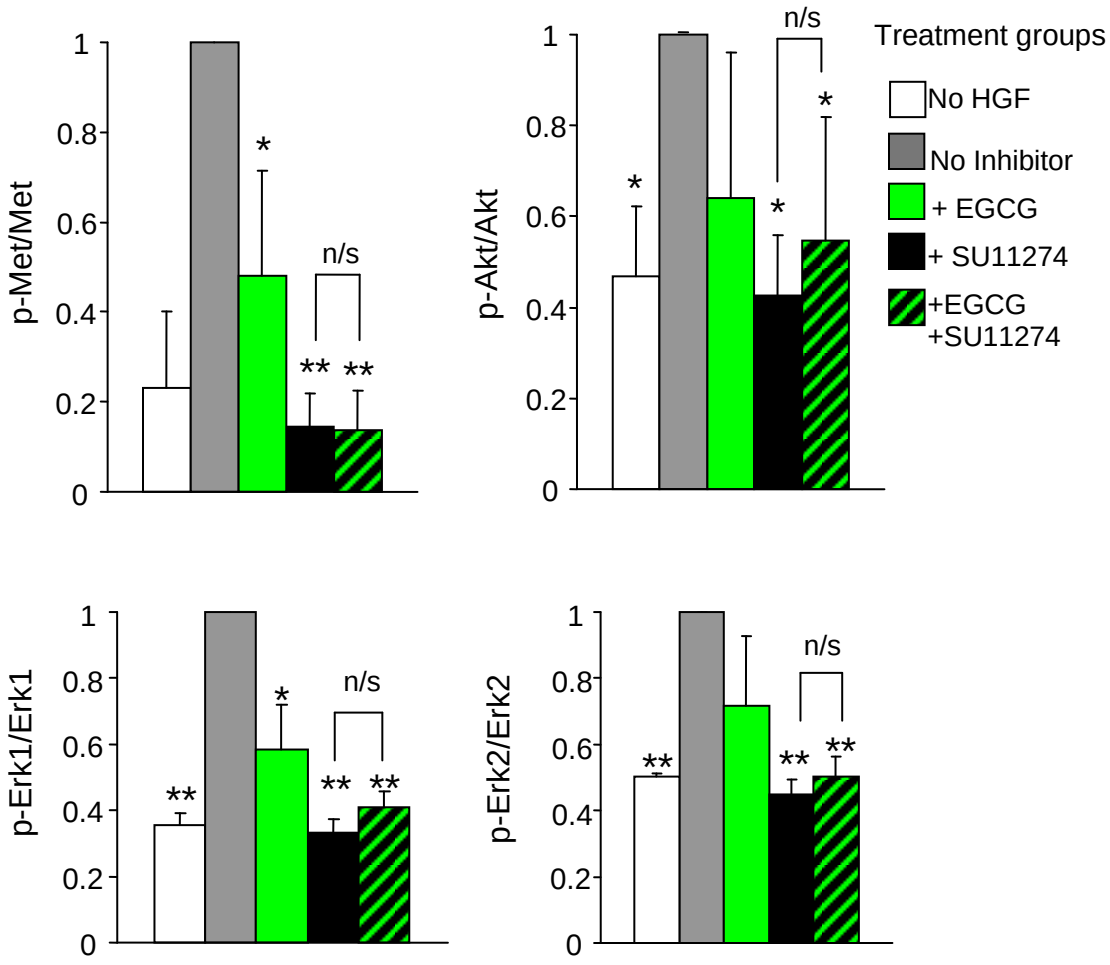

p-Met/Met
p-Akt/Akt
p-Erk1/Erk1
p-Erk2/Erk2
n/s
Treatment groups
No HGF
No Inhibitor
+ EGCG
+ SU11274
+EGCG
+SU11274

Figure 3.

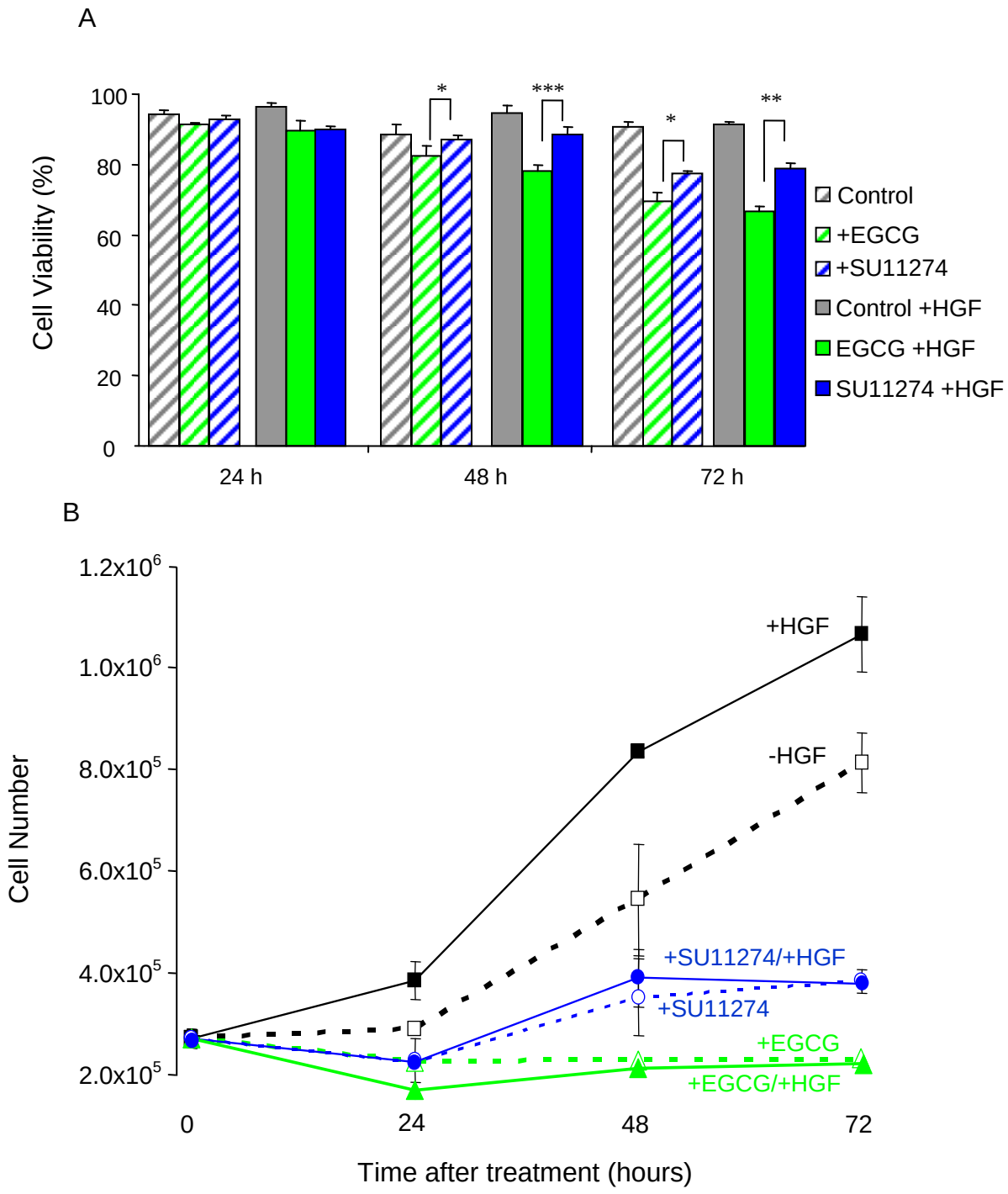
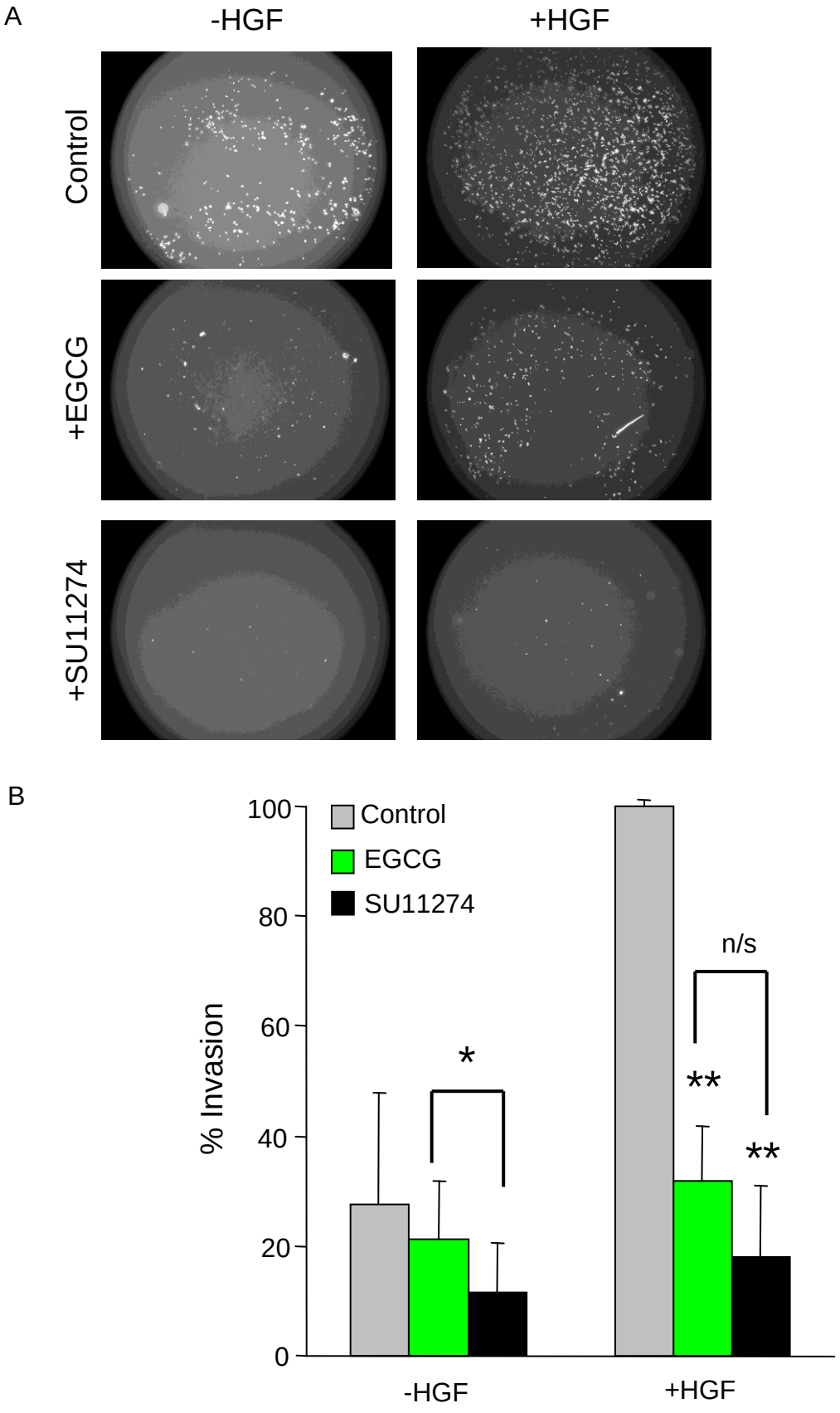

A
Cell Viability (%)
100
80
60
40
20
0
*
***
*
**
24 h
48 h
72 h
Control
+EGCG
+SU11274
Control +HGF
EGCG +HGF
SU11274 +HGF
B
Cell Number
1.2x10^6
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
+HGF
-HGF
+SU11274/+HGF
+SU11274
+EGCG
+EGCG/+HGF
0
24
48
72
Time after treatment (hours)

Figure 4.


A
-HGF
+HGF
Control
+EGCG
+SU11274
B
100
80
60
40
20
0
% Invasion
Control
EGCG
SU11274
*
**
n/s
**
-HGF
+HGF

Chapter 6: General Conclusion

Discussion, future studies and implications of work presented in this dissertation

Discussion

Met is dysregulated in many cancers and plays an important role in tumor progression [145]. Several studies have shown an association between Met overexpression and reduced survival time in a variety of cancers including breast [146, 147], renal [148] and esophageal [149]. Of particular interest, Met has been shown to be a prognostic indicator in colon cancer [10, 150]. In colon cancer patients, Met is associated with advanced stage and metastasis [9, 114].

Colon cancer is among the most common cancers in the United States. It has a high propensity to metastasize and a majority of deaths due to colon cancer are due to the metastatic disease. Thus strategies that target the metastatic process are expected to be an effective treatment approach that would lead to prolonged life-span of patients afflicted with this disease.

Previous studies have shown that tea catechins have the ability to suppress RTK activity. In particular, EGCG has been shown to suppress the activation of Met in breast [21] and hypopharyngeal [25] carcinoma cells. In this dissertation, the ability of EGCG to suppress Met signaling in colon cancer cells was investigated. In particular, the mechanism by which EGCG exerts its effects on Met signaling was studied.

In both HCT116 and HT-29 human colon cancer cells, EGCG suppressed Met activation at concentrations as low as 0.5 μM. Since it is well known that EGCG generates H_2O_2 in cell culture media [92, 121] and that

H_2O_2 can suppress Met signaling [14] H_2O_2 generation by EGCG was studied as a possible mechanism of action.

First, the amount of H_2O_2 generated by EGCG was measured. It was found that relatively low amounts of H_2O_2 was generated (< 1.5 μM) at the highest concentration of 10 μM EGCG. At these concentrations, H_2O_2 alone did not have an effect on receptor activation; however, at higher concentrations (> 2.5 μM) the receptor was activated rather than suppressed. To further differentiate the effects of H_2O_2 and EGCG on Met suppression, catalase was co-incubated with EGCG. Catalase is an enzyme that catalyzes the decomposition of H_2O_2 to water and oxygen. In the presence and absence of catalase, EGCG had an identical inhibitory effect on Met receptor activation. Taken together, these data ruled out the possibility of H_2O_2 generation as a mechanism for the suppression of Met activation by EGCG.

An *in vitro* kinase assay was used to look for the possible interaction between EGCG and the Met receptor. Both the Cornish-Bowden and Dixon plots produced results that are characteristic of competitive enzyme inhibition. The results of the kinetic analyses were confirmed by computer modeling. EGCG was found to dock in the kinase active site and had similar interactions with target amino acids as a known Met inhibitor was shown to have.

Interestingly, the non-gallated tea catechins, EC and EGC showed little or no inhibition of Met in both cell culture and *in vitro* kinase assays. In

molecular modeling, these compounds showed little interaction with the kinase domain of Met.

A result of Met kinase inhibition by EGCG was the reduction in downstream signaling of MAPK and PI3K pathways. EGCG is known to affect these pathways irrespective of RTK inhibition. To test whether the inhibition of these pathways was due to the inhibition of Met, the selective Met inhibitor SU11274 was used. When Met activation was blocked by SU11274, no effect of EGCG was seen for Erk and Akt kinases, suggesting that in the conditions used here, the inhibition of downstream MAPK and PI3K pathways by EGCG is mediated by the suppression of Met.

Both growth and invasive potential of HCT116 cells were inhibited by 5 μM EGCG. It is important to note that this is within the physiologically relevant concentration of EGCG. When compared to the selective Met inhibitor, SU11274 had a lower IC_{50} value. EGCG was more effective in attenuating the growth of HCT116 cells. And both compounds effectively inhibited invasion in a matrigel assay.

Future Studies

Based on the results presented in this dissertation, it will be important to confirm the proposed mechanism of competitive reversible binding of EGCG to the Met receptor. One way to do this would be to couple sepharose beads to EGCG, as described in He et al. [151]. An *in vitro* protein-binding assay could then be performed using the EGCG-sepharose beads and purified Met kinase. One could include mutant forms of Met to identify key amino acids important for EGCG-receptor binding. Proteins (wt-Met or mutant Met) that bind to the EGCG-sepharose beads could be analyzed by immunoblotting with anti-Met. It would be expected that EGCG-sepharose beads would bind wt-Met and that changes in amino acids identified here as important in EGCG binding in the kinase domain, namely P1158, M1160 and R1208, would show decreased or no binding to EGCG-sepharose beads. Perhaps more convincing would be the co-crystallization of Met with EGCG. It would also be of interest to perform parallel studies with known metabolites of EGCG, such as the –*O*-methylated intermediates, and possibly the ring open forms that occur in the gastrointestinal tract (valerolactones).

Further investigation using an animal model of colon cancer metastasis is warranted. One such model uses C57BL/6 mice subcutaneously implanted with MC-38 murine colon cancer cells. These cells express the Met receptor and metastasize to the liver when implanted in this mouse strain. Using this

preclinical model, the ability of EGCG or its metabolites to prevent liver metastasis of colon cancer cells could be tested.

A human trial could be performed using colon cancer patients undergoing surgical resection of colon tumors. In this study, patients could be given an oral dose of EGCG prior to surgery. The phosphorylation of Met in the tumors could be measured by Western blot analysis. This study would test the ability of EGCG to suppress Met signaling in human colon tumor samples.

Implications

This work is of importance because 5-year survival of patients diagnosed with colorectal cancer decreases to less than 10 percent for the metastatic disease [105]. By disrupting signaling pathways in cancer cells, in particular Met and perhaps other RTKs, which are involved in the metastatic process, one would expect less potential for metastasis to occur and therefore enhance patient survival. EGCG could thereby represent a therapeutic for use in patients with colon cancer. The colon would be an ideal organ to target because in these studies, concentrations of 5 μM EGCG have been demonstrated to disrupt Met signaling and inhibit cancer cell growth and invasion in a cell culture model. This gives further impetus to test EGCG in *in vivo* models of colon cancer metastasis It is important to note that concentrations as low as 7.5 μM EGCG has been detected in humans after oral pharmacological dosing [102]. Furthermore, despite extensive metabolism, by methylation, glucuronidation, sulfation or conversion to valerolactone breakdown products, it is expected that the concentrations of EGCG used in these studies would be achieved in the gastrointestinal tract [102]. However, the ability of EGCG to improve colon cancer patient survival via EGCG-mediated Met suppression remains to be tested.

Bibliography

[1] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57-70.

[2] B. Vogelstein, K.W. Kinzler, Cancer genes and the pathways they control, Nat Med 10 (2004) 789-799.

[3] P.A. Jones, S.B. Baylin, The fundamental role of epigenetic events in cancer, Nat Rev Genet 3 (2002) 415-428.

[4] C. Lengauer, K.W. Kinzler, B. Vogelstein, Genetic instabilities in human cancers, Nature 396 (1998) 643-649.

[5] S. Benvenuti, P.M. Comoglio, The MET receptor tyrosine kinase in invasion and metastasis, J Cell Physiol 213 (2007) 316-325.

[6] S. Rong, S. Segal, M. Anver, J.H. Resau, G.F. Vande Woude, Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation, Proc Natl Acad Sci U S A 91 (1994) 4731-4735.

[7] H. Takayama, W.J. LaRochelle, R. Sharp, T. Otsuka, P. Kriebel, M. Anver, S.A. Aaronson, G. Merlino, Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor, Proc Natl Acad Sci U S A 94 (1997) 701-706.

[8] R. Taulli, C. Scuoppo, F. Bersani, P. Accornero, P.E. Forni, S. Miretti, A. Grinza, P. Allegra, M. Schmitt-Ney, T. Crepaldi, C. Ponzetto, Validation of met as a therapeutic target in alveolar and embryonal rhabdomyosarcoma, Cancer Res 66 (2006) 4742-4749.

[9] Z.S. Zeng, M.R. Weiser, E. Kuntz, C.T. Chen, S.A. Khan, A. Forslund, G.M. Nash, M. Gimbel, Y. Yamaguchi, A.T.t. Culliford, M. D'Alessio, F. Barany, P.B. Paty, c-Met gene amplification is associated with advanced stage colorectal cancer and liver metastases, Cancer Lett 265 (2008) 258-269.

[10] U.S. Kammula, E.J. Kuntz, T.D. Francone, Z. Zeng, J. Shia, R.G. Landmann, P.B. Paty, M.R. Weiser, Molecular co-expression of the c-Met oncogene and hepatocyte growth factor in primary colon cancer predicts tumor stage and clinical outcome, Cancer Lett 248 (2007) 219-228.

[11] N.A. Lokker, M.R. Mark, E.A. Luis, G.L. Bennett, K.A. Robbins, J.B. Baker, P.J. Godowski, Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding, Embo J 11 (1992) 2503-2510.

[12] E. Gherardi, M.E. Youles, R.N. Miguel, T.L. Blundell, L. Iamele, J. Gough, A. Bandyopadhyay, G. Hartmann, P.J. Butler, Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor, Proc Natl Acad Sci U S A 100 (2003) 12039-12044.

[13] C. Birchmeier, W. Birchmeier, E. Gherardi, G.F. Vande Woude, Met, metastasis, motility and more, Nat Rev Mol Cell Biol 4 (2003) 915-925.

[14] A. Hashigasako, M. Machide, T. Nakamura, K. Matsumoto, T. Nakamura, Bi-directional regulation of Ser-985 phosphorylation of c-met via protein kinase C and protein phosphatase 2A involves c-Met activation and cellular responsiveness to hepatocyte growth factor, J Biol Chem 279 (2004) 26445-26452.

[15] K. Matsumoto, T. Nakamura, NK4 (HGF-antagonist/angiogenesis inhibitor) in cancer biology and therapeutics, Cancer Sci 94 (2003) 321-327.

[16] T. Burgess, A. Coxon, S. Meyer, J. Sun, K. Rex, T. Tsuruda, Q. Chen, S.Y. Ho, L. Li, S. Kaufman, K. McDorman, R.C. Cattley, J. Sun, G. Elliott, K. Zhang, X. Feng, X.C. Jia, L. Green, R. Radinsky, R. Kendall, Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors, Cancer Res 66 (2006) 1721-1729.

[17] A. Morotti, S. Mila, P. Accornero, E. Tagliabue, C. Ponzetto, K252a inhibits the oncogenic properties of Met, the HGF receptor, Oncogene 21 (2002) 4885-4893.

[18] S. Berthou, D.M. Aebersold, L.S. Schmidt, D. Stroka, C. Heigl, B. Streit, D. Stalder, G. Gruber, C. Liang, A.R. Howlett, D. Candinas, R.H. Greiner, K.E. Lipson, Y. Zimmer, The Met kinase inhibitor SU11274 exhibits a selective inhibition pattern toward different receptor mutated variants, Oncogene 23 (2004) 5387-5393.

[19] N. Puri, A. Khramtsov, S. Ahmed, V. Nallasura, J.T. Hetzel, R. Jagadeeswaran, G. Karczmar, R. Salgia, A selective small molecule inhibitor of c-Met, PHA665752, inhibits tumorigenicity and angiogenesis in mouse lung cancer xenografts, Cancer Res 67 (2007) 3529-3534.

[20] H.Y. Zou, Q. Li, J.H. Lee, M.E. Arango, S.R. McDonnell, S. Yamazaki, T.B. Koudriakova, G. Alton, J.J. Cui, P.P. Kung, M.D. Nambu, G. Los, S.L. Bender, B. Mroczkowski, J.G. Christensen, An orally available small-molecule inhibitor of c-Met, PF-2341066, exhibits cytoreductive antitumor efficacy through antiproliferative and antiangiogenic mechanisms, Cancer Res 67 (2007) 4408-4417.

[21] R.L. Bigelow, J.A. Cardelli, The green tea catechins, (-)-Epigallocatechin-3-gallate (EGCG) and (-)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells, Oncogene 25 (2006) 1922-1930.

[22] C.A. Larsen, W.H. Bisson, R.H. Dashwood, Tea catechins inhibit hepatocyte growth factor receptor (MET kinase) activity in human colon cancer cells: kinetic and molecular docking studies, J Med Chem 52 (2009) 6543-6545.

[23] C.A. Larsen, R.H. Dashwood, Suppression of Met activation in human colon cancer cells treated with (-)-epigallocatechin-3-gallate: minor role of hydrogen peroxide, Biochem Biophys Res Commun 389 (2009) 527-530.

[24] S.A. Milligan, P. Burke, D.T. Coleman, R.L. Bigelow, J.J. Steffan, J.L. Carroll, B.J. Williams, J.A. Cardelli, The green tea polyphenol EGCG potentiates the antiproliferative activity of c-Met and epidermal growth factor receptor inhibitors in non-small cell lung cancer cells, Clin Cancer Res 15 (2009) 4885-4894.

[25] Y.C. Lim, H.Y. Park, H.S. Hwang, S.U. Kang, J.H. Pyun, M.H. Lee, E.C. Choi, C.H. Kim, (-)-Epigallocatechin-3-gallate (EGCG) inhibits HGF-induced invasion and metastasis in hypopharyngeal carcinoma cells, Cancer Lett 271 (2008) 140-152.

[26] C.S. Yang, X. Wang, G. Lu, S.C. Picinich, Cancer prevention by tea: animal studies, molecular mechanisms and human relevance, Nat Rev Cancer 9 (2009) 429-439.

[27] H.N. Graham, Green tea composition, consumption, and polyphenol chemistry, Prev Med 21 (1992) 334-350.

[28] J.V. Higdon, B. Frei, Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions, Crit Rev Food Sci Nutr 43 (2003) 89-143.

[29] H. Mukhtar, N. Ahmad, Tea polyphenols: prevention of cancer and optimizing health, Am J Clin Nutr 71 (2000) 1698S-1702S; discussion 1703S-1694S.

[30] J. Ju, G. Lu, J.D. Lambert, C.S. Yang, Inhibition of carcinogenesis by tea constituents, Semin Cancer Biol 17 (2007) 395-402.

[31] N. Khan, F. Afaq, M. Saleem, N. Ahmad, H. Mukhtar, Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate, Cancer Res 66 (2006) 2500-2505.

[32] T. Kohri, F. Nanjo, M. Suzuki, R. Seto, N. Matsumoto, M. Yamakawa, H. Hojo, Y. Hara, D. Desai, S. Amin, C.C. Conaway, F.L. Chung, Synthesis of (-)-[4-3H]epigallocatechin gallate and its metabolic fate in rats after intravenous administration, J Agric Food Chem 49 (2001) 1042-1048.

[33] J.D. Lambert, M.J. Lee, H. Lu, X. Meng, J.J. Hong, D.N. Seril, M.G. Sturgill, C.S. Yang, Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice, J Nutr 133 (2003) 4172-4177.

[34] C. Li, X. Meng, B. Winnik, M.J. Lee, H. Lu, S. Sheng, B. Buckley, C.S. Yang, Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry, Chem Res Toxicol 14 (2001) 702-707.

[35] J.D. Lambert, S. Sang, C.S. Yang, Biotransformation of green tea polyphenols and the biological activities of those metabolites, Mol Pharm 4 (2007) 819-825.

[36] S. Kim, M.J. Lee, J. Hong, C. Li, T.J. Smith, G.Y. Yang, D.N. Seril, C.S. Yang, Plasma and tissue levels of tea catechins in rats and mice during chronic consumption of green tea polyphenols, Nutr Cancer 37 (2000) 41-48.

[37] L. Chen, M.J. Lee, H. Li, C.S. Yang, Absorption, distribution, elimination of tea polyphenols in rats, Drug Metab Dispos 25 (1997) 1045-1050.

[38] M. Natsume, N. Osakabe, M. Oyama, M. Sasaki, S. Baba, Y. Nakamura, T. Osawa, J. Terao, Structures of (-)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (-)-epicatechin: differences between human and rat, Free Radic Biol Med 34 (2003) 840-849.

[39] J.M. Van Amelsvoort, K.H. Van Hof, J.N. Mathot, T.P. Mulder, A. Wiersma, L.B. Tijburg, Plasma concentrations of individual tea catechins after a single oral dose in humans, Xenobiotica 31 (2001) 891-901.

[40] U. Ullmann, J. Haller, J.P. Decourt, N. Girault, J. Girault, A.S. Richard-Caudron, B. Pineau, P. Weber, A single ascending dose study of epigallocatechin gallate in healthy volunteers, J Int Med Res 31 (2003) 88-101.

[41] Z. Hou, J.D. Lambert, K.V. Chin, C.S. Yang, Effects of tea polyphenols on signal transduction pathways related to cancer chemoprevention, Mutat Res 555 (2004) 3-19.

[42] J.D. Lambert, J. Hong, G.Y. Yang, J. Liao, C.S. Yang, Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations, Am J Clin Nutr 81 (2005) 284S-291S.

[43] C.S. Yang, J.D. Lambert, Z. Hou, J. Ju, G. Lu, X. Hao, Molecular targets for the cancer preventive activity of tea polyphenols, Mol Carcinog 45 (2006) 431-435.

[44] C.S. Yang, S. Sang, J.D. Lambert, Z. Hou, J. Ju, G. Lu, Possible mechanisms of the cancer-preventive activities of green tea, Mol Nutr Food Res 50 (2006) 170-175.

[45] M.D. Brown, Green tea (Camellia sinensis) extract and its possible role in the prevention of cancer, Altern Med Rev 4 (1999) 360-370.

[46] S.K. Katiyar, H. Mukhtar, Tea consumption and cancer, World Rev Nutr Diet 79 (1996) 154-184.

[47] L. Kohlmeier, K.G. Weterings, S. Steck, F.J. Kok, Tea and cancer prevention: an evaluation of the epidemiologic literature, Nutr Cancer 27 (1997) 1-13.

[48] C.S. Yang, Z.Y. Wang, Tea and cancer, J Natl Cancer Inst 85 (1993) 1038-1049.

[49] C.S. Yang, J.D. Lambert, J. Ju, G. Lu, S. Sang, Tea and cancer prevention: molecular mechanisms and human relevance, Toxicol Appl Pharmacol 224 (2007) 265-273.

[50] C.S. Yang, P. Maliakal, X. Meng, Inhibition of carcinogenesis by tea, Annu Rev Pharmacol Toxicol 42 (2002) 25-54.

[51] Y. Tsubono, Y. Nishino, S. Komatsu, C.C. Hsieh, S. Kanemura, I. Tsuji, H. Nakatsuka, A. Fukao, H. Satoh, S. Hisamichi, Green tea and the risk of gastric cancer in Japan, N Engl J Med 344 (2001) 632-636.

[52] S. Bettuzzi, M. Brausi, F. Rizzi, G. Castagnetti, G. Peracchia, A. Corti, Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: a preliminary report from a one-year proof-of-principle study, Cancer Res 66 (2006) 1234-1240.

[53] L. Chang, M. Karin, Mammalian MAP kinase signalling cascades, Nature 410 (2001) 37-40.

[54] G.L. Johnson, R. Lapadat, Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases, Science 298 (2002) 1911-1912.

[55] S.G. Kennedy, A.J. Wagner, S.D. Conzen, J. Jordan, A. Bellacosa, P.N. Tsichlis, N. Hay, The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal, Genes Dev 11 (1997) 701-713.

[56] M.H. Cardone, N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, J.C. Reed, Regulation of cell death protease caspase-9 by phosphorylation, Science 282 (1998) 1318-1321.

[57] J.A. Romashkova, S.S. Makarov, NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling, Nature 401 (1999) 86-90.

[58] M. Karin, Y. Cao, F.R. Greten, Z.W. Li, NF-kappaB in cancer: from innocent bystander to major culprit, Nat Rev Cancer 2 (2002) 301-310.

[59] A. Richmond, Nf-kappa B, chemokine gene transcription and tumour growth, Nat Rev Immunol 2 (2002) 664-674.

[60] P. Dhawan, A. Richmond, A novel NF-kappa B-inducing kinase-MAPK signaling pathway up-regulates NF-kappa B activity in melanoma cells, J Biol Chem 277 (2002) 7920-7928.

[61] C. Albanese, J. Johnson, G. Watanabe, N. Eklund, D. Vu, A. Arnold, R.G. Pestell, Transforming p21ras mutants and c-Ets-2 activate the cyclin D1 promoter through distinguishable regions, J Biol Chem 270 (1995) 23589-23597.

[62] D.C. Guttridge, C. Albanese, J.Y. Reuther, R.G. Pestell, A.S. Baldwin, Jr., NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1, Mol Cell Biol 19 (1999) 5785-5799.

[63] T. Tanabe, N. Tohnai, Cyclooxygenase isozymes and their gene structures and expression, Prostaglandins Other Lipid Mediat 68-69 (2002) 95-114.

[64] S. Shishodia, B.B. Aggarwal, Nuclear factor-kappaB: a friend or a foe in cancer?, Biochem Pharmacol 68 (2004) 1071-1080.

[65] J.I. Jones, D.R. Clemmons, Insulin-like growth factors and their binding proteins: biological actions, Endocr Rev 16 (1995) 3-34.

[66] J.P. Liu, J. Baker, A.S. Perkins, E.J. Robertson, A. Efstratiadis, Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r), Cell 75 (1993) 59-72.

[67] M.N. Pollak, E.S. Schernhammer, S.E. Hankinson, Insulin-like growth factors and neoplasia, Nat Rev Cancer 4 (2004) 505-518.

[68] G.O. Hellawell, G.D. Turner, D.R. Davies, R. Poulsom, S.F. Brewster, V.M. Macaulay, Expression of the type 1 insulin-like growth factor receptor is up-regulated in primary prostate cancer and commonly persists in metastatic disease, Cancer Res 62 (2002) 2942-2950.

[69] A. Hakam, T.J. Yeatman, L. Lu, L. Mora, G. Marcet, S.V. Nicosia, R.C. Karl, D. Coppola, Expression of insulin-like growth factor-1 receptor in human colorectal cancer, Hum Pathol 30 (1999) 1128-1133.

[70] O. Larsson, A. Girnita, L. Girnita, Role of insulin-like growth factor 1 receptor signalling in cancer, Br J Cancer 92 (2005) 2097-2101.

[71] H. Huynh, P.K. Chow, L.L. Ooi, K.C. Soo, A possible role for insulin-like growth factor-binding protein-3 autocrine/paracrine loops in controlling hepatocellular carcinoma cell proliferation, Cell Growth Differ 13 (2002) 115-122.

[72] M. Shimizu, Y. Shirakami, H. Sakai, H. Tatebe, T. Nakagawa, Y. Hara, I.B. Weinstein, H. Moriwaki, EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells, Cancer Lett 262 (2007) 10-18.

[73] M. Li, Z. He, S. Ermakova, D. Zheng, F. Tang, Y.Y. Cho, F. Zhu, W.Y. Ma, Y. Sham, E.A. Rogozin, A.M. Bode, Y. Cao, Z. Dong, Direct inhibition of insulin-like growth factor-I receptor kinase activity by (-)-epigallocatechin-3-gallate regulates cell transformation, Cancer Epidemiol Biomarkers Prev 16 (2007) 598-605.

[74] Y.C. Liang, S.Y. Lin-shiau, C.F. Chen, J.K. Lin, Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells, J Cell Biochem 67 (1997) 55-65.

[75] Y. Fu, A. Chen, The phyto-chemical (-)-epigallocatechin gallate suppresses gene expression of epidermal growth factor receptor in rat hepatic stellate cells in vitro by reducing the activity of Egr-1, Biochem Pharmacol 72 (2006) 227-238.

[76] Z. Hou, S. Sang, H. You, M.J. Lee, J. Hong, K.V. Chin, C.S. Yang, Mechanism of action of (-)-epigallocatechin-3-gallate: auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells, Cancer Res 65 (2005) 8049-8056.

[77] S. Adachi, T. Nagao, H.I. Ingolfsson, F.R. Maxfield, O.S. Andersen, L. Kopelovich, I.B. Weinstein, The inhibitory effect of (-)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells, Cancer Res 67 (2007) 6493-6501.

[78] S. Adachi, T. Nagao, S. To, A.K. Joe, M. Shimizu, R. Matsushima-Nishiwaki, O. Kozawa, H. Moriwaki, F.R. Maxfield, I.B. Weinstein, (-)-Epigallocatechin gallate causes internalization of the epidermal growth factor receptor in human colon cancer cells, Carcinogenesis 29 (2008) 1986-1993.

[79] M.J. Cross, J. Dixelius, T. Matsumoto, L. Claesson-Welsh, VEGF-receptor signal transduction, Trends Biochem Sci 28 (2003) 488-494.

[80] Y.S. Ng, D. Krilleke, D.T. Shima, VEGF function in vascular pathogenesis, Exp Cell Res 312 (2006) 527-537.

[81] Y.K. Lee, N.D. Bone, A.K. Strege, T.D. Shanafelt, D.F. Jelinek, N.E. Kay, VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia, Blood 104 (2004) 788-794.

[82] T. Kondo, T. Ohta, K. Igura, Y. Hara, K. Kaji, Tea catechins inhibit angiogenesis in vitro, measured by human endothelial cell growth, migration and tube formation, through inhibition of VEGF receptor binding, Cancer Lett 180 (2002) 139-144.

[83] Y. Shirakami, M. Shimizu, S. Adachi, H. Sakai, T. Nakagawa, Y. Yasuda, H. Tsurumi, Y. Hara, H. Moriwaki, (-)-Epigallocatechin gallate suppresses the growth of human hepatocellular carcinoma cells by inhibiting activation of the vascular endothelial growth factor-vascular endothelial growth factor receptor axis, Cancer Sci 100 (2009) 1957-1962.

[84] H.Y. Ahn, K.R. Hadizadeh, C. Seul, Y.P. Yun, H. Vetter, A. Sachinidis, Epigallocathechin-3 gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblasts and human glioblastoma cells (A172), Mol Biol Cell 10 (1999) 1093-1104.

[85] A. Sachinidis, C. Seul, S. Seewald, H. Ahn, Y. Ko, H. Vetter, Green tea compounds inhibit tyrosine phosphorylation of PDGF beta-receptor and transformation of A172 human glioblastoma, FEBS Lett 471 (2000) 51-55.

[86] A. Sachinidis, R.A. Skach, C. Seul, Y. Ko, J. Hescheler, H.Y. Ahn, J. Fingerle, Inhibition of the PDGF beta-receptor tyrosine phosphorylation and its downstream intracellular signal transduction pathway in rat and human vascular smooth muscle cells by different catechins, Faseb J 16 (2002) 893-895.

[87] Y. Suzuki, S. Hattori, M. Isemura, Epigallocatechin-3-O-gallate inhibits fibroblast contraction of floating collagen gel: interaction between epigallocatechin-3-O-gallate and platelet derived growth factor, Biosci Biotechnol Biochem 68 (2004) 1817-1820.

[88] A.A. Weber, T. Neuhaus, R.A. Skach, J. Hescheler, H.Y. Ahn, K. Schror, Y. Ko, A. Sachinidis, Mechanisms of the inhibitory effects of epigallocatechin-3 gallate on platelet-derived growth factor-BB-induced cell signaling and mitogenesis, Faseb J 18 (2004) 128-130.

[89] S. Naran, X. Zhang, S.J. Hughes, Inhibition of HGF/MET as therapy for malignancy, Expert Opin Ther Targets 13 (2009) 569-581.

[90] P.C. Ma, G. Maulik, J. Christensen, R. Salgia, c-Met: structure, functions and potential for therapeutic inhibition, Cancer Metastasis Rev 22 (2003) 309-325.

[91] J.F. Sah, S. Balasubramanian, R.L. Eckert, E.A. Rorke, Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases, J Biol Chem 279 (2004) 12755-12762.

[92] L.H. Long, M.V. Clement, B. Halliwell, Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media, Biochem Biophys Res Commun 273 (2000) 50-53.

[93] D.A. Brown, E. London, Functions of lipid rafts in biological membranes, Annu Rev Cell Dev Biol 14 (1998) 111-136.

[94] A. Prinetti, V. Chigorno, G. Tettamanti, S. Sonnino, Sphingolipid-enriched membrane domains from rat cerebellar granule cells differentiated in culture. A compositional study, J Biol Chem 275 (2000) 11658-11665.

[95] L.J. Pike, X. Han, R.W. Gross, Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study, J Biol Chem 280 (2005) 26796-26804.

[96] M. Remacle-Bonnet, F. Garrouste, G. Baillat, F. Andre, J. Marvaldi, G. Pommier, Membrane rafts segregate pro- from anti-apoptotic insulin-like growth factor-I receptor signaling in colon carcinoma cells stimulated by members of the tumor necrosis factor superfamily, Am J Pathol 167 (2005) 761-773.

[97] L.J. Pike, L. Casey, Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking, Biochemistry 41 (2002) 10315-10322.

[98] C. Puri, D. Tosoni, R. Comai, A. Rabellino, D. Segat, F. Caneva, P. Luzzi, P.P. Di Fiore, C. Tacchetti, Relationships between EGFR signaling-competent and endocytosis-competent membrane microdomains, Mol Biol Cell 16 (2005) 2704-2718.

[99] C. Massie, I.G. Mills, The developing role of receptors and adaptors, Nat Rev Cancer 6 (2006) 403-409.

[100] F. Teillet, A. Boumendjel, J. Boutonnat, X. Ronot, Flavonoids as RTK inhibitors and potential anticancer agents, Med Res Rev 28 (2008) 715-745.

[101] B.K. Albrecht, J.C. Harmange, D. Bauer, L. Berry, C. Bode, A.A. Boezio, A. Chen, D. Choquette, I. Dussault, C. Fridrich, S. Hirai, D. Hoffman, J.F. Larrow, P. Kaplan-Lefko, J. Lin, J. Lohman, A.M. Long, J. Moriguchi, A. O'Connor, M.H. Potashman, M. Reese, K. Rex, A. Siegmund, K. Shah, R. Shimanovich, S.K. Springer, Y. Teffera, Y. Yang, Y. Zhang, S.F. Bellon, Discovery and optimization of triazolopyridazines as potent and selective inhibitors of the c-Met kinase, J Med Chem 51 (2008) 2879-2882.

[102] C.S. Yang, S. Sang, J.D. Lambert, M.J. Lee, Bioavailability issues in studying the health effects of plant polyphenolic compounds, Mol Nutr Food Res 52 Suppl 1 (2008) S139-151.

[103] F. Meric-Bernstam, M.C. Hung, Advances in targeting human epidermal growth factor receptor-2 signaling for cancer therapy, Clin Cancer Res 12 (2006) 6326-6330.

[104] I.B. Weinstein, Cancer. Addiction to oncogenes--the Achilles heal of cancer, Science 297 (2002) 63-64.

[105] D.W.P. Kufe, Raphael E.; Weichselbaum, Ralph R.; Bast, Robert C., Jr.; Gansler, Ted S.; Holland, James F.; Frei III, Emil, editors., Cancer Medicine, 6 ed., BC Decker Inc., Hamilton (Canada), 2003.

[106] Y.C. Liang, Y.C. Chen, Y.L. Lin, S.Y. Lin-Shiau, C.T. Ho, J.K. Lin, Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3'-digallate, Carcinogenesis 20 (1999) 733-736.

[107] M. Masuda, M. Suzui, I.B. Weinstein, Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines, Clin Cancer Res 7 (2001) 4220-4229.

[108] S. Guo, J. Lu, A. Subramanian, G.E. Sonenshein, Microarray-assisted pathway analysis identifies mitogen-activated protein kinase signaling as a mediator of resistance to the green tea polyphenol epigallocatechin 3-gallate in her-2/neu-overexpressing breast cancer cells, Cancer Res 66 (2006) 5322-5329.

[109] S. Pianetti, S. Guo, K.T. Kavanagh, G.E. Sonenshein, Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells, Cancer Res 62 (2002) 652-655.

[110] R. Sakata, T. Ueno, T. Nakamura, M. Sakamoto, T. Torimura, M. Sata, Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90, J Hepatol 40 (2004) 52-59.

[111] J.S. Park, M.H. Kim, H.J. Chang, K.M. Kim, S.M. Kim, B.A. Shin, B.W. Ahn, Y.D. Jung, Epigallocatechin-3-gallate inhibits the PDGF-induced VEGF expression in human vascular smooth muscle cells via blocking PDGF receptor and Erk-1/2, Int J Oncol 29 (2006) 1247-1252.

[112] A. Chen, L. Zhang, The antioxidant (-)-epigallocatechin-3-gallate inhibits rat hepatic stellate cell proliferation in vitro by blocking the tyrosine phosphorylation and

reducing the gene expression of platelet-derived growth factor-beta receptor, J Biol Chem 278 (2003) 23381-23389.

[113] G.P. Gupta, J. Massague, Cancer metastasis: building a framework, Cell 127 (2006) 679-695.

[114] Z. Zeng, M.R. Weiser, M. D'Alessio, A. Grace, J. Shia, P.B. Paty, Immunoblot analysis of c-Met expression in human colorectal cancer: overexpression is associated with advanced stage cancer, Clin Exp Metastasis 21 (2004) 409-417.

[115] H. Takeuchi, A. Bilchik, S. Saha, R. Turner, D. Wiese, M. Tanaka, C. Kuo, H.J. Wang, D.S. Hoon, c-MET expression level in primary colon cancer: a predictor of tumor invasion and lymph node metastases, Clin Cancer Res 9 (2003) 1480-1488.

[116] J.M. Otte, F. Schmitz, K. Kiehne, H.U. Stechele, T. Banasiewicz, P. Krokowicz, T. Nakamura, U.R. Folsch, K. Herzig, Functional expression of HGF and its receptor in human colorectal cancer, Digestion 61 (2000) 237-246.

[117] H. Kataoka, R. Hamasuna, H. Itoh, N. Kitamura, M. Koono, Activation of hepatocyte growth factor/scatter factor in colorectal carcinoma, Cancer Res 60 (2000) 6148-6159.

[118] K. Fazekas, O. Csuka, I. Koves, E. Raso, J. Timar, Experimental and clinicopathologic studies on the function of the HGF receptor in human colon cancer metastasis, Clin Exp Metastasis 18 (2000) 639-649.

[119] G.A. Orner, W.M. Dashwood, C.A. Blum, G.D. Diaz, Q. Li, R.H. Dashwood, Suppression of tumorigenesis in the Apc(min) mouse: down-regulation of beta-catenin signaling by a combination of tea plus sulindac, Carcinogenesis 24 (2003) 263-267.

[120] X. Hao, Y. Sun, C.S. Yang, M. Bose, J.D. Lambert, J. Ju, G. Lu, M.J. Lee, S. Park, A. Husain, S. Wang, Inhibition of intestinal tumorigenesis in Apc(min/+) mice by green tea polyphenols (polyphenon E) and individual catechins, Nutr Cancer 59 (2007) 62-69.

[121] W.M. Dashwood, G.A. Orner, R.H. Dashwood, Inhibition of beta-catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H_2O_2 at physiologically relevant EGCG concentrations, Biochem Biophys Res Commun 296 (2002) 584-588.

[122] J.K. Lin, Cancer chemoprevention by tea polyphenols through modulating signal transduction pathways, Arch Pharm Res 25 (2002) 561-571.

[123] N. Khan, H. Mukhtar, Multitargeted therapy of cancer by green tea polyphenols, Cancer Lett 269 (2008) 269-280.

[124] C. Li, M.J. Lee, S. Sheng, X. Meng, S. Prabhu, B. Winnik, B. Huang, J.Y. Chung, S. Yan, C.T. Ho, C.S. Yang, Structural identification of two metabolites of catechins and their kinetics in human urine and blood after tea ingestion, Chem Res Toxicol 13 (2000) 177-184.

[125] P.C. Hollman, L.B. Tijburg, C.S. Yang, Bioavailability of flavonoids from tea, Crit Rev Food Sci Nutr 37 (1997) 719-738.

[126] P. Chiarugi, F. Buricchi, Protein tyrosine phosphorylation and reversible oxidation: two cross-talking posttranslation modifications, Antioxid Redox Signal 9 (2007) 1-24.

[127] T.M. Abagyan RA, Kuznetsov DA, ICM: a new method for protein modeling and design: applications to docking and structure prediction from the distorted native conformation., J. Comp. Chem. 15 (1994) 488-506

[128] M. Totrov, R. Abagyan, Flexible protein-ligand docking by global energy optimization in internal coordinates, Proteins Suppl 1 (1997) 215-220.

[129] M. Totrov, Protein-ligand docking as an energy optimization problem. Drug-Receptor Thermodynamics: Introduction and Experimental Application, John Wiley & Sons, New York, 2001.

[130] A. Cornish-Bowden, A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors, Biochem J 137 (1974) 143-144.

[131] M. Dixon, The determination of enzyme inhibitor constants, Biochem J 55 (1953) 170-171.

[132] R.A. Abagyan, M.M. Totrov, D.A. Kuznetsov, ICM: A new method for protein modeling and design: applications to docking and structure prediction from the distorted native conformation., J. Comp. Chem. 15 (1994) 488-506

[133] T. Cardozo, M. Totrov, R. Abagyan, Homology modeling by the ICM method, Proteins 23 (1995) 403-414.

[134] A. Donnelly, B.S. Blagg, Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket, Curr Med Chem 15 (2008) 2702-2717.

[135] M.L. Peach, N. Tan, S.J. Choyke, A. Giubellino, G. Athauda, T.R. Burke, Jr., M.C. Nicklaus, D.P. Bottaro, Directed discovery of agents targeting the Met tyrosine kinase domain by virtual screening, J Med Chem 52 (2009) 943-951.

[136] C. Auger, W. Mullen, Y. Hara, A. Crozier, Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy, J Nutr 138 (2008) 1535S-1542S.

[137] J.G. Christensen, J. Burrows, R. Salgia, c-Met as a target for human cancer and characterization of inhibitors for therapeutic intervention, Cancer Lett 225 (2005) 1-26.

[138] S. Fujita, K. Sugano, Expression of c-met proto-oncogene in primary colorectal cancer and liver metastases, Jpn J Clin Oncol 27 (1997) 378-383.

[139] M. Jeffers, S. Rong, G.F. Woude, Hepatocyte growth factor/scatter factor-Met signaling in tumorigenicity and invasion/metastasis, J Mol Med 74 (1996) 505-513.

[140] M. Jeffers, L. Schmidt, N. Nakaigawa, C.P. Webb, G. Weirich, T. Kishida, B. Zbar, G.F. Vande Woude, Activating mutations for the met tyrosine kinase receptor in human cancer, Proc Natl Acad Sci U S A 94 (1997) 11445-11450.

[141] M. Sattler, Y.B. Pride, P. Ma, J.L. Gramlich, S.C. Chu, L.A. Quinnan, S. Shirazian, C. Liang, K. Podar, J.G. Christensen, R. Salgia, A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase, Cancer Res 63 (2003) 5462-5469.

[142] M. Shimizu, I.B. Weinstein, Modulation of signal transduction by tea catechins and related phytochemicals, Mutat Res 591 (2005) 147-160.

[143] J.K. Lin, Y.C. Liang, S.Y. Lin-Shiau, Cancer chemoprevention by tea polyphenols through mitotic signal transduction blockade, Biochem Pharmacol 58 (1999) 911-915.

[144] T. Raab, D. Barron, F.A. Vera, V. Crespy, M. Oliveira, G. Williamson, Catechin Glucosides: Occurrence, Synthesis, and Stability, J Agric Food Chem.

[145] C.F. Gao, G.F. Vande Woude, HGF/SF-Met signaling in tumor progression, Cell Res 15 (2005) 49-51.

[146] R.L. Camp, E.B. Rimm, D.L. Rimm, Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma, Cancer 86 (1999) 2259-2265.

[147] I. Tolgay Ocal, M. Dolled-Filhart, T.G. D'Aquila, R.L. Camp, D.L. Rimm, Tissue microarray-based studies of patients with lymph node negative breast carcinoma show that met expression is associated with worse outcome but is not correlated with epidermal growth factor family receptors, Cancer 97 (2003) 1841-1848.

[148] Y. Miyata, H. Kanetake, S. Kanda, Presence of phosphorylated hepatocyte growth factor receptor/c-Met is associated with tumor progression and survival in patients with conventional renal cell carcinoma, Clin Cancer Res 12 (2006) 4876-4881.

[149] M.R. Anderson, R. Harrison, P.A. Atherfold, M.J. Campbell, S.J. Darnton, J. Obszynska, J.A. Jankowski, Met receptor signaling: a key effector in esophageal adenocarcinoma, Clin Cancer Res 12 (2006) 5936-5943.

[150] F. Ginty, S. Adak, A. Can, M. Gerdes, M. Larsen, H. Cline, R. Filkins, Z. Pang, Q. Li, M.C. Montalto, The relative distribution of membranous and cytoplasmic met is a prognostic indicator in stage I and II colon cancer, Clin Cancer Res 14 (2008) 3814-3822.

[151] Z. He, F. Tang, S. Ermakova, M. Li, Q. Zhao, Y.Y. Cho, W.Y. Ma, H.S. Choi, A.M. Bode, C.S. Yang, Z. Dong, Fyn is a novel target of (-)-epigallocatechin gallate in the inhibition of JB6 Cl41 cell transformation, Mol Carcinog 47 (2008) 172-183.