Proteomic studies on anticancer activity of Green Tea Catechins (specifically EGCG) are suggesting a large set of protein targets that may directly interact with EGCG and alter the physiology of diseased cells, including cancer. Of notice, benign cells are usually left untouched. Lipid rafts have been recently recognized as signal processing hubs and suggested to be involved in drug uptake by means of endocytosis. These findings are suggesting new insights on the molecular mechanisms of anticancer drugs action. In the membrane, EGCG is hijacked by the laminin receptor (LamR), a lipid raft protein. Similar to aplidin and edelfosin, EGCG alters membrane domains composition also preventing EGF binding to EGFR, dimerization of EGFR and relocation of phosphorylated EGFR to lipid rafts. In vitro studies have recently shown that EGCG also binds both DNA and RNA in GpC-rich regions. This event may importantly affect genes function. Moreover, EGCG was shown to inhibit telomerase, topoisomerase II and DNA methyltransferase 1 (DNMT1), thus ultimately affecting chromatin maintenance and remodeling. But another important alternative pathway besides interaction with specific proteins may play an important role in EGCG action: direct targeting of bioactive membrane platforms, lipid rafts. Structural alteration of the platforms deeply impact (and often inactivates) important pathways involving MAP kinases. The key issue is that, important and specific differences in lipid rafts composition have been found in transformed versus benign cells and apoptotic versus non-apoptotic cells. We suggest here that the anticancer activity of Green Tea Catechins against different kind of cancers may find an explanation in direct targeting of lipid rafts by EGCG.

**Key words:** apoptosis, cancer, catechins, chemoprevention, clusterin, EGCG, gene expression, lipid rafts, signal transduction
**INTRODUCTION**

Green tea catechins (GTC) are the flavan-3-ols of flavonoids found in green tea leaves (*Camellia sinensis*). The major four catechins in green tea leaves are (-)-epigallocatechin gallate (EGCG), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC). Among catechins, EGCG is the most abundant in green tea leaves. Catechin has antiviral, anticarcinogenic and antioxidant activity and may prevent oxidative damage in many organs, including heart, kidney, lungs and spleen. Catechins have been found effective in the treatment of viral hepatitis and studies on animals show that catechins prevent oxidative damage to blood cells as well. Epidemiological studies suggest that GTC may have cancer preventive, as well as anti-atherogenic, properties (1-4). Polyphenols are strong antioxidants (5). EGCG appears to be the most potent antioxidant among GTC. But how important is the anti-oxidant power of GTC in the anticancer action? It has been already demonstrated that GTC extracts are safe for use in humans (3-7). Earlier studies, including ours, have demonstrated that GTC, or pure EGCG, specifically inhibited cancer cell growth in laboratory models (3, 5, 6-9). In a recent pilot clinical trial we found that GTC administration to high-risk men prevented cancer progression (10, 11). We have found that EGCG specifically targets prostate cancer cells by inducing apoptosis without damaging benign control cells (9). In our studies, we have identified Clusterin (CLU), an important gene involved in programmed cell death in the prostate, as a possible mediator of GTC action. In fact, EGCG induced pro-apoptotic CLU gene expression (13) and cell death only in cancer cells (8-11).

Current proteomic investigations on anticancer activity of GTC have identified a large number of proteins interacting with EGCG. Nearly all of them have been hypothesized to possibly mediate GTC action. The versatility of EGCG to interact with so many targets and particularly with MAP kinases, phosphatases, DNA, RNA, DNA methyltransferase, topoisomerases (hereafter, EGCG interactome/proteome) makes very difficult to propose a unifying hypothesis about its action. In particular, the interactions reported question whether the inhibitory action is relatively selective for a particular serine/threonine-specific protein kinases or not. Intriguingly, the vast majority of such interactions are reported to result in inhibitory effects, with the remarkable exception of CLU induction. Thus, this matter probably needs to be re-examined. For instance, KT5720, Rottlerin and Quercetin were found to inhibit many protein kinases, sometimes even much more potently than the original hypothesis on their presumed targets. The possibility that these experimental systems were detecting unspecific action as real, and a possible
conclusions drawn from cell systems-based experiments is that these data are likely to be erroneous or misleading (14). Thus, the legitimacy of the unidentified EGCG interactome/proteome is heavily debated. Even many Authors expert in the field (8-13, 15, 16) acknowledge that target molecules of EGCG are probably grossly overestimated because of underscoring the specificity of interaction. It is very likely that the many signaling pathways putatively affected by GTC in general or specifically by EGCG may not be relevant for in vivo action.

Herein, we will discuss the putative mechanism of GTC action in the chemoprevention of cancer, mostly taking the data obtained with EGCG as paradigm. Membrane lipid rafts assemble important signaling proteins into complexes prone to be activated by molecular triggers (15-23). Rafts may also have a higher-order role as signal-processing hubs, serving as targets of feedback loops that optimize signaling amplitude and timing (15, 17, 22, 23). It has been demonstrated that the raft is used as a platform by 67-kDa laminin receptor (LamR: OMIM, 150370). LamR may systematically reshape the rafts and affect uptake of EGCG (24,25). A possible unifying theory is that interaction and binding of EGCG to membranes alters the lipid order affecting MAP kinase pathway (4, 15, 17, 19, 22, 26). In this view, the MAPK pathway orchestrated by lipid rafts in cancer cells will be inactivated by incorporation of EGCG into membrane domains followed by destabilization. This event would inhibit cell proliferation. However, there is a two-fold relevance of EGCG binding to membranes concerning its bioavailability in vivo. On one hand, intracellular availability and localization (cytoplasmic or nuclear) of EGCG will depend on how fast lipid rafts are endocytosed and metabolized; on the other hand it also depends on its steady-state release from LamR. Bioavailability of EGCG with regard to interaction with membranes is also discussed further below.

**EGCG AND TUMOUR GROWTH**

Modulation of signaling pathways by EGCG from membrane to nucleus

Among GTCs, EGCG has received convincing confirmations for chemoprevention in nearly all types of cancers studied so far (1-4, 7-13, 19, 25-31, and supplementary list of References). While searching for specificity of interaction, different experimental studies have suggested a stunning list of enzymes, growth factors and their receptors as targets for EGCG. Similar to aplidin and edelfosin (reviewed in 15, see also 17), EGCG alters membrane domains composition (19). EGCG also inhibits epidermal growth factor (EGF) binding to receptor (EGFR), dimerization and relocation of phosphorylated EGFR to lipid rafts (19). Some in vitro model studies have suggested that EGCG can bind DNA and RNA in the GpC-rich regions modulating gene expression (33-35). EGCG can inhibits chromatin remodeling and modification enzymes like DNA methyltransferases (DNMTs), telomerases and topoisomerases (29, 36-41). Table 1
shows a list of cytosolic and nuclear proteins which are important in the membrane signaling pathways and nucleic acids that have been found involved in GTC action.

Table 1. Updated list of key molecules possibly modulated by EGCG. Abbreviations: B-cell chronic lymphocytic leukaemia, BCLL; Breast cancer, BrC; Prostate cancer, CaP; Colon cancer, CC; Plasma membrane, PM; lipid rafts, LR.

<table>
<thead>
<tr>
<th>Components</th>
<th>Sub-cellular localization and site of interaction:</th>
<th>Cell type studied:</th>
<th>References:</th>
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<tr>
<td>Plasma membrane (PM)/ Lipid rafts (LR)</td>
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<td></td>
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<tr>
<td>LR</td>
<td>PM and Organelle membranes</td>
<td>All types</td>
<td>15-19, 22, 23</td>
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<td>Laminin receptor, LamR</td>
<td>PM and LR</td>
<td>HepG2, HeLa, MCF-7, A375SM</td>
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<tr>
<td>FAS</td>
<td>PM and LR</td>
<td>LNCaP, Jurkat</td>
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<tr>
<td>Signaling molecules/enzymes and their locations</td>
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<td></td>
<td></td>
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<tr>
<td>EGFR/HER2</td>
<td>PM and LR</td>
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<td>4, 15, 16, 25, 26</td>
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<tr>
<td>HGFR/Met</td>
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<td>Dihydrofolate-reductase, folate binding protein (DHFR)</td>
<td>LR</td>
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<tr>
<td>NFκB</td>
<td>PM/LR</td>
<td>CC</td>
<td>4, 5, 15, 58</td>
</tr>
<tr>
<td>Insulin like Growth factor</td>
<td>PM/LR</td>
<td></td>
<td>4, 50</td>
</tr>
<tr>
<td>VEGF-R1/R2</td>
<td>LR</td>
<td>BAEC, HMEC-1, BCLL</td>
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<td>Cell junction proteinases</td>
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<tr>
<td>MMP-9</td>
<td>LR</td>
<td>BrC, CaP</td>
<td>4, 14, 58</td>
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<tr>
<td>uPAR</td>
<td>LR</td>
<td>BrC, CaP, Glioma</td>
<td>3, 4, 14, 15,</td>
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<tr>
<td>Proteasome subunit p38</td>
<td>LR</td>
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<td>4, 14, 50</td>
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<tr>
<td>Ubiquitin-activating enzyme E1</td>
<td>LR</td>
<td></td>
<td>4, 14</td>
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<tr>
<td>Nuclear components</td>
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<tr>
<td>DNA</td>
<td>Nucleus</td>
<td>Cell-free system, BrC</td>
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<tr>
<td>RNA</td>
<td>Nucleus, cytosol</td>
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<tr>
<td>AP1-</td>
<td>Nucleus</td>
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<td>4, 50</td>
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<tr>
<td>DNMT1/2/3</td>
<td>Nucleus</td>
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<tr>
<td>Clusterin, nCLU</td>
<td>Nucleus</td>
<td>CaP, CC</td>
<td>8,9,11,12</td>
</tr>
<tr>
<td>Telomerase</td>
<td>Nucleus</td>
<td>Lung cancer, BrC</td>
<td>29, 39</td>
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Thanks to the proteomics research approach, about 250 proteins have been validated as authentic raft proteins, excluding possible contaminations by non-raft, detergent-insoluble, membrane proteins (Reviewed in refs. 15, 16). Among such a large set of true raft proteins, we will review important signaling molecules (namely EGFR, VEGFR, LamR, TRAIL and FAS) whose functions are apparently modulated by EGCG (Table 1). Our review of “EGCG interactome” will also take into consideration that, besides the specific interactions with individual molecules, EGCG broadly targets their platforms in the membrane: the lipid rafts.

LamR

Human LamR precursor protein and the p40 ribosomal protein are encoded by the same gene, \(37LRP/p40\) (OMIM, *150370). Thus human LamR is a protein that has acquired dual function through evolution, acting as both a cell surface receptor and a ribosomal protein (42). Apart from its role as a ribosomal protein, LamR function as a nonintegrin cell surface protein and it has been identified as the receptor for the extracellular matrix molecule laminin-1. At the cell surface, LamR exists as both a monomer (37 kDa) and a dimer (67 kDa). The homo- or heterodimeric state of 67-kDa LamR has yet to be resolved, but its association with the cell surface is mediated by fatty acid acylation (42). It has been found that the dimeric 67-kDa LamR resides in lipid rafts as a cell surface receptor also binds EGCG, probably mediating the anticancer activity of EGCG (24). It was previously known that expression of 67 LamR confers EGCG responsiveness to tumor cells. However, the molecular basis for the anticancer activity of EGCG in vivo was not clearly understood. A recent study using a direct genetic screen has identified eukaryotic translation elongation factor 1A (eEF1A) as a component responsible for the anticancer activity of EGCG (25). It was further shown that, through both eEF1A and 67LR, EGCG induces the dephosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) at Thr-696 and activates myosin phosphatases. Silencing of 67 LamR, eEF1A, or MYPT1 in tumor cells resulted in abrogation of EGCG-induced tumor growth inhibition in vivo. Additionally, it was demonstrated that eEF1A is up-regulated by EGCG through 67 LamR (25). The structure-activity relationship by surface plasmon resonance analysis of major GTC (and their epimers) on cell-surface binding and the inhibitory effect on histamine release suggested that binding activities of pyrogallol-type catechins (EGCG and GCG) were higher than those of catechol-type catechins (ECG and CG). Similar observations were also reported on their histamine releasing inhibitory effects. LamR is a cancer metastasis associated protein expressed in a variety of tumor cells. Using a subtraction cloning strategy involving cDNA libraries constructed from cells treated or untreated with all trans-retinoic acid (RA), it has been observed that the anticancer action of EGCG is mediated by lipid raft associated LamR which hijack EGCG from the cell surface. It is assumed that
cell-laminin interaction via the LamR is involved in kinase-phosphatase cascades, since there is an association between LamR and the integrin α6 subunit, which is part of laminin-binding integrins α6β4 and α6β1. Evidences are also accumulating in favour of the involvement of MAP kinase in the laminin signaling pathway in metastatic human melanoma cells (16, 25, 43-45).

EGFR

The epidermal growth factor (EGF) acts through its receptors EGFR (HER2). EGFR-signaling involves small GTPases of the Rho family, while trafficking involves small GTPases of the Rab family. Only Rho and Rab family members are components of lipid rafts (15). Overexpression of EGFR produces a neoplastic phenotype in tumor cells. EGCG was shown to inhibit the activation of EGF receptor and downstream signaling in several human cancer cell lines (34), but the mechanism of action is still unknown (15, 19). Several plasma membrane-associated receptor tyrosine kinases (RTKs) including EGFR are localized in detergent-insoluble ordered membrane domains (lipid rafts). It is reasonable to believe that the inhibitory effect of EGCG on activation of the EGFR may be associated with changes in membrane lipid order. Adachi et al (19) found phosphorylated (activated) EGFR only in the lipid raft fraction, whereas total cellular non-phosphorylated EGFR was present in the non-raft membrane fraction. Pretreatment with EGCG inhibited the binding of Alexa Fluor 488-labeled EGF to the cells, also inhibiting EGF-induced dimerization of the EGFR (activation). To further study the possible effects of EGCG on membrane lipid organization, cells have been labeled with the fluorescent lipid analogue 1 (1’dihexadecyl-3,3’,3’-tetramethylindocarbocyanine perchlorate) which is preferentially incorporated into lipid rafts. With this experimental approach it has been found that treatment with EGCG caused a marked reduction in the EGFR content in rafts. Polyphenon E, a standardized GTC extract (19), had a similar effect but EC, a biologically inactive compound, did not significantly alter the Triton X-100 solubility of the membrane. Furthermore, they found that EGCG but not EC caused dramatic changes in the function of the gramicidin channels in the membrane bilayer. These findings suggested that EGCG inhibition of EGF binding to EGFR and dimerization/activation are caused by alteration of membrane organization. These effects may also explain the capability of EGCG to inhibit activation of other membrane-associated RTKs. Inhibition of RTKs by EGCG may play a critical role in the anticancer effects of GTC (Reviewed in more details in ref. 15).

DHFR

Structural modeling studies showed that EGCG can bind to human dihydrofolate reductase (DHFR) at the same site and in a similar orientation to that observed for some structurally well characterized DHFR inhibitor complexes
The response of lymphoma cells to EGCG was similar to that caused by well known antifolates. EGCG caused a dose-dependent inhibition of cell growth (IC50 = 20 µM), G0-G1 phase arrest of the cell cycle and induction of apoptosis. Folate depletion increased cell sensitivity to both antifolates and EGCG. These effects were attenuated by growing cells in a medium containing hypoxanthine-thymididine: this finding is consistent with DHFR being the site of action for EGCG (32).

**Lipid rafts and apoptosis**

Programmed cell death (apoptosis) is characterized by marked changes in cellular morphology, typically including chromatin condensation, membrane blebbing, nuclear breakdown, appearance of membrane associated apoptotic bodies, internucleosomal DNA fragmentation and cleavage of poly(ADP-ribose) polymerase (9, 12, 13, 15, 46-50). On the basis of the biochemical nature of lipid rafts a plethora of possible interactions with various subcellular structures has been suggested. Lipid rafts including sphingomyeline (SM), sphingosine and ceramide have been found specifically involved in apoptosis (15, 17, 18, 20, 21, 45, 46). The role of lipid rafts in apoptosis has been mostly studied in lymphocytes and endothelial cells, in which the physiological apoptotic program occurs after FAS triggering and *in situ* generation of sphingosine and ceramide by acid sphingomyelinase (aSMase) (15, 17, 46). Action of aSMase on membrane SM generates ceramide, which was found as a biochemical mediator of cell death induced by diverse stimuli such as ionizing radiation, chemotherapeutic agents, UVA light, EGCG and many others. Following stimuli, aSMase translocates into membranes and specifically generates ceramide in lipid rafts, which are plasma membrane sphingolipid-enriched microdomains (15, 45, 46). These structures serve as platforms for protein recruitment and oligomerization, thus allowing the signal transduction across the plasma membrane.

*A rationale for lipid rafts and drug therapy?*

Important quantitative differences in the lipid content of rafts have been found in normal/benign versus cancer cells (reviewed in ref, 15, see also 20, 21, 51-57). In particular, cholesterol and sphingomyelin are more abundant (51-55) and selected area membrane shedding has been observed in various tumours since many decades ago and confirmed more recently (16, 55, 57). It is known that an higher amount of cholesterol, sphingolipids and raft proteins, including LamR, are associated to cell growth induction and angiogenesis. Also, overall morphology of carcinoma cells is affected. LamR is usually upregulated in cancer cells (25, 42). A schematic view of how over expression of LamR might result in higher uptake of EGCG is provided in Fig. 1. This hypothesis provides a novel rationale for anticancer drug therapy, since cancer cells are more sensitive to cell surface-acting drugs often used in chemotherapy (17, 20, 21, 26, 46, 58).
Fig. 1. A feed-back loop for EGCG in inducing apoptosis in cancer cells mediated by lipid rafts. The lipid rafts, sphingolipid- and cholesterol-enriched membrane microdomains, are considered to be floating in an “ocean” of phospholipids and hence they have been termed “rafts”. Although represented in circles, rafts have to be imagined more realistically as either leaflets of the membrane and floats in the phospholipids ocean (See also refs 15, 22 and 23 for further insights on lipid raft structure and signaling). Upon binding of signaling molecules, rafts enclosed in the leaflets (sequestered by lateral diffusion and other mechanisms) and those coupled to receptors would collaborate for transducing the signals across the membrane bilayer (15-23). In the model presented here, EGCG will first be incorporated into the plasma membrane, and then re-loaded into the rafts where LamRs are present. In the rafts, EGCG interacts with EGFR, HER2, VEGFR, HGFR/Met and many other receptors (see text). The mechanism of endocytosis of EGCG has not yet been experimentally dissected. However, it is reasonable to believe that LamR, in association with rafts, may transport EGCG to cytosol, since human LamR is a protein that has acquired dual function through evolution, acting both as a cell surface receptor and a ribosomal protein (24, 25, 42). From cytosol, EGCG would enter the nucleus by a mechanism similar to other nuclear acting drugs (20, 21, 47). Upon binding to DNA, EGCG may recruit some important genome maintenance/DNA repair complexes, like DSB/NHEJ, and induce expression of apoptotic genes such as FAS and CLU (62-63). FAS is now loaded into lipid rafts after post translational modification by palmitoylation (15). Sequentially, the major steps are: 1, non-specific binding of EGCG in membrane lipid rafts would destabilize rafts structure and inactivate proliferative MAPK signaling; 2, uptake of EGCG by LamR would bring EGCG into cytosol and nucleus; 3, EGCG binding to DNA would activate DNA-repair based DNA-demethylation, resulting in gene activation; 4, over expression of tumor suppressor and pro-apoptotic genes (FAS, PAR4 and CLU) will inhibit step 3; 5, aSMase and FAS is now loaded into lipid rafts after post translational modification by palmitoylation. aSMase hydrolyses SM, and newly produced ceramide displaces cholesterol, transforming Chol-raft to Cer-raft (see also reference 15 for details) while, and simultaneously, aggregation of FAS-FASL complex with other factors would forms the FAS-DISC complex; 6, endocytosis of the death signal facilitated by Cer-rafts would finally cause cell death.
*Induction of apoptosis by EGCG*

A potent and novel cell-killing mechanism that involves the formation of FAS-driven scaffolds in membrane raft clusters, housing death receptors (DR) and apoptotic-related molecules is recently documented (15, 17, 46, 47, 58). FAS, tumor necrosis factor-receptor 1 (TNF-R1) and TNF-related apoptosis-inducing ligand (TRAIL)-R2/DR5 are clustered into lipid rafts in many cell lines, including leukemic Jurkat, M624 melanoma, and breast cancer cells following radiation or drug treatment, the presence of FAS being essential for apoptosis. Disruption of lipid rafts and interference with actin cytoskeleton prevents FAS clustering and apoptosis (Reviewed in ref. 15). The functions of SM in FAS-mediated apoptosis by FAS clustering through aggregation in lipid rafts have been demonstrated by cloning a gene responsible for SM synthesis (SMS1) and observing that the overproduction of membrane SM enhances FAS-mediated apoptosis through increasing death inducing signaling complex (DISC) formation, activation of caspases, efficient translocation of FAS into lipid rafts, FAS clustering and considerable increase in ceramide generation within lipid rafts upon FAS stimulation (18). TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a promising candidate for cancer therapy. A recent report showed that EGCG sensitizes TRAIL-resistant LNCaP cells to TRAIL-mediated apoptosis through modulation of intrinsic and extrinsic apoptotic pathways (58), *i.e.* upregulation of poly(ADP-ribose) polymerase cleavage and modulation of pro- and antiapoptotic Bcl-2 family of proteins (4, 15, 16, 46, 50, 58) with synergistic inhibition of apoptosis inhibitors and increase in caspase cleavage. A most striking observation was that the pretreatment of cells with EGCG resulted in modulation of FAS-DISC, including FADD, FLICE-inhibitory protein, and DR4 (15, 19, 46, 58).

*Angiogenesis and inhibition of metastasis diffusion by EGCG*

Long-term survival of cancer cells is dependent upon induction of neoangiogenesis. This process is tightly regulated by specific endogenous inhibitors and stimulators which regulate the process of endothelial cell proliferation, migration and differentiation. Endogenous stimulators of angiogenesis are fibroblast growth factors (FGF) and VEGFs. Recently described natural inhibitors include EGCG, endostatin and angiostatin (2, 4, 15, 30). The progression of human tumors involves the matrix metalloproteinase (MMP) family. Two members of the family, MMP2 and MMP9, seem to have a more important role in invasion and metastasis formation (4, 15, 59). They are involved in disruption of basal membrane collagen and extracellular matrix proteins during angiogenesis, tissue remodeling and repair but also play a role in tumorigenesis. DNA methylation has also been shown to affect MMP expression (16). EGCG has been shown to affect MMP activity both directly and indirectly. It has been shown that 0.1 % GTC in drinking water caused a
marked inhibition of MMP2 and MMP9 in the prostate in TRAMP mice as well as in endothelial cells (reviewed in ref. 4). EGCG (25-100 µM) also been reported to inhibit the MMP-2 and MMP-9 in endothelial cells (59). EGCG was found effective at inhibiting KS-IMM cell and endothelial cell growth, chemotaxis and invasion, over a wide range of doses; high concentrations also induced tumor cell apoptosis. EGCG also inhibited the metalloprotease-mediated gelatinolytic activity produced by endothelial cell supernatants and the formation of new capillary-like structures in vitro. Green tea or purified EGCG, when administered to mice in the drinking water, inhibited angiogenesis in vivo in the Matrigel sponge model and restrained KS tumor growth. Histological analysis of the tumors were consistent with an anti-angiogenic activity of EGCG and GTC. It seems that EGCG, through interaction with lipid rafts, could inhibit or delay cancer invasion, metastasis, and angiogenesis via modulations of MMPs (15, 16, 59).

Urokinase type plasminogen activator (uPA) is a trypsin-like protease that converts the zymogen plasminogen into active plasmin. It has been found to prevent apoptosis and stimulate angiogenesis, mitogenesis, and cell migration, also modulating cell adhesion. Inhibition of urokinase can decrease tumor size or even result in complete remission of cancer in mice (4, 50). It has been reported by molecular modeling that EGCG may block urokinase activity by interacting with the respective amino acids (Numbered with respect to the matured protein) His^{57} and Ser^{195} extending toward Arg^{35} from a positively charged loop of urokinase, thus preventing the formation of the catalytic triad of urokinase (3). Destabilization of uPAR function by disturbing its raft assembly may result in blocking uPA activity, one of the most frequently overexpressed enzymes in human cancers.

Epigenetic regulation of gene expression through EGCG signaling: modulation of CLU expression and induction of apoptosis

GTC and bioflavonoids (quercetin, fisetin, and myricetin) each inhibited SsSI DNMT- and DNMT1-mediated DNA methylation. The IC_{50} values for the in vitro inhibition of human DNMT1 by catechin, epicatechin and various flavonoids ranged from 1.0 to 8.4 µM. But EGCG was a more potent inhibitor, with IC_{50} values at 0.21 to 0.47 µM. Kinetic analyses suggested that catechol-containing dietary polyphenol could inhibit DNA methylation through two mechanisms: direct inhibition of the DNMTs plus indirect inhibition of the enzymes through increased formation of S-adenosylhomocysteine (a potent noncompetitive inhibitor of DNMTs) during catechol-O-methyltransferase (COMT)-mediated O-methylation of the catecholic dietary polyphenols. However, the strong inhibitory activity of EGCG for human DNMT1-mediated DNA methylation was largely independent from its own methylation and seems
to be essentially due to a Mg\(^{2+}\)-dependent direct inhibition of the enzyme (36-38, 60).

It was recently demonstrated that catechins may directly interact with nucleic acids in living cells \textit{in vitro} (33-35). Thanks to surface plasmon resonance (SPR) assay and cold spray ionization-mass spectrometry, Kuzuhara et al. (34) clearly showed that EGCG binds to both DNA and RNA. The assay also showed that all four major GTC bind DNA oligomers. Cold spray ionization-mass spectrometry analysis showed that one to three EGCG molecules bind a single-strand 18-mers of both DNA and RNA, and that one or two molecules of EGCG bind to double-strand AG-CT oligomers of different lengths. These results suggest that multiple binding sites for EGCG are present in DNA and RNA oligomers. Both galloyl and catechol groups of EGCG are essential for DNA binding through holding strands of DNA via their branching structure. By fluorescence and circular dichroism spectroscopic methods, Ghosh et al. (35) monitored the binding of copper complexes of ECG and EGCG with calf thymus DNA. This approaches revealed the close link between catechins and polynucleotides and will help our understanding of the mechanism of action of catechins with regard to cancer prevention.

In living cells EGCG may induce genotoxic stress, hence the genome maintenance protein team will be activated. Once incorporated into the DNA helix, EGCG may induces double strand break (DSB) mediated DNA demethylation (61-63) and re-expression of apoptotic genes such as Clusterin (CLU), FAS, Par4 and others (see Fig. 1 and refs. 9, 49, 61, 64, 65). Inhibition of extracellular signal-regulated kinase/mitogen-activated protein kinase pathway may also contribute to decreased DNA-methylation (66). We suggest that, once activated, CLU protein translocate to the nucleus and completing the feed-back loop CLU inactivates one or more components of DSB repair pathways, either RAD51 (and its partners) or Ku70/Ku80 complex formation. CLU may also inhibit Ku70–Bax (Bax is a Bcl2 anti-apoptotic family protein) interaction in the repair pathway (8-13, 62, 63, 66, 67). The formation of Double Strand Breaks (DSB) is a critical lesion that, if misrepaired, can promote genomic instability or cause lethality if not repaired. DSB can be formed in response to exo- or endogenously generated reactive oxygen species (ROS) and by some agents that mimic DNA structure (e.g. bleomycin). IR also produces unique DSB of high complexity (Reviewed in ref. 63). In some cellular systems the action of an inhibitor of chromatin silencing enzymes is required to initiate gene re-expression and activate promoters in densely methylated regions (16, 34-36, 53-66). EGCG induces CLU gene expression in prostate cancer cells (8, 9). Transient overexpression of the pro-apoptotic form of CLU (nuclear CLU, nCLU) decreases proliferation rate of SV40-immortalised human prostate epithelial cells by slowing down cell cycle progression (63, 67, 68). CLU over-expression in both malignant and non-malignant epithelial cells induces cell cycle arrest and apoptosis (12, 69). CLU-mediated apoptosis is regulated by
APC and is P21 dependent but P53 independent (70). Upstream promoter of CLU is regulated by acetylation/deacetylation or methylation, and actively repressed by oncogene, such as H-Ras (61, 66-68, 71, 72). CLU expression is lost during prostate cancer progression, being down-regulated in low and high grade human prostate cancer (8, 9). At difference, its expression is restored to high levels when EGCG inhibits prostate cancer progression in the TRAMP mice model (9).

**Structural evidences for EGCG interaction with other molecules**

A quick review into the available data on the EGCG interactome lead to the conclusion that most likely only lipid rafts, LamR, DNA, RNA, Topo II, DNMT1 and uPA may take active part by direct interaction with GTC and mediate biological relevant response. Interestingly, among them the LamR-EGCG complex formation was further confirmed and studied by crystal structure analyses (42). This finding links EGCG action to MAPK pathway (4) providing new insights into the mechanism of action of GTC. The affinity showed by EGCG at binding key proteins may also facilitate the design of novel therapeutic drugs targeting, for instance, LamR. Interaction of DNMT1 with EGCG can be rationally designed since GTCs has intrinsic affinities to methylases (60) Computational modeling of molecular docking of EGCG, homology studies on DNMT1s, and binding energy analyses showed that the gallic acid moiety of EGCG plays a crucial role in its high-affinity, direct inhibitory interaction with the catalytic site of the human DNMT1. Binding of enzyme is stabilized by Mg$^{2+}$ (34-36). Studies suggest that molecular interactions of EGCG with membranes is also possible (73, 74). Ishuzu et al. (73) have demonstrated that ECG and EGCG can be effluxed from cells with β-cyclodextrin, but in aqueous solvent there are diasteremeric preferences. Interestingly, β-cyclodextrin is also used to cause cholesterol efflux and lipid raft destabilization (16, 19-23). Small angle x-ray scattering experiments indicated that the intermembrane distance of multilamellar vesicles of phosphatidylcholine membrane greatly decreased when EGCG concentrations was above the threshold, suggesting that neighbouring membranes came in close contact with each other in the presence of EGCG (73).

EGCG was the most potent dose-dependent inhibitors of topoisomerase II (Topo II) catalytic activity isolated from hamster ovary AA8 cells. At active concentrations, a high level of endoreduplication (consisting in two successive rounds of DNA replication without mitosis) was observed (75). Maximum effect seems to require a pyrogallol structure at the B-ring. Additional substitution with a galloylic residue at the C3 hydroxyl group further potentiate the effect (76). It is known that some inhibitors of Topo II are redox-independent poisons, interacting with the enzyme in a non-covalent manner, while others enhance DNA cleavage in a redox-dependent manner that requires covalent addition to
topoisomerase II. GTC may inhibit Topo II by at least two different mechanisms. In a recent work, Bandele et al. (40) have tested GTC and flavonols (myricetin, quercetin, and kaempferol) on Topo II activity. Compounds were classified into four distinct groups: EGCG and EGC were redox-dependent Topo II poisons, kaempferol and quercetin acted as traditional redox-independent poisons, myricetin showed both mechanisms of action, and ECG and EC displayed no significant activity. On the basis of these findings, it was proposed that the C4'-OH on the B ring is critical for the compound to act as a redox-independent poison, while the addition of -OH groups at C3' and C5' increases the redox activity of the B ring allowing the compound to act in a redox-dependent fashion. The structure of the C ring in the flavonols is aromatic and planar and includes a C4-keto group. It has been proposed that it allows the formation of a pseudo ring with the C5-OH. Disruption of these structures abrogates enzyme binding blocking Topo II inhibition (40).

Bioavailability of EGCG

The bioavailability of EGCG is possibly increased by decreasing the presystemic elimination and stabilizing EGCG in the lumen. This would help its transfer across the intestinal apical membrane resulting in accumulation and thus increase in availability by inhibiting phase I and II enzymes and phase III transporters (76). In a crossover study, five human volunteers were given a single oral dose of GTE (A), nutrient mixture (NM) containing GTE (B) and formulation B along with black grapes 250 g (C). Blood samples were drawn at 0, 2, 4, 6 and 8 h. The pharmacokinetic parameters were analyzed by WinNonLin (Vs 5.0.1.) using a non-compartmental approach. Supplementation with nutrient mixture normally prescribed to cancer patients containing ascorbic acid, selenium, N-acetylcysteine and other nutrients (formulation B) resulted in an increase of the systemic availability of EGCG by 14%. Formulation C further increased its bioavailability by 13%, thus leading to a total increase of 27% (76). Approaches to increase the bioavailability of flavan-3-ols include the administration of green tea in combination with fruit juices, piperine and peracetylation of EGCG (77). Inclusion of genistein (from soy), at non-cytotoxic concentrations, increased the growth inhibitory effects of EGCG against HT-29 cells (up to 2-fold at 20 µM genistein). Intra-gastric co-administration of EGCG (75 mg/kg) and genistein (200 mg/kg) to CF-1 mice resulted in an increase in plasma half-life (t(1/2) 148.7±16.4 vs. 111.5±23.4 min) and exposure (AUC(0—>infinity) 183.9±20.2 vs. 125.8±26.4 µg/mL x min) of EGCG. Co-treatment with genistein also increased the maximal concentration (C(max)), AUC(0—>360min), and t(1/2) of EGCG in the small intestine by 2.0-, 4.7-, and 1.4-fold, respectively, compared to mice treated with EGCG only (78). To determine the effect of major flavonoids present in the diet, i.e. quercetin and EGCG, on the PK and bioavailability of biochanin A
(BCA, a flavonoid with chemopreventive properties), BCA was administered to rats intravenously (5 mg/kg) or orally (16.67 or 50 mg/kg) with or without concomitant EGCG and quercetin. In addition, *in vitro* studies with the human intestinal Caco-2 and human hepatic HepG2 cell lines were performed to evaluate the effects of quercetin and EGCG on the cellular metabolism of BCA, as well as in human breast cancer MCF-7 cells or MDCK cells to evaluate possible changes in cell efflux. These studies demonstrated that the administration of multiple flavonoids results in increased flavonoid bioavailability, as well as decreased clearance, potentially due to increased enterohepatic cycling (79).

It seems that clinically relevant questions are: how much of the membrane bound EGCG would be potentially an important bias in the essays in which bioavailability is studied? Is it possible that very low (sub-micromolar) concentration of EGCG in the blood stream would correspond to a much greater bioavailability in the membrane fraction? This issue has to be taken into particular consideration, because EGCG partitions in the lipid fractions, and particularly into membrane rafts, could be even more potent and specific against cancer cell. To our knowledge, published data addressing this issue from this point of view are not available, thus further future investigations are needed.

**CONCLUSION AND PERSPECTIVE**

Signaling proteins of specific pathways are often physically organized into complexes by lipid rafts (15-25). Structurally well-organized rafts promote proper signaling and prevent improper cross talk (15, 23). Rafts may also play a role in the quantitative response. Sequestering key proteins in rafts generates hubs for feedback loops that also modulate pathway activity regulation. Such feedback loops are necessary to finely tune up dose and time-course response to efficiently match the specific physiological function. Lipid rafts may therefore provide an active platform for optimal tuning of signaling response. In principle, the construction of “synthetic” feedback loops for dynamic regulation of signaling through recruitment of EGCG and/or structurally similar drugs targeting MAPK cascade or LamR would be fascinating. Alternative assembling of rafts may result in reprogramming cellular responses and could be exploited to engineer cells with novel therapeutic and biotechnological functions.

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