Doctoral Thesis

EGCG suppressed the expression of FLT3 by disruption

of FLT3/Hsp90 protein complex in AML cells

変異 **FLT3** を有する急性白血病細胞に対する **EGCG** の

作用機序の解析

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LIST OF ABBREVIATIONS

17-AAG	17-allylaminodemethoxygel danamycin
67LR	67 kDa laminin receptor
a.a	amino acid
AKT	v-akt murine thymoma viral oncogene homolog
ALL	Acute LymphoblasticLeukemia
AML	Acute Myeloid Leukemia
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
C/EBP	CCATT/enhancer binding protein alpha
Caspases	Cysteine-aspartic acid proteases
CML	Chronic myeloid leukemia
CN-AML	Cytogenetically normal-AML
CRC	Colorectal cancer
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethylsulfoxide
EC	Epicatechin
ECG	Epicatechin-3-gallate
EDTA	Ethylenediamine tetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor

ERK	Extracellular-regulated kinase
ER	Estrogen receptor
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
Flk-2	Fetal liver kinase -2
FLT3	Fms - like tyrosine kinase 3
FLT3-ITD	FLT3- internal tandem duplication
Fms	Feline McDonough sarcoma
GISTs	Gastrointestinal stromal tumors
GTCs	Green tea catechins
HA	Herbimycin A
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid
Hes1	The transcription factor hairy and enhancer of split-1
HO-1	Heme oxygenase-1
hTERT	Human telomerase reverse transcriptase
Hsp90	Heat shock protein 90
I/R	Ischemia-reperfusion
IB	Immunoblot
IGF-1R	Insulin-like growth factor 1 receptor
IL-3	Interleukin-3
IP	Immunoprecipitation
JMD	Juxtamembrane domain

KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSFR	Macrophage colony-stimulating-factor receptor
MEK	Mitogen-activated protein/extracellular signal-regulated kinase
mTOR	Mammalian target of rapamycin
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	platelet-derived growth factor receptor alpha
PI3K	Phosphoinositide-3 kinase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSK	The 90 kda ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SK6	p70-ribosomal-S6-kinase
STAT5	Signal transducer and activator of transcription 5
SYK	Spleen tyrosine kinase
TKD	Tyrosine kinase domain
VEGFR	Vascular endothelial growth factor receptor
WT	Wild- type
XIAP	X-linked inhibitor of apoptosis protein

SUMMARY

FMS-like tyrosine kinase 3 (FLT3) is a commonly mutated gene found in acute myeloid leukaemia (AML) patients and activated FLT3 is a promising molecular target for AML therapies. My data so far has shown that the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) suppresses cell proliferation, inducing apoptosis in AML cells with FLT3 mutations. Interestingly, I found that EGCG has the ability to down-regulate the expression of FLT3 and suppress the activity of its downstream signalling molecules such as AKT, MAPK and STAT5 [1]. However, the exact mechanism underlying FLT3 regulation by EGCG has not been completely clarified. In 2009, Yin et al. demonstrated that EGCG is a potential heat shock protein (Hsp) 90 inhibitor [2]. Moreover, mutant FLT3 has been found to be a *bona fide* client protein for Hsp90 in cell models and primary AML cells [3,4]. However, the chaperoning role of Hsp90 towards wild-type (WT) FLT3 is still controversial. Thus, the aims of my research are: (1) investigating the involvement of Hsp90 in the down-regulation of FLT3 levels by EGCG, (2) investigating the influence of EGCG on FLT3 promoter activity and gene expression and (3) evaluating the combination effect of EGCG and PKC412 (an FLT3 inhibitor) on AML cells with FLT3 mutations..

First, I clarified whether FLT3-WT requires Hsp90 as a molecular chaperone. The results showed that endogenous FLT3-WT was not co-IP with Hsp90 in THP-1 cells, but ectopic FLT3-WT expressed in 293FT was able to form a complex with Hsp90. Moreover, I found that endogenous FLT3-WT in THP-1 cells was not phosphorylated, whereas ectopically-expressed FLT3-WT in 293FT was phosphorylated. Using FLT3-WT/ITD/D835V-K644R mutants, which are constitutively de-phosphorylated, were not co-IP with Hsp90 in 293FT cells. These data indicated that Hsp90 selectively interacts with phosphorylated FLT3.

Upon confirming that Hsp90 selectively binds to phosphorylated FLT3, I postulated that the suppression of cellular FLT3 levels by EGCG is mediated through the inhibition of Hsp90 function by EGCG. Indeed, EGCG disrupted the interaction between Hsp90 and FLT3 in 293FT and MOLM-13 cells. Interestingly, the stability of endogenous FLT3-WT in THP-1 cells was not significantly influenced by EGCG treatment; I assume that this is mainly because FLT3-WT was not phosphorylated in THP-1 and thus there was no binding to Hsp90.

In contrast to EGCG, studies on other polyphenols such as ECG, EGC and catechin and the mechanism of underlying their biological activities have rarely been reported. In this study, I suggest for the first time that EGC and ECG but not catechin suppress FLT3 expression by inhibiting Hsp90 function in the same manner as that followed by EGCG.

I also found that EGCG reduced the transcription level of *FLT3* by suppressing its promoter activity.

Moreover, I obtained effective FLT3 down-regulation with a physiological serum concentration of EGCG (<10μM) on the combination with PKC412. For example, when combined with the FLT3 inhibitor- PKC412, the concentration of EGCG decreased to 10 μM (with 5 nM PKC412) and even 5 μM (with 7 nM PKC412) in MOLM-13 cells, suggesting that there exists a suitable strategy for using EGCG in clinical treatment.

Taken together, I clarified that EGCG, EGC and ECG destabilized FLT3 by disrupting its interaction with Hsp90. I have provided evidence that EGCG suppresses *FLT3* promoter activity and its transcription. Thus, I propose that EGCG could be a useful therapeutic agent for AML patients, particularly when used in combination with other drugs such as the FLT3 inhibitor, PKC412.

INTRODUCTION

1. FMS-like tyrosine kinase 3

1.1. FMS-like tyrosine kinase 3 receptor

FMS-like tyrosine kinase 3 *(FLT3)* is a commonly mutated gene in acute myeloid leukaemia (AML) [5]. Approximately 30 % AML patients harbour a FLT3 mutation [5]. FLT3 belongs to the class III receptor tyrosine kinase (RTK) family, along with the stem cell (Steel) factor receptor (KIT), macrophage colony-stimulating factor (M-CSF), receptor-FMS and the platelet-derived growth factor receptors PDGFR and . FLT3 shares approximately 30% homology with other family members. The *FLT3* gene was isolated using placenta cells by Rosnet *et al.* in 1991 [6,7] and cloning of the human *FLT3* gene followed a few years later [8]. At the same time, another group identified FLT3 from murine-enriched stem cell population of foetal liver cells and termed it foetal liver kinase-2 (Flk-2) [9]. The sequence of human *FLT3* is homologous to murine FLT3. The human *FLT3* gene is located on chromosome 13q12 and encompasses 24 exons. It encodes a membrane-bound glycosylated protein of 993 amino acids with a molecular weight of 158–160 kDa, as well as a non-glycosylated isoform of 130–143 kDa, which is not associated with the plasma membrane [7,10]. Similar to other members of the class III RTK family, FLT3 is characterized by an extracellular domain consisting of five immunoglobulin-like domains, a single transmembrane region, a cytoplasmic juxtamembrane domain (JMD) and a cytoplasmic tyrosine kinase domain (TKD) interrupted by a kinase insert domain (Fig. 1A) [11-14]. FLT3 receptor is normally expressed on the cell surface of haematopoietic progenitor cells, but its expression is lost upon cell maturation [15]. The expression of FLT3 has been also detected in the brain, bone marrow, placenta and gonads, where its function is unknown

[6,9,15]. FLT3 expression can be found in different human and mouse cell lines of both myeloid and lymphoid lineages, but the distribution is quite different [16-18]. Many studies indicate that FLT3 has a crucial role in the development, survival and proliferation of normal stem/progenitor cells [19].

Recent studies have indicated that approximately 10%–15% AML patients display high expression of wild-type (WT) FLT3 [20]. High FLT3 expression has a negative impact on overall and event-free survival in cytogenetically normal AML (CN-AML) patients lacking FLT3 mutations [21].

1.2. FLT3 ligand

FLT3 ligand (FL), first cloned in 1993-1994 by two independent groups [22,23], is one of the most important early-acting haematopoietic cytokines contributing to the maintenance, expansion and differentiation of haematopoietic stem cells [24]. FL is expressed as transmembrane molecules or remain attached to extracellular matrix components and can be converted to soluble factors by proteolytic processing or shedding, thus serving the need for local control of HSC stimulation [24,25]. Alternative splicing contributes to the generation of a soluble FL isoform by a stop codon inserted into the reading frame of the sixth exon [25]. Despite the widespread expression of *FL* mRNA in both haematopoietic and non-haematopoietic tissues [22,26], the FL protein has only been found in stromal fibroblasts present in the bone marrow microenvironment and T lymphocytes [27]. Both the membrane-bound and soluble isoforms of FL are biologically active and stimulate the tyrosine kinase activity of FLT3 [25]. Mice lacking FL as a result of targeted gene disruption are viable but have more severe defects than FLT3 receptor knockouts, including reduced cellularity in the haematopoietic organs, reduced number of myeloid and lymphoid progenitors in the bone marrow and a marked deficiency of natural killer and dendritic cells in the lymph nodes,

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spleen and thymus [28]. The co-expression of FL and FLT3 found in 40 of 110 cell lines indicated that the native FLT3 and FL may play a role in the survival or proliferation of leukaemic blasts [29].

1.3. FLT3 mutations in AML

Nakao *et al.* first reported the presence of internal tandem duplications (ITDs) in the JM of FLT3 in AML and suggested that these mutations play an important role in AML pathogenesis [30]. FLT3-ITD occur in approximately 25% younger adult patients with AML, predominantly in CN-AML [31]. ITD mutations result in amino acid sequence changes with intact coding frame that lead to a constitutive activation of FLT3, its downstream signalling pathways and dysregulation of cellular proliferation [32-34]. ITDs are located in exons 14 and 15 of *FLT3* and show a broad variation in the position of their insertion sites, from three to several hundred base pairs, with variable start and end points-, as well as in the number and sizes of the duplicated fragments [35-38]. The term 'FLT3-ITD' was derived from the attribute of an 'internal tandem duplication' but in reality, 'ITDs' can also include insertions of random extra nucleotides or be sole insertions of foreign sequences; therefore, a more appropriated term, 'length mutation' (FLT3-LM), was suggested [39]. It was previously assumed that FLT3-ITD localized in the JMD of FLT3, but Breitenbuecher *et al.* (2009) reported that approximately 30% FLT3-ITDs are situated in non-JMDs as well [38]. In 2001, the gain-of-function- missense point mutations in the activation loop domain of TKD of the FLT3 receptor (FLT3-D835) was first described at a frequency even lower than that of FLT3-ITD mutations—the mutations were from D835 to Y or H, or less frequently to V or E [40,41]. Subsequently, other FLT3 point mutations were also found in others domains including JMD, TKD1 particularly the secondary FLT3 point mutations that convey

resistance to FLT3 inhibitors [35,42,43]. FLT3-TKD point mutations occur in approximately 5%–10% AML patients [35,42].

Clinically, FLT3-ITD occurs most frequently in CN-AML, trisomy 8, t(6;9) and t(15;17), [39,44] and it is generally associated with leukocytosis on presentation [45-47]. While there are diverging data concerning the prognostic implications of FLT3-TKD [46,48,49], many studies have uniformly supported the view that the presence of FLT3-ITD is a major independent adverse prognostic indicator, associated with an increased risk of relapse and worse overall survival [37,44,50] particularly in those with high FLT3-ITD allelic ratios that predict for low complete remission rates and poor survival [35,51,52].

1.4.   FLT3-mediated signal transduction pathways

Upon stimulation by FL, FLT3-WT promotes receptor dimerization and subsequent signalling (Fig. 1B) through tyrosine-phosphorylated GAP, PLCg, Vav, Shc, and the p85 subunit of phosphoinositide-3 kinase (PI3K) and associate with GRB2, GAB2, SHIP, SHP2 (a protein tyrosine phosphatase), CBL (a proto-oncogene) and CBLB (a CBL-related protein) that ultimately act on the p85 subunit of PI3K [53-56]. FLT3 and these adaptor-protein complexes probably stimulate downstream effectors in the RAS–RAF–mitogen-activated protein/extracellular signal-regulated kinase (MEK)–extracellular-regulated kinase (ERK) pathway and PI3K pathway [54,55,57] (Fig. 2). Activated PI3K subsequently leads to the activation of a protein kinase cascade, including protein kinase B (PKB), mammalian target of rapamycin (mTOR), and p70-ribosomal-S6-kinase (SK6) [58]. FLT3-WT also causes phosphorylation of mitogen-activated protein kinase (MAPK) and minimal cell growth on FL stimulation in interleukin-3 (IL-3)-dependent haematopoietic progenitor Ba/F3 cells [54]. Consequently, both pathways have a strong influence on the regulation of

transcription [59] and/or translation [via the 90 kDa ribosomal S6 kinase (RSK) or mTOR/S6K] [58,60] of multiple regulatory genes.

FLT3-ITD and FLT3-TKD are constitutive activation proteins [61] (Fig. 1C and D). Together with modulating crucial downstream signal transducers such as Ras and MAPK, FLT3-ITD induces the activators of transcription, such as the signal transducer and activator of transcription 5 (STAT5) [32,33] (Fig. 2).

1.4.1. Negative regulation of FLT3 by the adaptor protein Lnk

Lnk (also known as SH2B3) is expressed in haematopoietic cells and plays a critical role in cytokine signaling and haematopoiesis [62-64]. Along with SH2-B (SH2B1) and APS (SH2B2), Lnk belongs to a family of adaptor proteins that modulate signalling of several cytokine and growth factor receptors. [65,66]. Lnk negatively modulates several important cytokine-induced signalling pathways, including the SCF/c-KIT, erythropoietin/JAK2 and thrombopoietin (TPO)/MPL-JAK2 pathways [63,67-69]. Lnk interacts with JMD, specifically to p-Tyr^{568} of c-KIT [70] and binds to PDGFRA, PDGFRB and FMS [71,72]. Interestingly, Lnk was found to physically interact with both FLT3-WT and FLT3-ITD through SH2 domains [73]. The tyrosine residues 572, 591, and 919 of FLT3 as phosphorylation sites are involved in direct binding to Lnk. Lnk itself is tyrosine phosphorylated by both FL-activated FLT3-WT and constitutively activated FLT3-ITD. It has been demonstrated that both shRNA-mediated depletion and forced over-expression of Lnk results in activation signals that emanate from both the forms of FLT3 and are under negative regulation by Lnk. Moreover, Lnk inhibited 32D cell proliferation driven by different FLT3 variants. Analysis of primary BM cells from Lnk-knockout mice showed that Lnk suppresses the expansion of FL-stimulated haematopoietic progenitors, including lymphoid-primed multipotent progenitors [73].

1.4.2. FLT3 is transactivated by spleen tyrosine kinase

Spleen tyrosine kinase (SYK) is a cytoplasmic tyrosine kinase critical in normal B cell development and haematopoietic signalling [74]; it was recently found to be aberrantly activated through translocations in T cell lymphoma [75] and myelodysplastic syndrome [76]. In 2014, Puissant *et al.* found that FLT3 is transactivated by SYK via direct binding [77]. This study demonstrated that FLT3-ITD was dependent on SYK for driving myeloid neoplasia in mice, despite the constitutive activation of the FLT3 receptor. Highly activated SYK is predominantly found in FLT3-ITD-positive AML and cooperates with FLT3-ITD to activate MYC transcriptional programs. Furthermore, activated SYK does not exert the same pro-oncogenic effect on other tyrosine kinase oncogenes, such as BCR-ABL, as it does on FLT3-ITD. However, the mechanism underlying SYK activation in FLT3-ITD-positive AML remains uncertain. FLT3-ITD AML cells are more vulnerable to SYK suppression than FLT3-WT counterparts. In an *in vivo* study of FLT3-ITD function, SYK was reported to be indispensable for myeloproliferative disease development, and SYK over-expression promoted overt transformation to AML and resistance to FLT3-ITD-targeted therapy [77].

1.4.3. Mutant FLT3 is stabilized by heat shock protein 90

Heat shock protein 90 (Hsp90) is one of the most abundant chaperone proteins in cells, comprising 1%–2% cellular proteins under non-stress conditions [78]. It interacts with a large and diverse group of substrate proteins; it interacts with almost 400 proteins including human kinases, transcription factors, and E3 ligases, commonly referred to as 'clients', promoting their folding and function [79]. Hsp90 seemingly focuses on metastable proteins that are regulatory hubs in biological networks [80].

Mutant FLT3 has been demonstrated to be a *bona fide* client protein for Hsp90 in cell models and primary AML cells [3,4,81,82]. Hsp90 inhibitors including herbimycin A (HA);

17-allylaminodemethoxygel danamycin (17-AAG), geldanamycin were shown to disrupt the chaperone association of Hsp90 with mutant FLT3 resulting in the degradation of FLT3-ITD and apoptosis of myeloid cell lines transfected with mutant FLT3 as well as of primary AML cells expressing FLT3-ITD [1,3,4,81-83]. However, in the literature, for the chaperoning of FLT3-WT by Hsp90 is still a controversial topic. The results obtained using 32D/FLT3-WT cells [81] and AML blasts [3] showed that FLT3-WT did not bind to Hsp90. The absence of FLT3-WT from the Hsp90-immunoprecipitated complex in FLT3-WT AML blasts was assumed to be not because of its low expression but perhaps because of Hsp90 having a low affinity for FLT3-WT [3]. On the other hand, others reports indicated that Hsp90 could form a complex with FLT3-WT in SEMK2 leukaemic cells, Ba/F3 and 32D expressing FLT3-WT [4,82].

1.5.   Transcriptional regulation of FLT3 expression

The transcription of *FLT3* is regulated by Hoxa9, Meis1, c-Myb and C/EBP   [84-86]. Hox proteins are homeodomain-containing transcription factors that play a vital role in establishing body plan during development, limb regeneration, wound healing, adipogenesis, and haematopoietic stem cell self-renewal [87]. Hoxa9 in particular is expressed at high levels in early haematopoietic progenitor cells and promotes stem cell expansion; in contrast, Hoxa9 down-regulation is associated with haematopoietic differentiation [88,89]. The Hox cofactor- Meis1 cooperates with *Hox* genes to accelerate the onset of AML in mouse models [90]. Interestingly, *Meis1* is frequently found to be up-regulated along with *Hox* genes in human leukaemias, and this is associated with particularly high levels of *FLT3* mRNA [91]. Chromatin immunoprecipitation (IP) has been used to confirm co-occupancy of Hoxa9 and Meis1 on the *FLT3* promoter in myeloid leukaemogenesis and lymphohaematopoietic models [84-86]. *Hoxa9* knockdown has been reported to significantly reduce *FLT3*

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transcription and expression [84]. Conversely, forced expression of *Hoxa9* was found to increase FLT3 transcription and expression in a Pro-B cell line that expressed low levels of FLT3 [84].

The CCATT/enhancer-binding protein alpha (C/EBP) is a leucine zipper transcription factor that has a pivotal role in granulocyte and neutrophil development [92] and also acts as a tumours suppressor in the haematopoietic system [93]. So far, a few hints of a connection between FLT3 and C/EBP markers have been demonstrated [94,95]. These studies showed that the favourable outcome associated with bi-allelic C/EBP mutations was cancelled out by the presence of FLT3-ITD mutations [94,95]; FLT3-ITD signalling inhibits C/EBP differentiating function by promoting C/EBP phosphorylation [96], and C/EBP with a C-terminal mutation collaborates with FLT3-ITD in inducing AML [97]. Such collaborations may rely on the ability of FLT3 signalling to support myeloid commitment of the expanding *C/EBP* -mutated cells [94]. A study pertaining to the profiling arrays from AML patients with differing *C/EBP* status linked FLT3 expression and C/EBP activity and also showed that bi-allelic *C/EBP* mutations associated with lower levels of *FLT3* transcript in AML patients, whereas no significant differences in FLT3 expression between patients with WT or mono-allelic *C/EBP* mutations were noted [86].

c-Myb is a leucine zipper transcription factor; its expression is associated with immature and proliferative cellular stages and turned off during the maturation of the haematopoietic lineage [98,99]. Silencing strategies in mice have shown that c-Myb plays a major role in haematopoiesis, including lineage commitment, proliferation and differentiation [100-103]. c-Myb activity also associates with a broad spectrum of haematological malignancies, including chronic myeloid leukaemia, T-acute lymphoblastic leukaemia and AML [104-106]. c-Myb has a critical role in the transforming potential of the AML-inducing fusion protein

MLL-ENL [106]. Notably, c-Myb was identified as an important downstream target of Hoxa9 and Meis1 [106]. The cooperative activities of c-Myb and C/EBP in activating the promoter of myeloid genes including *FLT3* have been previously reported [86,107,108]. Together with Hoxa9 and Meis1, C/EBP and c-Myb are important elements of the combinatorial binding of leukaemia-related transcription factors that regulate *FLT3* expression [86].

The transcription factor hairy and enhancer of split 1 (Hes1) was recently found to directly bind to the promoter region of the *FLT3* gene and down-regulated its promoter activity [109]. FLT3 was consequently up-regulated in MLL–AF9-expressing immortalized and leukaemia cells with a *Hes1*- or *RBPJ*-null background. MLL–AF9-expressing *Hes1*-null AML cells showed enhanced proliferation and ERK phosphorylation following FL stimulation. FLT3 inhibition efficiently abrogated the proliferation of MLL–AF9-induced Hes1-null AML cells. Furthermore, an agonistic anti-Notch2 antibody induced apoptosis of MLL–AF9-induced AML cells in a *Hes1*-WT but not a *Hes1*-null background. Furthermore, on referring to two independent databases containing messenger RNA (mRNA) expression profiles, it has been observed that the expression levels of *FLT3* mRNA are negatively correlated with those of Hes1 in AML patient samples. These observations demonstrate that Hes1 mediates tumour suppressive roles of Notch signalling in AML development, probably by down-regulating FLT3 expression [109].

1.6.   Targeting FLT3: small molecule FLT3 inhibitors

AMLs with FLT3-ITD have higher relapse rates [110] and therefore inferior disease-free and overall survivals [111], particularly in AMLs with larger ITD size [112], higher allelic burden [113] and multiple ITDs [114]. Therefore, FLT3 inhibition has become a legitimate therapeutic option, and clinical trials of FLT3 inhibitors in AML have been ongoing for a

decade [115-117]. To date, more than 20 small molecule inhibitors against FLT3 have been reported; some of them have been evaluated in clinical trials [118]. These include sorafenib (BAY 43-9006), sunitinib (SU11248), midostaurin (PKC412), lestaurtinib (CEP-701), tandutinib (MLN518), ABT-869, AKN-032, KW-2449, and AC220 [116,119-125]. Structurally, most of these inhibitors are heterocyclic compounds, inhibiting FLT3 activity by competing with adenosine triphosphate (ATP) for binding to the ATP-binding pocket of TKD [126]. Functionally, they are generally multikinase inhibitors. Their clinical activities appear to be mediated by FLT3 inhibition, so that their efficacies are limited to AML carrying FLT3-ITD, and correlated with the inhibition of FLT3 phosphorylation and hence its downstream signalling effectors [127-131]. Quizartinib (AC220), a second-generation FLT3 inhibitor, has recently shown activity in AML in phase II clinical trials [132]. FLT3-targeted antibody therapy (IMC-EB10) has just completed safety investigations in a phase 1 clinical trial (NCT00887926).

Although the inhibition of FLT3 may be achievable, clinical efficacy is less than convincing, being limited by the invariable leukaemia progression despite continuous treatment [116]. Comprehensive reviews on the mechanisms underlying drug resistance have been published [41,116,133-140]. The proposed mechanisms include persistent activation of FLT3 signalling due to over-expression of FL and FLT3, activation of anti-apoptotic signals and protection of leukaemia-initiating cells by the bone marrow niche [41,116,133-140]. Recently, there is compelling evidence that leukaemia clones carrying both ITD and TKD mutations appear when resistance to FLT3 inhibitors occurs [41]. Interestingly, the emergence of double ITD and TKD mutants can be recapitulated *in vitro* when FLT3-ITD+ leukaemia cell lines are treated with mutagens and FLT3 inhibitors [141]. Furthermore, murine xenotransplantation models also suggest that, in some cases, FTL3-ITD and TKD

double mutants actually exist in minute amounts before treatment with FLT3 inhibitors, expand under the selection pressure of FLT3 inhibition and become the predominant resistant clone(s) during the drug-refractory phase [142]. On the basis of this model of clonal evolution, a multipronged strategy using more potent FLT3 inhibitors, and a combinatorial approach targeting both FLT3-dependent and -independent pathways are needed to improve outcome.

2. (−)-epigallocatechin-3-gallate (EGCG)

2.1. Tea catechins and cancer chemoprevention

Since ancient times, green tea (*Camellia sinensis*) has been considered to be a health-promoting beverage. Green tea catechins (GTCs), including epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and (−)-epigallocatechin-3-gallate (EGCG), are the major polyphenolic compounds of green tea (Fig. 3). The dry weights of EC, ECG, EGC and EGCG are 792 ± 3, 1702 ± 16, 1695 ± 1 and 8295 ± 92 mg/100 g, in green tea, and 240 ± 1, 761 ± 4, 1116 ± 24 and 1199 ± 0.12 mg/100 g, in black tea [143]. Several properties of GTCs have been implicated in their chemo-preventive effects, such as their anti-oxidant [144], pro-oxidant [145] and anti-inflammatory [146] effects. Accounting for 505-80% of the total GTCs content, EGCG is the most abundant catechin in green tea and a traditional anti-oxidative free radical scavenger [144,147-149].

The anti-oxidant properties of EGCG are due to the presence of phenolic groups that are sensitive to oxidation and can generate quinone [150]. The phenolic groups of EGCG serve as electron donors that can give up an electron or a hydrogen atom, and the phenolic molecule is capable of making an internal adjustment to stabilize the unpaired electron that results from the loss [151]. EGCG can increase the levels of enzymes related to oxidative

stress, including glutathione-S-transferase, glutamate cysteine ligase, superoxide dismutases, and haeme oxygenase-1 (HO-1) both *in vitro* and *in vivo* [150,152]. EGCG-treated cancer cells were found to have up-regulated PPAR activation that leads to the suppression of the cytoprotective enzyme HO-1 and induction of cell death [153].

Lipopolysaccharide (LPS) is the important pathogenic substance that induces inflammatory responses and angiogenesis, and it is a potent activator for MAPKs, which are expressed in many inflammatory cells by stimulating the release of intermediary growth factors/cytokines [154,155]. EGCG blocks LPS-induced production of several inflammatory factors, including TNF- , VEGF, MCP-1 and NO [156,157]. Moreover, EGCG effectively attenuates LPS - induced activation and phosphorylation of MAPK pathways (ERK1/2 and p38) and NF B pathways (p-IkB , p65 and p-p65) [156,157]. Moreover, EGCG inhibits the JAK/STAT signalling cascade and suppresses the cytokine-induced expression of inducible nitric oxide synthase and intercellular adhesion molecule-1, the key molecules involving in inflammatory and tumourigenesis processes in cholangiocarcinoma [158].

Many studies have shown that the nuclear activities of EGCG inhibit inflammatory responses that are usually accompanied by increased oxidative stress [159,160]. Thus, one may claim that this anti-inflammatory action is mainly due to the direct anti-oxidant activity of EGCG. However, it is not clear whether the anti-oxidative effects of EGCG are a major mechanism underlying the anti-inflammatory actions of EGCG [159,160]. Interestingly, EGCG-stimulated production of reactive oxygen species (ROS) causes the activation of NF- B and NF-E2-related factor 2 (Nrf2), leading to the increased expression of HO-1 and glutathione [161,162]. Scavenging ROS by using various anti-oxidants abolishes EGCG-stimulated induction of HO-1, while pre-treatment with EGCG has protective effects against hydrogen peroxide-induced cytotoxicity [162]. Protective mechanisms used by EGCG to

defend against oxidative stress may be secondary to the induction of various endogenous anti-oxidant proteins [162].

2.2. Tea catechin and anti-tumours effects: the possible mechanisms

Several properties of GTCs have been implicated in their anti-cancer effects including anti-mutagenic [152] and anti-angiogenic effects [154]. Among GTCs, EGCG is the most potent catechins capable of inhibiting cell proliferation and inducing apoptosis in cancer cells [144,147-149]. EGCG has been found to inhibit the development of cancer including lung [163], prostate [164], colon [165], skin [166], and breast cancers [167].

A study involving breast cancer MCF7 and MDA-MB-231 cells showed that EGCG and a pro-drug of EGCG (pEGCG, EGCG octaacetate) caused the hypomethylation of the human telomerase reverse transcriptase (hTERT) gene via the inhibition of histone deacetylase (HDAC) and histone acetyltransferase activity [168]. hTERT is a catalytic subunit of telomerase, an important enzyme required for the maintenance of telomere length and tumourigenesis. Demethylation of hTERT establishes a transcription-repressing environment to prevent aberrant hTERT expression and leads to tumour suppression [168]. pEGCG was synthesized by the modulation of hydroxyl groups with peracetate groups to enhance the bioavailability and stability of EGCG. The same research group also reported that combining EGCG and the HDAC inhibitor trichostatin synergistically re-activated a functional oestrogen receptor in MDA-MB-231 cells via altering the binding transcription repressor complex pRb2/p130–E2F4/5–HDAC–DNMT1–SUV39H1 to the oestrogen receptor (ERα) promoter. This induction of ERα expression could sensitize ERα-negative breast cancers to anti-hormone therapy [169].

EGCG increases hepatic autophagy by promoting the formation of autophagosomes, increasing lysosomal acidification, and stimulating autophagic flux in hepatic cells and *in*

vivo. EGCG also increases the phosphorylation of AMPK, one of the major regulators of autophagy. Importantly, siRNA knockdown of AMPK has been reported to abrogate EGCG-induced autophagy [170].

An important mechanism frequently overlooked in considering the biological effects of EGCG and its derivatives is their potential interaction with tyrosine kinase oncoproteins that are capable of initiating cell signalling [1,171-179]. EGCG inhibits the activation or expression of some RTKs including epidermal growth factor receptor (ErbB) family members: EGFR [171,172], HER2, HER3 [173,174], vascular endothelial growth factor receptor (VEGFR) [175], PDGFR [176], fibroblast growth factor receptors (FGFR) [172] and insulin-like growth factor 1 receptor (IGF-1R) [177]. Many possible mechanisms have been proposed and tested to account for the effects of EGCG on RTKs.

Our research group's previous studies have provided evidence that EGCG can directly target the activation or expression of RTKs and their intracellular signalling pathways [1,178,179]. KIT has been an excellent molecular target for treating gastrointestinal stromal tumour (GISTs) as it is over-expressed in almost all GISTs including imatinib-resistant cases (approximately 95% GISTs over-express KIT) [180]. EGCG treatment inhibits the proliferation of GIST-T1 cells and induces apoptosis by inhibiting the phosphorylation of KIT and KIT downstream signalling molecules including MAPK and AKT [179]. The phosphorylation of both focal adhesion kinase (FAK) and IGF-1R receptor was reported to decrease after incubated these cell with EGCG in pancreatic cancer cells (AsPC-1 and BxPC-3) [178]. In AML with mutant FLT3, EGCG has been reported to down-regulate FLT3 expression and inhibit AKT, MAPK and STAT5 activities [1]. The possible mechanism underlying this is explained in the Results and Discussion sections of this thesis. Additional evidence from other research groups also indicate that EGCG suppresses the

expression of ErbB1 and ErbB2 proteins in mammary and epidermoid carcinoma cells, and its inhibitory effect on cell viability is mediated by the 67-kDa laminin receptor (67LR) [171]. ErbB2 is known to be an internalization-resistant receptor; therefore, agents capable of removing it from the membrane have therapeutic potential [181]. Furthermore, EGCG has been shown to decrease the homoclustering of a lipid raft marker, glycosylphosphatidylinositol-anchored GFP, and reduce the co-localization between lipid rafts and 67LR [171]. Therefore, the main conclusion that can be derived is that the primary target of EGCG is the lipid raft component of the plasma membrane followed by secondary changes in the expression of ErbB proteins [171].

Recently, EGCG has been demonstrated as an inhibitor of Hsp90 [2] . EGCG acts by binding at or near a C-terminal ATP binding site to inhibit dimerization and promote an Hsp90 conformation that interferes with its chaperone activity for client proteins [2]. EGCG also inhibits Hsp90 function by impairing its association with co-chaperones including Hsc70 and p23 in the pancreatic cancer cell line MIA PaCa-2 [182]. In 2010, Tran *et al.* reported that in MCF-7 human breast cancer cells, EGCG specifically inhibited the expression of Hsp90 by inhibiting the promoter activity of Hsp90 [183].

EGCG has also been shown to competitively bind to the ATP binding site of IGF-1R and block downstream signalling [184]. Sah *et al.* demonstrated that EGCG directly inhibits ERK and AKT kinases in immortalized human cervical cells [185]. In addition, EGCG was shown to play a role in the direct inhibition of the activation of ERK and MEK1 and of the association between RAF with MEK1 as well as in the inhibition of AP-1 activity in *H-Ras-* transformed mouse epidermal cells [186,187]. EGCG also exertes antiproliferative effects on *H-Ras-*transformed rat intestinal epithelial cells [188]. These reports seem to be significant when considering the prevention of colorectal cancer (CRC) by GTCs because

Ras (KRAS) gene mutations occur frequently in this malignancy [189]. Moreover, EGCG administration through drinking water has been reported to significantly decrease small intestinal tumour formation in adenomatous polyposis coli (APC)$^{Min/1}$ mice, a recognized model for human intestinal cancer, by reducing the expression of the phosphorylated form of AKT and ERK proteins in small intestinal tumours [190]. EGCG administration through drinking water also suppressed tumour formation in APC$^{Min/1}$ mice by decreasing the levels of basic fibroblast growth factor in small intestinal tissue samples [191]. These reports are important because mutations of the APC gene, a tumour suppressor gene, are critically implicated in human colorectal carcinogenesis [192].

Examining the cellular uptake and distribution of EGCG in cells has revealed that approximately 75% radioactively labelled EGCG was found in the cytosolic compartment, while some radioactivity was found in the membrane fraction [193]. This suggests that EGCG at part directly binds to membrane components, including proteins and lipids. At the cell surface, it directly interrupts the binding of EGF to VEGF or interferes with the binding of EGF to EGFR [172] thus preventing these growth factors from interacting with their corresponding receptors and activating downstream signalling cascades [172,194]. However, Weinstein *et al.* found that EGCG did not inhibit EGF binding when assays were performed at 4°C, suggesting the involvement of others mechanism rather than evidence for competition between EGCG and EGF [195]. In addition, EGCG may inhibit the activation of RTKs by affecting the expression levels of their ligands. The expression levels of the EGFR family ligands EGF and heregulin have been shown to be down-regulated by EGCG treatment in CRC cells [196]. EGCG also decreases the levels of IGF-1, IGF-2, and VEGF, which may be associated with decreased ERK and AKT activities, in CRC and hepatocellular carcinoma cells [177,196,197].

EGCG was found to modulate gene expression by inhibiting various transcription factors including Sp1, NF- B, AP-1, STAT1, STAT3 and FOXO1 [198-206]. The expression of NF- B and AP-1 is inhibited by EGCG in rats exposed to ischaemia-reperfusion (I/R) injury [207]. EGCG inhibits STAT-1 to mediate its own protective effects in myocardial I/R injury [205,208]. Inhibition of the transcription factor FOXO1 by EGCG leads to the suppression of the basal levels of endothelin-1 and the differentiation of adipocytes [201,209]. Both these mechanisms are linked to the activation of AKT that inhibits FOXO1 by direct phosphorylation of FOXO1 and may have cardiometabolic implications [210,211].

2.3.   Possible clinical applications of EGCG

EGCG possesses several potential clinical advantages compared to other traditional cancer drugs as it is readily available in tea, a common beverage worldwide, inexpensive to isolate and can be orally administered [212]. While traditional cancer drugs often harm some healthy cells along with cancer cells and usually have toxic adverse effects [213], EGCG appears to specifically target cancer cells, without harming healthy cells, and has an acceptable safety profile [214]. These benefits support further development of EGCG as a potentially useful anti-carcinogenic agent. A prospective cohort study with over 8000 individuals revealed that the daily consumption of green tea resulted in delayed cancer onset, and a follow-up study of breast cancer patients found that stages I and II breast cancer patients experienced a lower recurrence rate and longer disease-free period [215]. Moreover, EGCG delivered in the form of a capsule (200 mg p.o.) for 12 weeks has been reported to be effective in patients with human papilloma virus-infected cervical lesions [216].

The issues underlying the clinical application of EGCG include its poor stability and poor oral bioavailability [217]. The octanol/buffer partition coefficient P of EGCG is 0.86 ± 0.03. EGCG has high hydrophilicity and has difficulty in penetrating the cell membrane [218].

Maximum catechin plasma concentration has been shown to be achieved 2 h after consumption, and this was followed by rapid clearance [219,220]. Two factors considered to be contributing to the limited oral bioavailability are sensitivity of catechin to the digestive system and absorption barriers in the human gastrointestinal tract; both play a role in the overall very poor intrinsic permeability of catechin and EGCG across the intestinal epithelium [221]. Currently, using colloidal delivery systems such as liposomes [222-225], micro-/nano-particles [226,227] or synthesized EGCG prodrugs such as peracetylated EGCG (AcEGCG) [228] to increase lipophilicity and membrane permeability and to improve the bioefficacy of EGCG are being investigated and have so far achieved promising results.

STUDY OBJECTIVES

The main objectives of my study are:

1)   Identifying determinants involved in the binding of FLT3 to Hsp90.

2)   Identifying the mechanism by which EGCG down-regulates FLT3 expression.

3)   Investigating the influence of EGCG on *FLT3* promoter activity and *FLT3* transcription, and

4)   Evaluating the combined effects of EGCG and the FLT3 inhibitor PKC412 on AML cells with mutant FLT3.

MATERIALS AND METHODS

1. Cell lines and culture conditions

Experiments were conducted using human leukaemia cell lines: cell lines MOLM-13 and MOLM-14 were established from a patient with acute monocytic leukaemia (M5a) carrying t(9;11) [229], MV4-11 from a patient with AML carrying t(4;11) [230] and KOCL-48 from an leukaemic infant carrying t(4;11) [231].

In MOLM-13 and MOLM-14 cells, two mutations within *FLT3* exon 14 were detected: ITD of 21 bps corresponding to codons Phe594-Asp600 and a novel missense nucleotide substitution at the codon 599 (Tyr599Phe) [232,233]. Two types of mutations were located on the same allele [233]. In MV4-11 cells, there are an ITD of 30 bps within *FLT3* exon 14 corresponding to codons Tyr591-Asp600, and a Tyr591His mutation [232,233]. In KOCL-48 cell line, *FLT3*-Asp835Glu mutation was detected [232]. The cell line THP-1 came from the peripheral blood of a one-year old male infant with monocytic AML [234]. They do not contain any known FLT3 mutations and have high endogenous FLT3-WT expression. These cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, Japan K.K., Tokyo, Japan) supplemented with 10% heat-inactivated feetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS, USA), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin (Nakalai Tesque, Kyoto, Japan) in a humidified incubator with 5% CO_2 and 37°C.

The parental 32D cells were cultured in RPMI 1640 medium supplemented with 1 ng/ml IL-3 (R&D Systems).

The 293FT and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Japan K.K., Tokyo, Japan) supplemented with 10% FBS, 100 IU/ml

penicillin, 0.1 mg/ml streptomycin, 1% sodium pyruvate (Wako Pure. Chemical Industries, Osaka, Japan) and 1% L-glutamine (Nakalai Tesque, Kyoto, Japan) in a humidified incubator with 5% CO_2 and 37°C.

2. Plasmids constructs

The gene encoding full-length human FLT3-WT or FLT3-ITD [12 amino acids (a.a) were inserted in JMD] was PCR-amplified using pAL-FLT3-WT or pAL-FLT3-ITD [kindly provided by Dr. Masao Mizuki (Osaka University Medical School, Osaka, Japan)] vectors as templates using the forward primer FLT3(NotI)-F:GCGGCCGCATGCCGGCGTTGGCG and the reverse primer FLT3(SpeI)-R: ACTAGTCTACGAATCTTCGACCTG. The fragments were inserted into the *NotI* and *SpeI* sites of pME-FLAG (a FLAG tag vector) to generate pME-FLAG–FLT3-WT or pME-FLAG–FLT3-ITD12 vectors. The pME-FLAG–FLT3-D835V, pME-FLAG–FLT3-D835Y, pME-FLAG–FLT3-D835N, pME-FLAG–FLT3-D835E, pME-FLAG–FLT3-D835H, pME-FLAG–FLT3-WT-K644R, pME-FLAG–FLT3-ITD12-K644R and pME-FLAG–FLT3-D835V-K644R vectors were created using the PrimeSTAR Mutagenesis Basal kit (Takara, Tokyo, Japan) according to manufacturer's instructions.

The pMKITNeo-FLT3-ITD7 (7 a.a. inserted in JMD) vector was kindly provided by Prof. Toshio Kitamura (the University of Tokyo, Tokyo, Japan).

The *FLT3* promoter was amplified using the forward primer 5 -TAATCTCGAGCGTGGAATTCCTAGAATTGG-3 and the reverse primer 5 -ACGCAAGCTTGGCCTCCGGAGCCCGGGGT-3 . The amplified DNA fragments were digested using *XhoI* and *HindIII* and inserted into the *XhoI/HindIII* site of the pGL4-10 plasmid (Promega, Tokyo, Japan).

All constructs were verified by restriction enzyme digestion and DNA sequencing.

3.   Generation of 32D cells stably expressing FLT3-WT, FLT3-ITD or FLT3-D835V

32D cells stably expressing FLT3-WT, FLT3-ITD or FLT3-D835V were generated using pME-FLAG–FLT3-WT, pME-FLAG–FLT3-ITD12 or pME-FLAG–FLT3-D835V vectors, respectively, using Lipofectamine 2000 (Invitrogen, Tokyo, Japan) according to manufacturer's instructions. These cells were selected in the presence of 0.8 mg/ml G418 (GIBCO BRL, Gaithersburg, MD, USA) for 2 weeks to establish 32D-FLT3-WT/FLT3-ITD or FLT3-D835V.   32D transfectants expressing FLT3-ITD or FLT3-D835V were maintained in RPMI 1640 medium containing 10% FBS in the absence of rmIL-3, while 32D-FLT3-WT-expressing cells were maintained in the presence of 10 ng/ml rhFL (Peprotech, Tokyo, Japan).

4.   Transient transfection

For transient expression in 293FT cells, cells were transfected with the indicated plasmids using the Lipofectamine reagent, according to manufacturer's instructions. Cells were harvested 48 h after transfection for IP and immunoblotting.

5.   Reagents

EGCG, EGC, ECG and catechin (purified powder) were generously gifted by Dr. Yukihiko Hara (Japan), PKC412 was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan) and 17-allylamino-17-desmethoxygeldanamycin (17-AAG) was purchased from Calbiochem (Darmstadt, Germany). All reagents were dissolved in dimethylsulfoxide (DMSO) (Wako Pure. Chemical Industries, Osaka, Japan). Control cells were cultured with the same concentration of carrier DMSO as used in the highest dose of reagents. DMSO concentration was kept under 0.1% for all experiments to avoid cytotoxicity.

6.　Cell proliferation assays

Cell proliferation was determined using the trypan blue dye exclusion test as previously described [179]. Briefly, cells were seeded in 6-well plates at a density of 1×10^5 cells/ml in the presence of different concentrations of EGCG, EGC, ECG and catechin for 72 h. After treatment, 10 µl cell suspensions was mixed with 10 µl 0.4 % trypan blue, and viable cells were manually counted using a haemocytometer. Results were calculated as the percentage of values measured when cells were grown in the absence of reagents.

7.　Western blot analysis

Cells were plated onto 10-cm dishes at a density of 1×10^5 cells/ml in the presence of various concentrations of reagents. After incubation for indicated durations, cells were collected and washed twice with phosphate buffered saline (PBS) (−). Cells were then dissolved in a protein lysis buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, 10 mM $Na_2H_2P_2O_7$, 0.01% Triton X-100, 5 mM N-2-hydroxyethyl piperazine-N -2-ethanesulfonic acid (HEPES), 150 mM NaCl, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 75 µg/mL aprotinin on ice for 30 min with brief vortexing 4 times with every 10 min. After centrifugation at 13,000 rpm at 4°C for 10 min, total cell lysates were collected. Protein samples were electrophoresed through a polyacrylamide gel and transferred to a Hybond-P membrane (Amersham, Buckinghamshire, UK) by electro-blotting. After washing, the membrane was probed with antibodies, and antibody binding was detected using BCIP/NBT substrate (Promega). The following antibodies were obtained from Santa Cruz Biotechnology (CA, USA): FLT-3/FLK-2 (S-18) (sc-480), STAT5 (C-17) (sc-835), Hsp90α/β (F-8) (sc-13119) and survivin (sc-17779). Phosphotyrosine-clone 4G10 antibody was purchased from Millipore (Tokyo, Japan). IgG

from murine serum (I5381-5MG) and anti-actin (A2066) were from Sigma-Aldrich. FLT3 (8F2) and normal rabbit IgG (#2729S), p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Thr202/Tyr204), AKT, phospho-AKT (Ser473), phospho-STAT5 (Tyr694), caspase-3, caspase-9 (C9), XIAP and c-Myb (D2R4Y) antibodies were from Cell Signaling Technology Japan (Tokyo, Japan). Anti-PARP antibody was from WAKO Chemicals (Osaka, Japan).

8.   Co-immunoprecipitation

For IP, cells were treated with EGCG for indicated hours and then harvested. Cells were lysed as indicated above. Then, cell lysates were pre-clearned for reducing the amount of non-specific contaminants with 50 μl protein G-Sepharose 4 fast flow (Amersham Pharmacia Biosciences, Tokyo, Japan) to a total volume of 500 μl. After incubation on a rotator for 1h at 4°C, supernatants were collected by centrifuging at 2500 ×g for 2–3 min at 4°C. A portion of the lysed samples was immunoprecipitated with FLT3 (F-8) or Hsp90 antibodies in an incubation buffer (10 mM Tris, pH 7.5; 5 mM $MgCl_2$; 50 mM KCl and 0.01% Nonidet P-40) for 1 h or overnight at 4°C. Protein G-Sepharose 4 fast flow (Amersham Pharmacia Biosciences, Tokyo, Japan) was then added for 1 h. The immunoprecipitates were washed five times with Tris-buffered saline–Tween. The bound proteins were resolved by SDS-PAGE and analysed by western blotting.

9.   Determination of apoptosis

MOLM-13 and MOLM-14 cells were treated with EGCG, EGC or ECG for 16 h. Apoptotic cells were evaluated by PE Annexin V (BD PharMingen) and analysed by FACS Calibur (Becton, Dickinson). Collected data were analysed by FlowJo software (Tree Star) by Dr. Makoto Yamagishi (the University of Tokyo, Tokyo, Japan).

10. Morphologic assessment to detect apoptotic cells

For detecting fragmented nuclei and condensed chromatin, MOLM-13 cells at a density of 1×10^5 cells/ml were treated with 60 µM EGCG. After the indicated duration, cells were harvested and fixed onto slides using a cytospin (Shandon, Shandon Southern Products Ltd., Cheshire, UK). Cells then were stained with Wright-Giemsa stain. Cell morphology was observed under an inverted microscope.

11. Luciferase reporter assay

To check *FLT3* promoter activity, each reporter (100 ng) plasmid and 5 ng Renilla luciferase plasmid (internal control) were transfected into 293T cells (1×10^5 cells/well in 48-well plates) by Lipofectamine, following manufacturer's instructions. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Tokyo, Japan). Promoter activities were expressed as the ratio of *Firefly* luciferase to *Renilla* luciferase activities.

12. Semi-quantitative reverse transcription-PCR.

Total RNA was extracted from cells treated with or without 60 µM EGCG for 8 h using Sepasol (Nakalai). First-strand cDNA was synthesized from 1 µg of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers. A segment of *FLT3* spanning exons 14 and 15 was amplified using primers *FLT3*.1675F (5′-GACAACATCTCATTCTATGCAAC-3′) and *FLT3*.18R1 (5′-TCTGAACTTCTCTTGAACCA-3′). The thermal cycling profile was 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were separated by electrophoresis on a 1.5% agarose gel.

13. Isobologram

The dose-response interactions between EGCG and PKC412 in MOLM-13, MOLM-14,

MV4-11 and KOCL-48 cells were evaluated at IC_{50} by the isobologram of Steel and Peckham [235]. IC_{50} was defined as the reagent concentration that resulted in 50% cell growth inhibition. The concept of isobologram has been described in detail elsewhere [235]. I used this method as it can cope with agents with unclear cytotoxic mechanisms and a variety of dose-response curves of anti-cancer agents [235].

14.   Statistical analysis

Data for isobologram were analysed as described elsewhere [236]. When the observed data points of the combinations mainly fell in the area of supraadditivity or in the areas of subadditivity and protection, i.e. the mean value of the data was smaller than that of the predicted minimum values or greater than that of the predicted maximum values, the combinations were considered to have a synergistic or antagonistic effect, respectively. To determine whether the condition of synergism (or antagonism) truly existed, a statistical analysis was performed. The Wilcoxon signed-ranks test was used for comparing the observed data with the predicted minimum or maximum values for additive effects, which were closest to the observed data. Probability (P) values of <0.05 were considered significant. Combinations with P   0.05 were regarded as indicating additive to synergistic or additive to antagonistic effects. The other data were analysed using Student's t test.

RESULTS

1. Destabilization of FLT3 by EGCG

1.1. Phosphorylation status of FLT3 is critical for its interaction with Hsp90

Mutant FLT3 has been demonstrated to be a *bona fide* client protein for Hsp90 in cell models and primary AML cells [3,4,79,81,82]. On the other hand, in the literature, the chaperoning of FLT3-WT by Hsp90 is still controversial topic [3,4]. To address whether FLT3-WT requires Hsp90 as a molecular chaperone, I performed co-IP using AML cells carrying FLT3-WT (THP-1), FLT3-ITD (MOLM-13; MV4-11) and FLT3-D835E (KOCL-48 cells) as well as 293FT cells transiently over-expressing FLT3-WT or FLT3 mutations. Cell lysates from these cells were co-immunoprecipitated with Hsp90 antibody and then immunoblotted (IB) with FLT3 antibody. Notably, parental 293FT did not express FLT3 (Fig. 4A, lane 6). In agreement with previous reports, mutant FLT3s (FLT3-ITD and FLT3-TKD) were shown to interact with Hsp90 in both AML cell lines and 293FT cells expressing FLT3-ITD and FLT3-TKD (Fig. 4A), indicating that mutant FLT3s are client proteins of Hsp90. Interestingly, endogenous FLT3-WT did not co-immunoprecipitate with Hsp90 in THP-1 cells (Fig. 4A, lane 4) but ectopically-expressed FLT3-WT in 293FT associated with Hsp90 (Fig. 4A, lane 7). Figure 4B shows that endogenous FLT3-WT in THP-1 was not phosphorylated, whereas ectopically-expressed FLT3-WT in 293FT was phosphorylated. Furthermore, constitutively de-phosphorylated form of FLT3/K644R did not co-immunoprecipitate with Hsp90 in 293FT cells (Fig.4C, lane 2, 4 and 6). These data indicated that Hsp90 selectively interacts with phosphorylated FLT3. Thus, it is highly possible that the phosphorylation status of FLT3 is critical for its interaction with Hsp90.

1.2. EGCG suppressed cell growth of FLT3-over-expressing AML cells

To examine whether EGCG can suppress the growth of AML cell lines, MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells were incubated either with DMSO alone (control) or with different concentrations of EGCG for 72 h. Cell proliferation was evaluated using the trypan blue exclusion test. The results showed that cells carrying mutant FLT3 (MOLM-13, MOLM-14, MV4-11 and KOCL-48) were more sensitivity to EGCG in comparison with THP-1 cells expressing FLT3-WT (Fig. 5A). Interestingly, 32D cells stably expressing FLT3-WT were as sensitive to EGCG as those stably expressing mutant FLT3 (Fig. 5B). Along with the data presented in Fig. 4B, these data indicated that the sensitivity of FLT3-expressing cells to EGCG could be dependent on FLT3 phosphorylation status. Altogether, EGCG exerted the anti-proliferation effects FLT3-phosphorylated cells.

1.3. EGCG induced apoptosis in FLT3-mutated AML cells

EGCG not only exerts a growth-inhibitory effect but also induces apoptosis in many cancer cell lines [178,179]. Next, I demonstrated that EGCG induced apoptosis in FLT3-mutated cell lines. First, Annexin V labeling analysis indicated that EGCG was significantly triggered apoptotic cell death in MOLM-14 cells (Fig. 6A). Second, I checked the appearance of apoptotic markers after treatment with or without EGCG using western blot analysis in MOLM-14 and MOLM-13 cells. The bands of cleaved-C9, cleaved-caspase-3 and cleaved-PARP were observed after 8 h treatment with EGCG (Fig. 6B and C). Moreover, EGCG treatment caused suppressed expression of anti-apoptotic molecules such as survivin and XIAP in MOLM-14 cells (Fig. 6B). Finally, nuclei fragmentation was observed in MOLM-13 cells after 8 h treatment with 60 μM EGCG (Fig. 6D). Overall, EGCG induced apoptosis in MOLM-14 and MOLM-13 cells.

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1.4. Down-regulation of FLT3 expression in EGCG-treated AML cells

FLT3 plays a pivotal role in the proliferation and survival of leukaemic cells. To investigate whether EGCG treatment may affect the expression of FLT3, I analysed the expression of FLT3 by western blotting in MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells treated with or without EGCG. Interestingly, the expression of FLT3 significantly decreased after treating MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells (FLT3-mutated cells) for 8 h with different concentrations of EGCG (Fig. 7A). EGCG also suppressed FLT3 expression in 293FT and 32D cells expressing FLT3-ITD and FLT3-D835V (Fig. 7B and C) Interestingly, in THP-1 cells (FLT3-WT cells), the expression of FLT3 did not change even at high EGCG concentrations (180 µM) (Fig. 7A). However, EGCG inhibited FLT3-WT expression in 293FT and 32D cells expressing FLT3-WT as effectively as in those expressing FLT3-ITD and FLT3-D835V in both dose- and time-dependent manners (Fig. 7B, C and D). These data indicated that EGCG exerts its inhibitory effect on FLT3 expression.

1.5. EGCG disrupts interaction between FLT3 and Hsp90

EGCG has been demonstrated to be an inhibitor of Hsp90 [2]. In this study, I demonstrated that phosphorylated FLT3 is a client of Hsp90 (Fig. 4) and that EGCG treatment affects FLT3 expression (Fig. 7). These data suggest that Hsp90 is involved in down-regulating FLT3 expression by EGCG. To confirm this hypothesis, total cell lysates of MOLM-13 and 293FT expressing FLT3-ITD or WT were collected after EGCG treatment and immunoprecipitated with antibodies as shown in Fig. 8B. IP results showed that EGCG treatment disrupted the association between Hsp90 with FLT3 and that it resulted in reducing FLT3 expression (Fig. 8B). I have confirmed that Hsp90 expression was not affected by EGCG treatment (Fig. 8A). The results confirmed aforementioned hypothesis.

1.6. Phosphorylation status of FLT3 downstream molecules in EGCG-treated AML cells

It is assumed that the down-regulation of FLT3 could lead to the inhibition of its activity, and subsequently suppress the activity of its downstream molecules. To confirm this, I measured the phosphorylation status of MAPK, AKT and STAT5 in MOLM-13, MOLM-14, MV4-11, KOCL-48 and THP-1 cells after treatment with or without EGCG. The inhibition of MAPK, AKT and STAT5 phosphorylation (p-MARK, p-AKT and p-STAT5) was observed in MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells after 8 h EGCG treatment (Fig. 9). Although EGCG caused the suppression of cell growth in THP-1 cells ($IC_{50} \approx 60$ µM EGCG, Fig. 5A), phosphorylation of MAPK but not that of AKT or STAT5 decreased at very high concentrations of EGCG (180 µM) (Fig. 9).

1.7. Growth inhibitory effects of EGC, ECG and catechin on FLT3-mutated AML cells

In contrast to EGCG, studies on other GTCs such as ECG, EGC and catechin have rarely been reported, particularly regarding their biological activity mechanisms. In this study, I attempted to test the inhibitory effects of EGC, ECG and catechin on the growth of AML cell lines. MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells were incubated with either DMSO alone (control) or different concentrations of reagents for 72 h. Cell proliferation was evaluated using the trypan blue exclusion test. The results showed that EGC and ECG significantly inhibited the cell proliferation of MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells in a dose-dependent manner (Fig. 10B and C). Whereas, catechin had no significant affect cell growth inhibition (Fig. 10D). Among GTCs, EGCG along with EGC had stronger

anti-cancer effects than others (EGCG ≈ EGC > ECG > catechin). These data indicated that the cell growth inhibitory activity of GTCs is polyphenol-dependent.

1.8.   EGC and ECG induced apoptosis in FLT3-mutant AML cells.

Next, I demonstrated that EGC and ECG induced apoptosis in FLT3-mutated cell lines. I investigated the appearance of apoptotic markers after EGC or ECG treatment by western blotting in MOLM-14 cells. The bands of cleaved-C9, cleaved-caspase-3 and cleaved-PARP were observed after 8 h EGC or ECG treatment (Fig. 11A). Moreover, EGC or ECG treatment resulted in the inhibition of anti-apoptotic molecules such as survivin and XIAP in MOLM-14 cells (Fig. 11A).

The results from PE-Annexin V staining (Fig. 11B) indicated that EGC and ECG induced apoptosis in MOLM-14 cells treated with EGC or ECG. Overall, as with EGCG, EGC and ECG also induced apoptosis in MOLM-14 cells.

1.9.   Down-regulation of FLT3 expression and its downstream molecules in EGC- and ECG-treated AML cells

To examine whether EGC and ECG suppress the expression of FLT3, MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells were treated with 100 µM EGC or 100/200 µM ECG for 8 h. Western blotting was performed to analyse the expression of FLT3. EGC-treated cells showed not only the suppression of FLT3 expression, but also the suppression of phosphorylation of MAPK, AKT and STAT5 (Fig. 12A). Similarly, the suppression of FLT3 expression and phosphorylation of its downstream molecules were also observed in ECG-treated cells (Fig. 12B).

1.10. EGC and ECG suppressed FLT3 expression through Hsp90

Total cell lysates of MOLM-13 cells after EGC or EGC treatment were collected and immunoprecipitated with antibodies as shown in Fig. 13. IP results showed that EGC and ECG treatment disrupted the association between Hsp90 and FLT3 resulting in a reduction of FLT3 expression (Fig. 13).

2. Suppression of FLT3 gene expression by EGCG

Next, I investigated the effects of EGCG on transcriptional regulation of *FLT3*. *FLT3* mRNA level was evaluated in MOLM-13, MOLM-14, MV4-11, KOCL-48 and THP-1 cells with or without EGCG (60 µM) treatment for 8 h. *FLT3* mRNA level significantly reduced on EGCG treatment in MOLM-13 cells (Fig. 14A). Using the luciferase-based *FLT3* promoter reporter system, I also demonstrated that EGCG significantly inhibited FLT3 promoter activity (Fig. 14B).

3. Cytotoxic effects of EGCG in combination with PKC412

EGCG is one of the most studied polyphenol of green tea and shows the strongest anti-cancer effects among other GTCs. In this study, I evaluated the combined effects of EGCG and the FLT3 inhibitor PKC412. Interestingly, the results showed that the combination of EGCG and PKC412 treatment strongly suppressed cell growth in MOLM-13, KOCL-48, MV4-11 and MOLM-14 cells compared with EGCG or PKC412 treatment alone (Fig. 15). However, the combined effects of simultaneous exposure to these differed among the cell lines. In MOLM-13 and MOLM-14 cells, the combined data points fell within the envelope of additivity. The mean values of the data (0.423 and 0.414, respectively) were greater than those of the predicted minimum values (0.379 and 0.121, respectively) and smaller than those of the predicted maximum values for the additive effect (0.766 and 0.549, respectively; Table 1), indicating that simultaneous exposure to EGCG and PKC412

produced an additive effect. In KOCL-48 and MV4-11 cells, the combined data points fell mainly in the area of subadditivity, and the mean values of the data (0.887 and 0.803, respectively) were greater than those of the predicted maximum additive values (0.410 and 0.579, respectively; Table 1), which were regarded as the antagonism effect.

DISCUSSION

1.   Phosphorylation status of FLT3 is critical for its interaction with Hsp90

In this study, I showed for the first time that FLT3 phosphorylation is required for its interaction with Hsp90, regardless of FLT3-mutants or -WT (Fig. 4A and B). De-phosphorylated FLT3 was unable to bind to Hsp90 (Fig. 4C). This study sheds light onto the controversy surrounding the chaperoning of FLT3-WT by Hsp90. Although a study revealed the association between FLT3-ITD and Hsp90 in 2002 [81], the interaction between FLT3-WT and Hsp90 has not yet been clarified because of the lack of reliable evidence regarding their interaction.

It is noteworthy that approximately 10%–15% AML patients display high FLT3-WT expression [20]. The high levels of FLT3-WT receptor may promote constitutive activation of the WT receptor in malignant cells [237]. In primary AML cells, the tyrosine phosphorylated of over-expression FLT3-WT is rarely detected (3/27 patients with FLT3-WT over-expression) [238]. The phosphorylation of FLT3-WT can be detected using western blotting if its transcription level is up to 200,000 copies in 1 μg RNA [238]. Therefore, it is too difficult to observe the Hsp90–FLT3-WT complex in blast cells of AML patients [3]; however, this is achievable in cell lines over-expressing FLT3-WT, e.g. SEMK2 cells [82]. SEMK2 cells have extremely high level of FLT3 expression and also have a high level of phosphorylated FLT3 due to FLT3 locus amplification [237]. Therefore, the phosphorylation status of FLT3-WT could endow it to form a complex with Hsp90 in SEMK2 cells [82]. Moreover, the Hsp90–FLT3-WT complex has been detected in Ba/F3- and 32D-expressing FLT3-WT cells [4,82]. In these cells, the growth of FLT3-WT-

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transduced Ba/F3 and 32D cells was conferred by FLT3-WT signalling (phosphorylated FLT3) in the presence of FL ligand [239].

Interestingly, there is no controversy regarding the existence of an interaction between FLT3-ITD or FLT3-TKD and Hsp90. Most reports have detected the binding complex between mutant FLT3 and Hsp90 [1,3,4,79,81-83]. This might because mutant FLT3 is closely associated with high expression levels of the FLT3 transcript [238] and is highly activated [240].

Moreover, treating cells carrying FLT3-ITD with the FLT3 inhibitor sorafenib, which inhibits FLT3-ITD activation, reduced the interaction between Hsp90 and FLT3 [4]. These data indicated that FLT3 activity may be necessary for its interaction with Hsp90.

Previous reports have shown that the auto-phosphorylation of FLT3-WT caused by its over-expression is inhibited by a potent FLT3 kinase inhibitor at the same sensitivity as that of mutated FLT3 [238]. Other studies have provided clear evidence that high FLT3 expression has a negative impact on overall and event-free survival in cytogenetically normal AML patients lacking FLT3 mutations [21,238]. More recently, high transcript levels of *FLT3* were associated with high risk of relapse in paediatric AML patients [241]. In this study, I found that phosphorylated FLT3-WT was stabilized by Hsp90 and so was mutant FLT3 (Fig. 4). These data indicated that the over-expressed phosphorylated FLT3-WT possesses the same clinical characteristics as those possessed by FLT3-ITD. Therefore, my findings suggest that the over-expression of phosphorylated FLT3-WT might consider as the same group with mutant FLT3.

Another study revealed that the association between Hsp90 and kinase proteins is determined by the intrinsic stability of the kinase domain [79]. A two-step model for the recognition of Hsp90 client proteins has been developed: first, CDC37 provides recognition

of the kinase family and then, thermodynamic parameters determine client binding within the family [79]. This model is consistent with known differences in the Hsp90 machinery between prokaryotic and eukaryotic cells: prokaryotes do not have CDC37 and prokaryotic Hsp90 does not chaperone kinases [242]; CDC37 is universally employed by Hsp90 for all kinases [79]. In addition, the difference in thermal stability of the kinase constructs were correlated with the interaction score of kinases to Hsp90, for example the T_m of Abl-WT was 41.5°C; for the Abl-M472I mutant, it was 38.0°C, and for the Abl-M244I mutant, it was 45.4°C. The interaction score of them to Hsp90 have been reported to be 3.44, 1.72 and 6.88, respectively [79]. Therefore, the thermodynamic stability but not the primary amino acid sequence is the key determinant in the association of kinase clients with Hsp90 [79]. However, there is no evidence about the difference in thermal stability of the phosphorylated and non-phosphorylated kinases particularly FLT3-WT, therefore to conclude whether thermal stability is the only determinant for interaction between FLT3 and Hsp90 demands further studies.

The majority of Hsp90-interacting tyrosine kinases are activating; mutant kinases with decreased activity when compared to their WT counterparts have also been reported to be Hsp90 clients. For example, B-RAF mutants that have reduced kinase activity displayed enhanced sensitivity towards Hsp90 inhibitor-mediated degradation [243]. Similarly, kinase-defective ERBB2 is an Hsp90 client, indicating that the activation status may not be the sole determining factor for the recognition of client proteins by Hsp90 [244]. Interestingly, in my experiments, the FLT3-WT/ITD/D835V-K644R mutations that lack the kinase activity did not form complexes with Hsp90 (Fig. 4C). The limitation of my data is that the exact determinant factor for the interaction between FLT3 and Hsp90 was not so clear due to the confusion between the phosphorylation status and kinase activity of FLT3-K644R mutation.

The FLT3 WT/ITD/D835V-K644R mutations lack kinase activity; thus, they could not phosphorylate themselves. Further examinations need to be performed for confirmation. Regardless of this limitation, the discrepancy in my observations once again indicated that the determinant for binding to Hsp90 varies among different kinases. In case of FLT3, I found that the phosphorylation status of FLT3 is a determinant for its interaction with Hsp90 (Fig. 4).

2. EGCG-regulated FLT3 expression: the possible mechanism

So far, my group has demonstrated that EGCG suppressed cell proliferation and caused apoptotic cell death in GIST cells by the inhibition of KIT activity [179]. In this study, I have reported that polyphenols of green tea caused apoptotic cell death in *FLT3*-mutated cell lines (Fig. 5, 6, 10 and 11) by disrupting the interaction between Hsp90 and FLT3 (Fig. 8 and 13), leading to down-regulating the expression of FLT3 (Fig. 7 and 12) and ultimately suppressing the activity of AKT, MAPK and STAT5 (Fig. 9 and 12).

Recent studies have indicated that RTKs are one of the critical targets of EGCG to inhibit cancer cell growth. Previous studies have provided evidence that EGCG inhibited the activation or expression of some RTKs including EGFR [172], HER2, HER3 [173,174], VEGFR [175], PDGFR [245], FGFR [172] and IGF-1R [177,184]. In this study, I have shown that EGCG suppressed FLT3 expression in cell lines harbouring FLT3 mutations (Fig. 7) but not in THP-1 cells carrying FLT3-WT (Fig. 7A). However, in transient 293FT- and 32D-stably expressing FLT3-WT, EGCG could inhibit FLT3-WT expression as effectively as in those expressing FLT3-ITD and FLT3-D835V (Fig. 7 B, C and D).

EGCG has been demonstrated as a potential inhibitor of Hsp90 [2]. EGCG acts by binding at or near a C-terminal ATP binding site to inhibit dimerization and promote an Hsp90 conformation that interferes with its chaperone activity for client proteins [2]. EGCG was

shown to inhibit Hsp90 function by impairing Hsp90 association with co-chaperones including Hsc70 and p23 in the pancreatic cancer cell line MIA PaCa-2 [182]. In 2010, Tran *et al.* reported that EGCG specifically inhibits the expression of Hsp90 by inhibiting the promoter activity of Hsp90 in MCF-7 human breast cancer cells [183]. Figure 8A shows that EGCG did not affect the expression of Hsp90 but disrupted the interaction between Hsp90 and FLT3 (Fig. 8B). Regarding the insensitivity of EGCG in THP-1 cells, I assume that this is mainly because FLT3-WT in THP-1 cells is not phosphorylated, and thereby, it is not able to bind or has little binding effect to Hsp90 (Fig. 4). As a result, inhibition of Hsp90 function by EGCG could not effectively suppress FLT3 expression in THP-1 cells.

Another study found that short-term exposure to EGCG (within 2 h) inhibited EGF-induced EGFR phosphorylation and its downstream signalling pathways but long-term exposure to EGCG (> 24 h) decreased the expression levels of EGFR in human lung cancer cells [246]. Although, EGFR is not phosphorylated in A549 lung cancer cells, EGFR-WT has been reported to interact with Hsp90 [247] and non-activated EGFR is inhibited by EGCG [246]; this indicates that EGCG tends to target Hsp90 functions rather than the activity of kinase proteins. In the current study, short-term incubation (2 h) of MOLM-13 cells with 60 µM EGCG did not significantly affect the phosphorylation of FLT3 (data not shown). Therefore, reduction in kinase activation along with down-regulation of kinase protein levels on long-term incubation with EGCG may be due to the dissociation of kinase clients from Hsp90 as a result of Hsp90 function disruption by EGCG.

Recent studies have reported that EGCG can bind to its receptor 67LR, which is related to cancerometastasis [248-252]. 67LR has been originally identified as a non-intergrin cell-surface receptor for the extracellular matrix molecule laminin, over-expressed in various cancer types [253]. Further, EGCG has been shown to induce cell death in HL-60 cells by

inducing the 67LR/Akt/ eNOS/cGMP pathway [254]. However, Adachi *et al.* indicated that EGCG may not directly bind to the cell surface receptor [195]. Moreover, when EGCG is incubated with cells, 75% radioactively labelled EGCG was found in the cytosolic compartment while some radioactivity was found in the membrane fraction [193]. This suggests that the majority of EGCG permeates into the cytoplasm, where it directly interacts with Hsp90 and other cytoplasmic molecules with a high possibility. Furthermore, only EGCG but not EGC, ECG and other GTCs was shown to bind to 67LR [248]; my report found that other GTCs have anti-tumour effects similar to those possessed by EGCG (Fig. 10). Therefore, 67LR is not the sole pathway for EGCG and other GTCs mediated its effect. In contrast to EGCG, studies on ECG, EGC and EC have rarely been reported, particularly pertaining to their biological activity mechanism. In my data, I for the first time suggest that EGC and ECG also suppress FLT3 expression through inhibition of Hsp90 function in the same manner as that by EGCG (Fig. 10-13).

The presence of the three adjacent hydroxyl (OH) groups in the molecule was suggested as a key factor for enhancing the activity of EGCG in cancer cells [255,256]. Compounds having a galloyl moiety show more potent activity [255]. In my study, I found that the cell growth inhibitory activity of GTCs is structure-dependent (Fig. 10). The three adjacent OH groups at position C-3´, 4´ and 5´ on the B ring of EGCG and EGC are more effective in suppressing cell proliferation and inducing cell death than the two adjacent OH groups at C-3´ and 4´ of ECG and catechin (Fig. 10 and 11). Moreover, the additional gallate moiety in ECG at C-3 generally endows it with stronger anti-tumour effects than non-gallate compound such as catechin (Fig. 10C and D).

Previous studies have shown that the anti-cancer effects of various GTCs vary depending on the type and stage of malignancy [257,258]. Among GTCs, ECG is more effective than

EGCG in suppressing the growth of gender-based carcinomas [257]. In contrast, recent studies of human melanoma cell lines have indicated that EGCG is more potent than other GTCs [258]. The results of my present investigation demonstrated that EGCG along with EGC has stronger anti-cancer effects than other GTCs (EGCG ≈ EGC > ECG > catechin) in leukaemic cells (Fig. 10). These investigations indicated that anti-cancer action of various GTCs vary with the type of malignancy and provides a model for tumour cell heterogeneity based on susceptibility and resistance of tumour cells to different GTCs. Therefore, this information is critical for undertaking chemopreventive or chemotherapeutic trials against cancers.

To examine whether the down-regulation of FLT3 by EGCG was partly caused by reducing its transcriptional level, a transient transfection study using the *FLT3* promoter-reporter construct was performed. A portion of the 5 -flanking region of *FLT3* was cloned. As shown in Fig. 14B, EGCG inhibited *FLT3* promoter activity. However, the mRNA of *FLT3* was only significantly suppressed by EGCG in MOLM-13 cells but not in other cells (Fig. 14A).

3.   Cytotoxic effects of EGCG in combination with PKC412

AML patients of all World Health Organization (WHO) subtypes other than t(15;17) received 7+3 induction therapy that combines Ara-C (cytarabine; 7 days, continuous intravenous) with an anthracycline such as daunorubicin or doxorubicin (adriamycin; 3 days, intravenous push) [259]. Consolidation therapy followed in a cytogenetically-directed manner, with the preferred treatment being either further intensive chemotherapy for good-prognosis cases, such as Core Binding Factor rearrangements, allogenic stem-cell transplantation for poor-prognosis cases such as those with complex cytogenetic, or a less clearly defined set of options for intermediate-prognosis cases, including normal cytogenetic

AML [260-262]. Maintenance therapy for AML is not the current standard of care because of a lack of efficacy. Patients in relapse have limited treatment options, and outcome is uniformly poor [263,264]. However, the high-dose cytarabine plus anthracycline/anthracenedione treatment can become ineffective because of increased drug resistance [265]. In some cases, chemotherapy has to be discontinued because of adverse effects, which include bone marrow depression induced by indiscriminate killing of cells.

Most patients respond to induction therapy and enter remission with undetectable levels of leukaemic blasts in the bone marrow. However, without further treatment, relapse is inevitable for the vast majority of patients. Additional therapeutics, in particular those that target known oncogenic signals in individual AML (personalized therapy), could potentially delay or prevent relapse [266,267]. New treatment regimens are needed for relapse cases, particularly in cases in which the leukaemia has developed resistance to chemotherapy. In addition, even in those patients with relapsed leukaemia who remain sensitive to standard chemotherapy, treatment may be complicated by toxicities associated with exceeding safe cumulative doses of individual compounds, particularly anthracyclines [268]. This further highlights the need for novel agents.

FLT3 inhibition has become a legitimate therapeutic option, and clinical trials of FLT3 inhibitors in AML have been ongoing for a decade [115-117]. To date, more than 20 small molecule inhibitors against FLT3 have been reported; some of them have been evaluated in clinical trials [118]. Although these inhibitors have been shown to be efficacious against FLT3-activated cell lines *in vitro* and in preclinical efficacy models, clinical results to date have been disappointing due to undesirable drug properties, dose-limiting toxicity and lack of durable responses in patients with AMLs [119,269].

One of the potential effective strategies for treating AML patients with *FLT3* mutations is combination therapy with inhibitors of FLT3, and alternative pathways [41] to target multiple pathogenetic signalling pathways have been reported with a view to overcoming resistance of the FLT3-ITD/TKD double mutant clones. For instance, resistance to FLT3 inhibition has been associated with the up-regulation of STAT5-mediated PIM (proviral integration site for Moloney-murine leukaemia virus 1) expression, which is an anti-apoptotic effector. Recent reports have demonstrated that PIM inhibition suppresses the growth of FLT3-ITD+ cell lines and may function synergistically with FLT3 inhibitors *in vitro* [270]. On the basis of the inhibitory activity of arsenic trioxide on ERK activity, a downstream effector of FLT3 activation, combination of arsenic trioxide and the FLT3 inhibitor AG1296 has been evaluated [271]. The combination synergistically suppresses the growth and induces apoptosis in FLT3-ITD+ cell lines. Although these therapeutic concepts have yet to be tested in clinical trials, they provide proof-of-principle observations that combinatorial targeting of multiple pathways may be exploited for the treatment of drug-resistant double mutants. More candidate targets should be identified either by rational design of regimen or high-throughput chemical screening.

The advantage of studying the effects of EGCG lies in knowing that the Japanese customarily ingest EGCG in green tea every day. It is thought to be a non-toxic agent and is now developing as a cancer preventive drug in the USA and Europe [272,273].

A number of publications have reported the effectiveness of using EGCG in combination with other drugs [153,249]. For example, in many cancer cell types, EGCG was shown to up-regulate the expression of PPAR [153]; therefore, the combination of EGCG and the agonist of PPAR clofibrate improved the anti-cancer effects of EGCG in comparison with treatment using EGCG or clofibrate alone [153]. Other reports have found that PDE5, a

cGMP negative regulator, was over-expressed in AML cell;, therefore, combination with PDE5 inhibitor significantly enhanced the anti-AML effects of EGCG at plasma levels, as observed in clinical trials [253,274]. Moreover, phase I combination between EGCG with standard chemo-radiation in surgically unresectable stage III non-small-cell lung cancer has been reported [275].

In this study, I combined EGCG with PKC412 to evaluate their combined effects on *FLT3*-mutated cell lines. The concentration of both drugs significantly reduced (Fig. 15) as compared with using EGCG or PKC412 alone. For example, the concentration of EGCG was 15 µM for reaching 50% cell-growth inhibition in MOLM-13 cells, however, when combined with PKC412, it reduced to 10 µM (with 5 nM PKC412) and even 5 µM (with 7 nM PKC412). Similarly, the concentration of PKC412 alone for reaching 50% cell-growth inhibition in MOLM-13 cells was 20 nM, however, when combined with EGCG, it remarkably reduced to 5 nM (with 10 µM EGCG).

Taken together, the combination of EGCG and PKC412 fetched good but not so impressive results; further candidates should be tested in combination with EGCG.

CONCLUSION

In conclusion, I clarified that EGCG, EGC and ECG destabilized FLT3 by disrupting its interaction with the molecular chaperone Hsp90 (Fig. 16). I also provided evidence that EGCG suppresses *FLT3* promoter activity and its transcription. Thus, EGCG is an effective inhibitor of FLT3 at transcription and protein levels. Here I propose that EGCG can be a useful therapeutic agent for AML patients particularly when used in combination with other drugs such as the FLT3 inhibitor PKC412. My data suggest that EGCG is a promising candidate for the treatment of AML or for combination with other drugs such as FLT3 inhibitors to improve the overall treatment efficacy.

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REFERENCES

1. Ly BT, Chi HT, Yamagishi M, Kano Y, Hara Y, et al. (2013) Inhibition of FLT3 expression by green tea catechins in FLT3 mutated-AML cells. PLoS One 8: e66378.
2. Yin Z, Henry EC, Gasiewicz TA (2009) (-)-Epigallocatechin-3-gallate is a novel Hsp90 inhibitor. Biochemistry 48: 336-345.
3. Al Shaer L, Walsby E, Gilkes A, Tonks A, Walsh V, et al. (2008) Heat shock protein 90 inhibition is cytotoxic to primary AML cells expressing mutant FLT3 and results in altered downstream signalling. Br J Haematol 141: 483-493.
4. Oshikawa G, Nagao T, Wu N, Kurosu T, Miura O (2011) c-Cbl and Cbl-b ligases mediate 17-allylaminodemethoxygeldanamycin-induced degradation of autophosphorylated Flt3 kinase with internal tandem duplication through the ubiquitin proteasome pathway. J Biol Chem 286: 30263-30273.
5. Network CGAR (2013) Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. N Engl J Med 368: 2059-2074.
6. Rosnet O, Marchetto S, deLapeyriere O, Birnbaum D (1991) Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family. Oncogene 6: 1641-1650.
7. Rosnet O, Mattei MG, Marchetto S, Birnbaum D (1991) Isolation and chromosomal localization of a novel FMS-like tyrosine kinase gene. Genomics 9: 380-385.
8. Rosnet O, Schiff C, Pebusque MJ, Marchetto S, Tonnelle C, et al. (1993) Human FLT3/FLK2 gene: cDNA cloning and expression in hematopoietic cells. Blood 82: 1110-1119.
9. Matthews W, Jordan CT, Wiegand GW, Pardoll D, Lemischka IR (1991) A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. Cell 65: 1143-1152.
10. Markovic A, MacKenzie KL, Lock RB (2005) FLT-3: a new focus in the understanding of acute leukemia. Int J Biochem Cell Biol 37: 1168-1172.
11. Agnes F, Shamoon B, Dina C, Rosnet O, Birnbaum D, et al. (1994) Genomic structure of the downstream part of the human FLT3 gene: exon/intron structure conservation among genes encoding receptor tyrosine kinases (RTK) of subclass III. Gene 145: 283-288.
12. Rosnet O, Birnbaum D (1993) Hematopoietic receptors of class III receptor-type tyrosine kinases. Crit Rev Oncog 4: 595-613.
13. Scheijen B, Griffin JD (2002) Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease. Oncogene 21: 3314-3333.
14. Griffith J, Black J, Faerman C, Swenson L, Wynn M, et al. (2004) The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell 13: 169-178.
15. Maroc N, Rottapel R, Rosnet O, Marchetto S, Lavezzi C, et al. (1993) Biochemical characterization and analysis of the transforming potential of the FLT3/FLK2 receptor tyrosine kinase. Oncogene 8: 909-918.
16. Brasel K, Escobar S, Anderberg R, de Vries P, Gruss HJ, et al. (1995) Expression of the flt3 receptor and its ligand on hematopoietic cells. Leukemia 9: 1212-1218.

17. Turner A, Lin N, Issarachai S, Lyman S, Broudy V (1996) FLT3 receptor expression on the surface of normal and malignant human hematopoietic cells. Blood 88: 3383-3390.
18. Kikushige Y, Yoshimoto G, Miyamoto T, Iino T, Mori Y, et al. (2008) Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. J Immunol 180: 7358-7367.
19. Stirewalt DL, Radich JP (2003) The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer 3: 650-665.
20. Riccioni R, Pelosi E, Riti V, Castelli G, Lo-Coco F, et al. (2011) Immunophenotypic features of acute myeloid leukaemia patients exhibiting high FLT3 expression not associated with mutations. Br J Haematol 153: 33-42.
21. Kuchenbauer F, Kern W, Schoch C, Kohlmann A, Hiddemann W, et al. (2005) Detailed analysis of FLT3 expression levels in acute myeloid leukemia. Haematologica 90: 1617-1625.
22. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, et al. (1994) Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature 368: 643-648.
23. Lyman SD, James L, Vanden Bos T, de Vries P, Brasel K, et al. (1993) Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells. Cell 75: 1157-1167.
24. Wodnar-Filipowicz A (2003) Flt3 ligand: role in control of hematopoietic and immune functions of the bone marrow. News Physiol Sci 18: 247-251.
25. Lyman SD, James L, Escobar S, Downey H, de Vries P, et al. (1995) Identification of soluble and membrane-bound isoforms of the murine flt3 ligand generated by alternative splicing of mRNAs. Oncogene 10: 149-157.
26. Lyman SD, James L, Johnson L, Brasel K, de Vries P, et al. (1994) Cloning of the human homologue of the murine flt3 ligand: a growth factor for early hematopoietic progenitor cells. Blood 83: 2795-2801.
27. Pfister O, Chklovskaia E, Jansen W, Meszaros K, Nissen C, et al. (2000) Chronic overexpression of membrane-bound flt3 ligand by T lymphocytes in severe aplastic anaemia. Br J Haematol 109: 211-220.
28. McKenna HJ, Stocking KL, Miller RE, Brasel K, De Smedt T, et al. (2000) Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood 95: 3489-3497.
29. Meierhoff G, Dehmel U, Gruss HJ, Rosnet O, Birnbaum D, et al. (1995) Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines. Leukemia 9: 1368-1372.
30. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, et al. (1996) Internal tandem duplication of the flt3 gene found in acute myeloid leukemia. Leukemia 10: 1911-1918.
31. Marcucci G, Haferlach T, Dohner H (2011) Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. J Clin Oncol 29: 475-486.
32. Hayakawa F, Towatari M, Kiyoi H, Tanimoto M, Kitamura T, et al. (2000) Tandem-duplicated Flt3 constitutively activates STAT5 and MAP kinase and introduces autonomous cell growth in IL-3-dependent cell lines. Oncogene 19: 624-631.

33. Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, et al. (2000) Flt3 mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. Blood 96: 3907-3914.

34. Brandts CH, Sargin B, Rode M, Biermann C, Lindtner B, et al. (2005) Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res 65: 9643-9650.

35. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, et al. (2002) Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood 99: 4326-4335.

36. Schnittger S, Bacher U, Haferlach C, Alpermann T, Kern W, et al. (2012) Diversity of the juxtamembrane and TKD1 mutations (exons 13-15) in the FLT3 gene with regards to mutant load, sequence, length, localization, and correlation with biological data. Genes Chromosomes Cancer 51: 910-924.

37. Kayser S, Schlenk RF, Londono MC, Breitenbuecher F, Wittke K, et al. (2009) Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood 114: 2386-2392.

38. Breitenbuecher F, Schnittger S, Grundler R, Markova B, Carius B, et al. (2009) Identification of a novel type of ITD mutations located in nonjuxtamembrane domains of the FLT3 tyrosine kinase receptor. Blood 113: 4074-4077.

39. Schnittger S, Schoch C, Dugas M, Kern W, Staib P, et al. (2002) Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood 100: 59-66.

40. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, et al. (2001) Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood 97: 2434-2439.

41. Leung AY, Man CH, Kwong YL (2013) FLT3 inhibition: a moving and evolving target in acute myeloid leukaemia. Leukemia 27: 260-268.

42. Reindl C, Bagrintseva K, Vempati S, Schnittger S, Ellwart JW, et al. (2006) Point mutations in the juxtamembrane domain of FLT3 define a new class of activating mutations in AML. Blood 107: 3700-3707.

43. Williams AB, Nguyen B, Li L, Brown P, Levis M, et al. (2013) Mutations of FLT3/ITD confer resistance to multiple tyrosine kinase inhibitors. Leukemia 27: 48-55.

44. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, et al. (2002) Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. Blood 100: 4372-4380.

45. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, et al. (1997) Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). Leukemia 11: 1447-1452.

46. Mead AJ, Linch DC, Hills RK, Wheatley K, Burnett AK, et al. (2007) FLT3 tyrosine kinase domain mutations are biologically distinct from and have a significantly more favorable prognosis than FLT3 internal tandem duplications in patients with acute myeloid leukemia. Blood 110: 1262-1270.

47. Scholl C, Gilliland DG, Frohling S (2008) Deregulation of signaling pathways in acute myeloid leukemia. Semin Oncol 35: 336-345.
48. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S (2008) Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. Blood 111: 2527-2537.
49. Whitman SP, Ruppert AS, Radmacher MD, Mrozek K, Paschka P, et al. (2008) FLT3 D835/I836 mutations are associated with poor disease-free survival and a distinct gene-expression signature among younger adults with de novo cytogenetically normal acute myeloid leukemia lacking FLT3 internal tandem duplications. Blood 111: 1552-1559.
50. Mrozek K, Marcucci G, Paschka P, Whitman SP, Bloomfield CD (2007) Clinical relevance of mutations and gene-expression changes in adult acute myeloid leukemia with normal cytogenetics: are we ready for a prognostically prioritized molecular classification? Blood 109: 431-448.
51. Whitman SP, Archer KJ, Feng L, Baldus C, Becknell B, et al. (2001) Absence of the wild-type allele predicts poor prognosis in adult de novo acute myeloid leukemia with normal cytogenetics and the internal tandem duplication of FLT3: a cancer and leukemia group B study. Cancer Res 61: 7233-7239.
52. Schlenk RF, Kayser S, Bullinger L, Kobbe G, Casper J, et al. (2014) Differential impact of allelic ratio and insertion site in FLT3-ITD positive AML with respect to allogeneic hematopoietic stem cell transplantation. Blood.
53. Dosil M, Wang S, Lemischka IR (1993) Mitogenic signalling and substrate specificity of the Flk2/Flt3 receptor tyrosine kinase in fibroblasts and interleukin 3-dependent hematopoietic cells. Mol Cell Biol 13: 6572-6585.
54. Zhang S, Mantel C, Broxmeyer HE (1999) Flt3 signaling involves tyrosyl-phosphorylation of SHP-2 and SHIP and their association with Grb2 and Shc in Baf3/Flt3 cells. J Leukoc Biol 65: 372-380.
55. Zhang S, Broxmeyer HE (2000) Flt3 ligand induces tyrosine phosphorylation of gab1 and gab2 and their association with shp-2, grb2, and PI3 kinase. Biochem Biophys Res Commun 277: 195-199.
56. Zhang S, Broxmeyer HE (1999) p85 subunit of PI3 kinase does not bind to human Flt3 receptor, but associates with SHP2, SHIP, and a tyrosine-phosphorylated 100-kDa protein in Flt3 ligand-stimulated hematopoietic cells. Biochem Biophys Res Commun 254: 440-445.
57. Srinivasa SP, Doshi PD (2002) Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways cooperate in mediating cytokine-induced proliferation of a leukemic cell line. Leukemia 16: 244-253.
58. Anjum R, Blenis J (2008) The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol 9: 747-758.
59. Bhaskar PT, Hay N (2007) The two TORCs and Akt. Dev Cell 12: 487-502.
60. Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 10: 307-318.
61. Kiyoi H, Towatari M, Yokota S, Hamaguchi M, Ohno R, et al. (1998) Internal tandem duplication of the FLT3 gene is a novel modality of elongation mutation which causes constitutive activation of the product. Leukemia 12: 1333-1337.

62. Ema H, Sudo K, Seita J, Matsubara A, Morita Y, et al. (2005) Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. Dev Cell 8: 907-914.

63. Takaki S, Morita H, Tezuka Y, Takatsu K (2002) Enhanced hematopoiesis by hematopoietic progenitor cells lacking intracellular adaptor protein, Lnk. J Exp Med 195: 151-160.

64. Velazquez L, Cheng AM, Fleming HE, Furlonger C, Vesely S, et al. (2002) Cytokine signaling and hematopoietic homeostasis are disrupted in Lnk-deficient mice. J Exp Med 195: 1599-1611.

65. Qian X, Ginty DD (2001) SH2-B and APS are multimeric adapters that augment TrkA signaling. Mol Cell Biol 21: 1613-1620.

66. Wakioka T, Sasaki A, Mitsui K, Yokouchi M, Inoue A, et al. (1999) APS, an adaptor protein containing Pleckstrin homology (PH) and Src homology-2 (SH2) domains inhibits the JAK-STAT pathway in collaboration with c-Cbl. Leukemia 13: 760-767.

67. Buza-Vidas N, Antonchuk J, Qian H, Mansson R, Luc S, et al. (2006) Cytokines regulate postnatal hematopoietic stem cell expansion: opposing roles of thrombopoietin and LNK. Genes Dev 20: 2018-2023.

68. Seita J, Ema H, Ooehara J, Yamazaki S, Tadokoro Y, et al. (2007) Lnk negatively regulates self-renewal of hematopoietic stem cells by modifying thrombopoietin-mediated signal transduction. Proc Natl Acad Sci U S A 104: 2349-2354.

69. Tong W, Zhang J, Lodish HF (2005) Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways. Blood 105: 4604-4612.

70. Gueller S, Gery S, Nowak V, Liu L, Serve H, et al. (2008) Adaptor protein Lnk associates with Tyr(568) in c-Kit. Biochem J 415: 241-245.

71. Gueller S, Goodridge HS, Niebuhr B, Xing H, Koren-Michowitz M, et al. (2010) Adaptor protein Lnk inhibits c-Fms-mediated macrophage function. J Leukoc Biol 88: 699-706.

72. Gueller S, Hehn S, Nowak V, Gery S, Serve H, et al. (2011) Adaptor protein Lnk binds to PDGF receptor and inhibits PDGF-dependent signaling. Exp Hematol 39: 591-600.

73. Lin DC, Yin T, Koren-Michowitz M, Ding LW, Gueller S, et al. (2012) Adaptor protein Lnk binds to and inhibits normal and leukemic FLT3. Blood 120: 3310-3317.

74. Mocsai A, Ruland J, Tybulewicz VL (2010) The SYK tyrosine kinase: a crucial player in diverse biological functions. Nat Rev Immunol 10: 387-402.

75. Pechloff K, Holch J, Ferch U, Schweneker M, Brunner K, et al. (2010) The fusion kinase ITK-SYK mimics a T cell receptor signal and drives oncogenesis in conditional mouse models of peripheral T cell lymphoma. J Exp Med 207: 1031-1044.

76. Kuno Y, Abe A, Emi N, Iida M, Yokozawa T, et al. (2001) Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12)(q22;p12). Blood 97: 1050-1055.

77. Puissant A, Fenouille N, Alexe G, Pikman Y, Bassil CF, et al. (2014) SYK is a critical regulator of FLT3 in acute myeloid leukemia. Cancer Cell 25: 226-242.

78. Sreedhar AS, Kalmar E, Csermely P, Shen YF (2004) Hsp90 isoforms: functions, expression and clinical importance. FEBS Lett 562: 11-15.

79. Taipale M, Krykbaeva I, Koeva M, Kayatekin C, Westover KD, et al. (2012) Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. Cell 150: 987-1001.

80. Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. Nat Rev Mol Cell Biol 11: 515-528.

81. Minami Y, Kiyoi H, Yamamoto Y, Yamamoto K, Ueda R, et al. (2002) Selective apoptosis of tandemly duplicated FLT3-transformed leukemia cells by Hsp90 inhibitors. Leukemia 16: 1535-1540.

82. Yao Q, Nishiuchi R, Li Q, Kumar AR, Hudson WA, et al. (2003) FLT3 expressing leukemias are selectively sensitive to inhibitors of the molecular chaperone heat shock protein 90 through destabilization of signal transduction-associated kinases. Clin Cancer Res 9: 4483-4493.

83. George P, Bali P, Annavarapu S, Scuto A, Fiskus W, et al. (2005) Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. Blood 105: 1768-1776.

84. Gwin K, Frank E, Bossou A, Medina KL (2010) Hoxa9 regulates Flt3 in lymphohematopoietic progenitors. J Immunol 185: 6572-6583.

85. Wang GG, Pasillas MP, Kamps MP (2006) Persistent transactivation by meis1 replaces hox function in myeloid leukemogenesis models: evidence for co-occupancy of meis1-pbx and hox-pbx complexes on promoters of leukemia-associated genes. Mol Cell Biol 26: 3902-3916.

86. Volpe G, Walton DS, Del Pozzo W, Garcia P, Dasse E, et al. (2013) C/EBPalpha and MYB regulate FLT3 expression in AML. Leukemia 27: 1487-1496.

87. Argiropoulos B, Humphries RK (2007) Hox genes in hematopoiesis and leukemogenesis. Oncogene 26: 6766-6776.

88. Pineault N, Helgason CD, Lawrence HJ, Humphries RK (2002) Differential expression of Hox, Meis1, and Pbx1 genes in primitive cells throughout murine hematopoietic ontogeny. Exp Hematol 30: 49-57.

89. Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J, et al. (2002) Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 99: 121-129.

90. Abramovich C, Humphries RK (2005) Hox regulation of normal and leukemic hematopoietic stem cells. Curr Opin Hematol 12: 210-216.

91. Roche J, Zeng C, Baron A, Gadgil S, Gemmill RM, et al. (2004) Hox expression in AML identifies a distinct subset of patients with intermediate cytogenetics. Leukemia 18: 1059-1063.

92. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, et al. (1997) Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. Proc Natl Acad Sci U S A 94: 569-574.

93. Hasemann MS, Damgaard I, Schuster MB, Theilgaard-Monch K, Sorensen AB, et al. (2008) Mutation of C/EBPalpha predisposes to the development of myeloid leukemia in a retroviral insertional mutagenesis screen. Blood 111: 4309-4321.

94. Reckzeh K, Bereshchenko O, Mead A, Rehn M, Kharazi S, et al. (2012) Molecular and cellular effects of oncogene cooperation in a genetically accurate AML mouse model. Leukemia 26: 1527-1536.

95. Renneville A, Boissel N, Gachard N, Naguib D, Bastard C, et al. (2009) The favorable impact of CEBPA mutations in patients with acute myeloid leukemia is only observed in the absence of associated cytogenetic abnormalities and FLT3 internal duplication. Blood 113: 5090-5093.

96. Radomska HS, Alberich-Jorda M, Will B, Gonzalez D, Delwel R, et al. (2012) Targeting CDK1 promotes FLT3-activated acute myeloid leukemia differentiation through C/EBPalpha. J Clin Invest 122: 2955-2966.

97. Kato N, Kitaura J, Doki N, Komeno Y, Watanabe-Okochi N, et al. (2011) Two types of C/EBPalpha mutations play distinct but collaborative roles in leukemogenesis: lessons from clinical data and BMT models. Blood 117: 221-233.

98. Weston K (1998) Myb proteins in life, death and differentiation. Curr Opin Genet Dev 8: 76-81.

99. Oh IH, Reddy EP (1999) The myb gene family in cell growth, differentiation and apoptosis. Oncogene 18: 3017-3033.

100. Mucenski ML, McLain K, Kier AB, Swerdlow SH, Schreiner CM, et al. (1991) A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell 65: 677-689.

101. Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, et al. (2003) Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. Embo j 22: 4478-4488.

102. Sandberg ML, Sutton SE, Pletcher MT, Wiltshire T, Tarantino LM, et al. (2005) c-Myb and p300 regulate hematopoietic stem cell proliferation and differentiation. Dev Cell 8: 153-166.

103. Sakamoto H, Dai G, Tsujino K, Hashimoto K, Huang X, et al. (2006) Proper levels of c-Myb are discretely defined at distinct steps of hematopoietic cell development. Blood 108: 896-903.

104. Lidonnici MR, Corradini F, Waldron T, Bender TP, Calabretta B (2008) Requirement of c-Myb for p210(BCR/ABL)-dependent transformation of hematopoietic progenitors and leukemogenesis. Blood 111: 4771-4779.

105. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, et al. (2007) The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. Blood 110: 1251-1261.

106. Hess JL, Bittner CB, Zeisig DT, Bach C, Fuchs U, et al. (2006) c-Myb is an essential downstream target for homeobox-mediated transformation of hematopoietic cells. Blood 108: 297-304.

107. Oelgeschlager M, Nuchprayoon I, Luscher B, Friedman AD (1996) C/EBP, c-Myb, and PU.1 cooperate to regulate the neutrophil elastase promoter. Mol Cell Biol 16: 4717-4725.

108. Burk O, Mink S, Ringwald M, Klempnauer KH (1993) Synergistic activation of the chicken mim-1 gene by v-myb and C/EBP transcription factors. Embo j 12: 2027-2038.

109. Kato T, Sakata-Yanagimoto M, Nishikii H, Ueno M, Miyake Y, et al. (2014) Hes1 suppresses acute myeloid leukemia development through FLT3 repression. Leukemia.

110. Kiyoi H, Yanada M, Ozekia K (2005) Clinical significance of FLT3 in leukemia. Int J Hematol 82: 85-92.

111. Sheikhha MH, Awan A, Tobal K, Liu Yin JA (2003) Prognostic significance of FLT3 ITD and D835 mutations in AML patients. Hematol J 4: 41-46.

112. Meshinchi S, Stirewalt DL, Alonzo TA, Boggon TJ, Gerbing RB, et al. (2008) Structural and numerical variation of FLT3/ITD in pediatric AML. Blood 111: 4930-4933.

113. Santos FP, Jones D, Qiao W, Cortes JE, Ravandi F, et al. (2011) Prognostic value of FLT3 mutations among different cytogenetic subgroups in acute myeloid leukemia. Cancer 117: 2145-2155.

114. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, et al. (2008) The impact of FLT3 internal tandem duplication mutant level, number, size, and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. Blood 111: 2776-2784.

115. Pemmaraju N, Kantarjian H, Ravandi F, Cortes J (2011) FLT3 inhibitors in the treatment of acute myeloid leukemia: the start of an era? Cancer 117: 3293-3304.

116. Kindler T, Lipka D, Fischer T (2010) FLT3 as a therapeutic target in AML: still challenging after all these years. Blood 116: 5089-5102.

117. Knapper S (2007) FLT3 inhibition in acute myeloid leukaemia. Br J Haematol 138: 687-699.

118. Knapper S (2011) The clinical development of FLT3 inhibitors in acute myeloid leukemia. Expert Opin Investig Drugs 20: 1377-1395.

119. Wiernik PH (2010) FLT3 inhibitors for the treatment of acute myeloid leukemia. Clin Adv Hematol Oncol 8: 429-436, 444.

120. Eriksson A, Hoglund M, Lindhagen E, Aleskog A, Hassan SB, et al. (2010) Identification of AKN-032, a novel 2-aminopyrazine tyrosine kinase inhibitor, with significant preclinical activity in acute myeloid leukemia. Biochem Pharmacol 80: 1507-1516.

121. O'Farrell AM, Abrams TJ, Yuen HA, Ngai TJ, Louie SG, et al. (2003) SU11248 is a novel FLT3 tyrosine kinase inhibitor with potent activity in vitro and in vivo. Blood 101: 3597-3605.

122. Auclair D, Miller D, Yatsula V, Pickett W, Carter C, et al. (2007) Antitumor activity of sorafenib in FLT3-driven leukemic cells. Leukemia 21: 439-445.

123. Kelly LM, Yu JC, Boulton CL, Apatira M, Li J, et al. (2002) CT53518, a novel selective FLT3 antagonist for the treatment of acute myelogenous leukemia (AML). Cancer Cell 1: 421-432.

124. Pratz KW, Cortes J, Roboz GJ, Rao N, Arowojolu O, et al. (2009) A pharmacodynamic study of the FLT3 inhibitor KW-2449 yields insight into the basis for clinical response. Blood 113: 3938-3946.

125. Shankar DB, Li J, Tapang P, Owen McCall J, Pease LJ, et al. (2007) ABT-869, a multitargeted receptor tyrosine kinase inhibitor: inhibition of FLT3 phosphorylation and signaling in acute myeloid leukemia. Blood 109: 3400-3408.

126. Pratz K, Levis M (2008) Incorporating FLT3 inhibitors into acute myeloid leukemia treatment regimens. Leuk Lymphoma 49: 852-863.

127. Fiedler W, Mesters R, Tinnefeld H, Loges S, Staib P, et al. (2003) A phase 2 clinical study of SU5416 in patients with refractory acute myeloid leukemia. Blood 102: 2763-2767.

128. Fischer T, Stone RM, Deangelo DJ, Galinsky I, Estey E, et al. (2010) Phase IIB trial of oral Midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and

multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. J Clin Oncol 28: 4339-4345.

129. Levis M, Brown P, Smith BD, Stine A, Pham R, et al. (2006) Plasma inhibitory activity (PIA): a pharmacodynamic assay reveals insights into the basis for cytotoxic response to FLT3 inhibitors. Blood 108: 3477-3483.

130. Smith BD, Levis M, Beran M, Giles F, Kantarjian H, et al. (2004) Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. Blood 103: 3669-3676.

131. Stone RM, De Angelo J, Galinsky I, Estey E, Klimek V, et al. (2004) PKC 412 FLT3 inhibitor therapy in AML: results of a phase II trial. Ann Hematol 83 Suppl 1: S89-90.

132. Cortes JE, Kantarjian H, Foran JM, Ghirdaladze D, Zodelava M, et al. (2013) Phase I study of quizartinib administered daily to patients with relapsed or refractory acute myeloid leukemia irrespective of FMS-like tyrosine kinase 3-internal tandem duplication status. J Clin Oncol 31: 3681-3687.

133. Kojima K, McQueen T, Chen Y, Jacamo R, Konopleva M, et al. (2011) p53 activation of mesenchymal stromal cells partially abrogates microenvironment-mediated resistance to FLT3 inhibition in AML through HIF-1alpha-mediated down-regulation of CXCL12. Blood 118: 4431-4439.

134. Parmar A, Marz S, Rushton S, Holzwarth C, Lind K, et al. (2011) Stromal niche cells protect early leukemic FLT3-ITD+ progenitor cells against first-generation FLT3 tyrosine kinase inhibitors. Cancer Res 71: 4696-4706.

135. Sato T, Yang X, Knapper S, White P, Smith BD, et al. (2011) FLT3 ligand impedes the efficacy of FLT3 inhibitors in vitro and in vivo. Blood 117: 3286-3293.

136. Stolzel F, Steudel C, Oelschlagel U, Mohr B, Koch S, et al. (2010) Mechanisms of resistance against PKC412 in resistant FLT3-ITD positive human acute myeloid leukemia cells. Ann Hematol 89: 653-662.

137. Weisberg E, Sattler M, Ray A, Griffin JD (2010) Drug resistance in mutant FLT3-positive AML. Oncogene 29: 5120-5134.

138. Weisberg E, Ray A, Nelson E, Adamia S, Barrett R, et al. (2011) Reversible resistance induced by FLT3 inhibition: a novel resistance mechanism in mutant FLT3-expressing cells. PLoS One 6: e25351.

139. Zhou J, Bi C, Janakakumara JV, Liu SC, Chng WJ, et al. (2009) Enhanced activation of STAT pathways and overexpression of survivin confer resistance to FLT3 inhibitors and could be therapeutic targets in AML. Blood 113: 4052-4062.

140. Breitenbuecher F, Markova B, Kasper S, Carius B, Stauder T, et al. (2009) A novel molecular mechanism of primary resistance to FLT3-kinase inhibitors in AML. Blood 113: 4063-4073.

141. Moore AS, Faisal A, Gonzalez de Castro D, Bavetsias V, Sun C, et al. (2012) Selective FLT3 inhibition of FLT3-ITD+ acute myeloid leukaemia resulting in secondary D835Y mutation: a model for emerging clinical resistance patterns. Leukemia 26: 1462-1470.

142. Man CH, Fung TK, Ho C, Han HH, Chow HC, et al. (2012) Sorafenib treatment of FLT3-ITD(+) acute myeloid leukemia: favorable initial outcome and mechanisms of subsequent nonresponsiveness associated with the emergence of a D835 mutation. Blood 119: 5133-5143.

143. Belsville M (2003) U.S. Department of Agriculture; . USDA Database for the Flavonoid Content of Selected Foods, Prepared by the Nutrient Data Laboratory, Food Composition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service.

144. Yang CS, Maliakal P, Meng X (2002) Inhibition of carcinogenesis by tea. Annu Rev Pharmacol Toxicol 42: 25-54.

145. Hou Z, Sang S, You H, Lee MJ, Hong J, et al. (2005) 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: 8049-8056.

146. Shirakami Y, Shimizu M, Tsurumi H, Hara Y, Tanaka T, et al. (2008) EGCG and Polyphenon E attenuate inflammation-related mouse colon carcinogenesis induced by AOM plus DDS. Mol Med Rep 1: 355-361.

147. Yang CS, Wang X, Lu G, Picinich SC (2009) Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. Nat Rev Cancer 9: 429-439.

148. Shimizu M, Weinstein IB (2005) Modulation of signal transduction by tea catechins and related phytochemicals. Mutat Res 591: 147-160.

149. Yin ST, Tang ML, Su L, Chen L, Hu P, et al. (2008) Effects of Epigallocatechin-3-gallate on lead-induced oxidative damage. Toxicology 249: 45-54.

150. Liu TT, Liang NS, Li Y, Yang F, Lu Y, et al. (2003) Effects of long-term tea polyphenols consumption on hepatic microsomal drug-metabolizing enzymes and liver function in Wistar rats. World J Gastroenterol 9: 2742-2744.

151. Zhou L, Elias RJ (2012) Factors influencing the antioxidant and pro-oxidant activity of polyphenols in oil-in-water emulsions. J Agric Food Chem 60: 2906-2915.

152. Lambert JD, Elias RJ (2010) The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Arch Biochem Biophys 501: 65-72.

153. Zhang S, Yang X, Luo J, Ge X, Sun W, et al. (2014) PPARalpha activation sensitizes cancer cells to epigallocatechin-3-gallate (EGCG) treatment via suppressing heme oxygenase-1. Nutr Cancer 66: 315-324.

154. Kim HJ, Park GM, Kim JK (2013) Anti-inflammatory effect of pristimerin on lipopolysaccharide-induced inflammatory responses in murine macrophages. Arch Pharm Res 36: 495-500.

155. Lin CM, Chang H, Chen YH, Li SY, Wu IH, et al. (2006) Protective role of wogonin against lipopolysaccharide-induced angiogenesis via VEGFR-2, not VEGFR-1. Int Immunopharmacol 6: 1690-1698.

156. Zhang HY, Wang JY, Yao HP (2014) Epigallocatechin-3-gallate attenuates lipopolysaccharide-induced inflammation in human retinal endothelial cells. Int J Ophthalmol 7: 408-412.

157. Liu Q, Qian Y, Chen F, Chen X, Chen Z, et al. (2014) EGCG attenuates pro-inflammatory cytokines and chemokines production in LPS-stimulated L02 hepatocyte. Acta Biochim Biophys Sin (Shanghai) 46: 31-39.

158. Senggunprai L, Kukongviriyapan V, Prawan A, Kukongviriyapan U (2014) Quercetin and EGCG Exhibit Chemopreventive Effects in Cholangiocarcinoma Cells via Suppression of JAK/STAT Signaling Pathway. Phytother Res 28: 841-848.

159. Bae YS, Lee JH, Choi SH, Kim S, Almazan F, et al. (2009) Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein:

toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. Circ Res 104: 210-218, 221p following 218.

160. Picchi A, Gao X, Belmadani S, Potter BJ, Focardi M, et al. (2006) Tumor necrosis factor-alpha induces endothelial dysfunction in the prediabetic metabolic syndrome. Circ Res 99: 69-77.

161. Pullikotil P, Chen H, Muniyappa R, Greenberg CC, Yang S, et al. (2012) Epigallocatechin gallate induces expression of heme oxygenase-1 in endothelial cells via p38 MAPK and Nrf-2 that suppresses proinflammatory actions of TNF-alpha. J Nutr Biochem 23: 1134-1145.

162. Wu CC, Hsu MC, Hsieh CW, Lin JB, Lai PH, et al. (2006) Upregulation of heme oxygenase-1 by Epigallocatechin-3-gallate via the phosphatidylinositol 3-kinase/Akt and ERK pathways. Life Sci 78: 2889-2897.

163. Zhou DH, Wang X, Yang M, Shi X, Huang W, et al. (2013) Combination of Low Concentration of (-)-Epigallocatechin Gallate (EGCG) and Curcumin Strongly Suppresses the Growth of Non-Small Cell Lung Cancer in Vitro and in Vivo through Causing Cell Cycle Arrest. Int J Mol Sci 14: 12023-12036.

164. Chuu CP, Chen RY, Kokontis JM, Hiipakka RA, Liao S (2009) Suppression of androgen receptor signaling and prostate specific antigen expression by (-)-epigallocatechin-3-gallate in different progression stages of LNCaP prostate cancer cells. Cancer Lett 275: 86-92.

165. Sanchez-Tena S, Vizan P, Dudeja PK, Centelles JJ, Cascante M (2013) Green tea phenolics inhibit butyrate-induced differentiation of colon cancer cells by interacting with monocarboxylate transporter 1. Biochim Biophys Acta 1832: 2264-2270.

166. Singh T, Katiyar SK (2013) Green tea polyphenol, (-)-epigallocatechin-3-gallate, induces toxicity in human skin cancer cells by targeting beta-catenin signaling. Toxicol Appl Pharmacol 273: 418-424.

167. Belguise K, Guo S, Sonenshein GE (2007) Activation of FOXO3a by the green tea polyphenol epigallocatechin-3-gallate induces estrogen receptor alpha expression reversing invasive phenotype of breast cancer cells. Cancer Res 67: 5763-5770.

168. Meeran SM, Patel SN, Chan TH, Tollefsbol TO (2011) A novel prodrug of epigallocatechin-3-gallate: differential epigenetic hTERT repression in human breast cancer cells. Cancer Prev Res (Phila) 4: 1243-1254.

169. Li Y, Yuan YY, Meeran SM, Tollefsbol TO (2010) Synergistic epigenetic reactivation of estrogen receptor-alpha (ERalpha) by combined green tea polyphenol and histone deacetylase inhibitor in ERalpha-negative breast cancer cells. Mol Cancer 9: 274.

170. Zhou J, Farah BL, Sinha RA, Wu Y, Singh BK, et al. (2014) Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, stimulates hepatic autophagy and lipid clearance. PLoS One 9: e87161.

171. Mocanu MM, Ganea C, Georgescu L, Varadi T, Shrestha D, et al. (2014) Epigallocatechin 3-O-gallate induces 67 kDa laminin receptor-mediated cell death accompanied by downregulation of ErbB proteins and altered lipid raft clustering in mammary and epidermoid carcinoma cells. J Nat Prod 77: 250-257.

172. Liang YC, Lin-shiau SY, Chen CF, Lin JK (1997) Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. J Cell Biochem 67: 55-65.

173. Guo S, Lu J, Subramanian A, Sonenshein GE (2006) 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: 5322-5329.

174. Pianetti S, Guo S, Kavanagh KT, Sonenshein GE (2002) Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/neu signaling, proliferation, and transformed phenotype of breast cancer cells. Cancer Res 62: 652-655.

175. Lee YK, Bone ND, Strege AK, Shanafelt TD, Jelinek DF, et al. (2004) 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: 788-794.

176. Sachinidis A, Skach RA, Seul C, Ko Y, Hescheler J, et al. (2002) 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: 893-895.

177. Shimizu M, Shirakami Y, Sakai H, Tatebe H, Nakagawa T, et al. (2008) EGCG inhibits activation of the insulin-like growth factor (IGF)/IGF-1 receptor axis in human hepatocellular carcinoma cells. Cancer Lett 262: 10-18.

178. Vu HA, Beppu Y, Chi HT, Sasaki K, Yamamoto H, et al. (2010) Green tea epigallocatechin gallate exhibits anticancer effect in human pancreatic carcinoma cells via the inhibition of both focal adhesion kinase and insulin-like growth factor-I receptor. J Biomed Biotechnol 2010: 290516.

179. Chi HT, Vu HA, Iwasaki R, Thao le B, Hara Y, et al. (2009) Green tea (-)-epigalocatechin-3-gallate inhibits KIT activity and causes caspase-dependent cell death in gastrointestinal stromal tumor including imatinib-resistant cells. Cancer Biol Ther 8: 1934-1939.

180. Miettinen M, Lasota J (2001) Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. Virchows Arch 438: 1-12.

181. Hommelgaard AM, Lerdrup M, van Deurs B (2004) Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. Mol Biol Cell 15: 1557-1567.

182. Li Y, Zhang T, Jiang Y, Lee HF, Schwartz SJ, et al. (2009) (-)-Epigallocatechin-3-gallate inhibits Hsp90 function by impairing Hsp90 association with cochaperones in pancreatic cancer cell line Mia Paca-2. Mol Pharm 6: 1152-1159.

183. Tran PL, Kim SA, Choi HS, Yoon JH, Ahn SG (2010) Epigallocatechin-3-gallate suppresses the expression of HSP70 and HSP90 and exhibits anti-tumor activity in vitro and in vivo. BMC Cancer 10: 276.

184. Li M, He Z, Ermakova S, Zheng D, Tang F, et al. (2007) Direct inhibition of insulin-like growth factor-I receptor kinase activity by (-)-epigallocatechin-3-gallate regulates cell transformation. Cancer Epidemiol Biomarkers Prev 16: 598-605.

185. Sah JF, Balasubramanian S, Eckert RL, Rorke EA (2004) Epigallocatechin-3-gallate inhibits epidermal growth factor receptor signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases. J Biol Chem 279: 12755-12762.

186. Chung JY, Park JO, Phyu H, Dong Z, Yang CS (2001) Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (-)-epigallocatechin-3-gallate and theaflavin-3,3'-digallate. Faseb j 15: 2022-2024.

187. Chung JY, Huang C, Meng X, Dong Z, Yang CS (1999) Inhibition of activator protein 1 activity and cell growth by purified green tea and black tea polyphenols in H-ras-

transformed cells: structure-activity relationship and mechanisms involved. Cancer Res 59: 4610-4617.

188. Peng G, Wargovich MJ, Dixon DA (2006) Anti-proliferative effects of green tea polyphenol EGCG on Ha-Ras-induced transformation of intestinal epithelial cells. Cancer Lett 238: 260-270.

189. Lievre A, Bachet JB, Le Corre D, Boige V, Landi B, et al. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. Cancer Res 66: 3992-3995.

190. Ju J, Hong J, Zhou JN, Pan Z, Bose M, et al. (2005) Inhibition of intestinal tumorigenesis in Apcmin/+ mice by (-)-epigallocatechin-3-gallate, the major catechin in green tea. Cancer Res 65: 10623-10631.

191. Sukhthankar M, Yamaguchi K, Lee SH, McEntee MF, Eling TE, et al. (2008) A green tea component suppresses posttranslational expression of basic fibroblast growth factor in colorectal cancer. Gastroenterology 134: 1972-1980.

192. Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, et al. (1992) APC mutations occur early during colorectal tumorigenesis. Nature 359: 235-237.

193. Hong J, Lu H, Meng X, Ryu JH, Hara Y, et al. (2002) Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (-)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. Cancer Res 62: 7241-7246.

194. Kondo T, Ohta T, Igura K, Hara Y, Kaji K (2002) 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: 139-144.

195. Adachi S, Nagao T, To S, Joe AK, Shimizu M, et al. (2008) (-)-Epigallocatechin gallate causes internalization of the epidermal growth factor receptor in human colon cancer cells. Carcinogenesis 29: 1986-1993.

196. Shimizu M, Shirakami Y, Sakai H, Yasuda Y, Kubota M, et al. (2010) (-)-Epigallocatechin gallate inhibits growth and activation of the VEGF/VEGFR axis in human colorectal cancer cells. Chem Biol Interact 185: 247-252.

197. Shirakami Y, Shimizu M, Adachi S, Sakai H, Nakagawa T, et al. (2009) (-)-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: 1957-1962.

198. Aktas O, Prozorovski T, Smorodchenko A, Savaskan NE, Lauster R, et al. (2004) Green tea epigallocatechin-3-gallate mediates T cellular NF-kappa B inhibition and exerts neuroprotection in autoimmune encephalomyelitis. J Immunol 173: 5794-5800.

199. Chen A, Zhang L (2003) 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: 23381-23389.

200. Dong Z, Ma W, Huang C, Yang CS (1997) Inhibition of tumor promoter-induced activator protein 1 activation and cell transformation by tea polyphenols, (-)-epigallocatechin gallate, and theaflavins. Cancer Res 57: 4414-4419.

201. Kim H, Sakamoto K (2012) (-)-Epigallocatechin gallate suppresses adipocyte differentiation through the MEK/ERK and PI3K/Akt pathways. Cell Biol Int 36: 147-153.

202. Lee IT, Lin CC, Lee CY, Hsieh PW, Yang CM (2013) Protective effects of (-)-epigallocatechin-3-gallate against TNF-alpha-induced lung inflammation via ROS-dependent ICAM-1 inhibition. J Nutr Biochem 24: 124-136.

203. Reiter CE, Kim JA, Quon MJ (2010) Green tea polyphenol epigallocatechin gallate reduces endothelin-1 expression and secretion in vascular endothelial cells: roles for AMP-activated protein kinase, Akt, and FOXO1. Endocrinology 151: 103-114.

204. Ren F, Zhang S, Mitchell SH, Butler R, Young CY (2000) Tea polyphenols down-regulate the expression of the androgen receptor in LNCaP prostate cancer cells. Oncogene 19: 1924-1932.

205. Townsend PA, Scarabelli TM, Pasini E, Gitti G, Menegazzi M, et al. (2004) Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. Faseb j 18: 1621-1623.

206. Yang F, de Villiers WJ, McClain CJ, Varilek GW (1998) Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr 128: 2334-2340.

207. Aneja R, Hake PW, Burroughs TJ, Denenberg AG, Wong HR, et al. (2004) Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats. Mol Med 10: 55-62.

208. Stephanou A (2004) Role of STAT-1 and STAT-3 in ischaemia/reperfusion injury. J Cell Mol Med 8: 519-525.

209. Kim H, Hiraishi A, Tsuchiya K, Sakamoto K (2010) (-) Epigallocatechin gallate suppresses the differentiation of 3T3-L1 preadipocytes through transcription factors FoxO1 and SREBP1c. Cytotechnology 62: 245-255.

210. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, et al. (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 389: 994-999.

211. Kuriyama S, Shimazu T, Ohmori K, Kikuchi N, Nakaya N, et al. (2006) Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: the Ohsaki study. Jama 296: 1255-1265.

212. Sartippour MR, Shao ZM, Heber D, Beatty P, Zhang L, et al. (2002) Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. J Nutr 132: 2307-2311.

213. Ahn WS, Huh SW, Bae SM, Lee IP, Lee JM, et al. (2003) A major constituent of green tea, EGCG, inhibits the growth of a human cervical cancer cell line, CaSki cells, through apoptosis, G(1) arrest, and regulation of gene expression. DNA Cell Biol 22: 217-224.

214. Hastak K, Gupta S, Ahmad N, Agarwal MK, Agarwal ML, et al. (2003) Role of p53 and NF-kappaB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells. Oncogene 22: 4851-4859.

215. Fujiki H, Suganuma M, Okabe S, Sueoka E, Suga K, et al. (1999) Mechanistic findings of green tea as cancer preventive for humans. Proc Soc Exp Biol Med 220: 225-228.

216. Ahn WS, Yoo J, Huh SW, Kim CK, Lee JM, et al. (2003) Protective effects of green tea extracts (polyphenon E and EGCG) on human cervical lesions. Eur J Cancer Prev 12: 383-390.

217. Baba S, Osakabe N, Natsume M, Muto Y, Takizawa T, et al. (2001) In vivo comparison of the bioavailability of (+)-catechin, (-)-epicatechin and their mixture in orally administered rats. J Nutr 131: 2885-2891.

218. Song Q, Li D, Zhou Y, Yang J, Yang W, et al. (2014) Enhanced uptake and transport of (+)-catechin and (-)-epigallocatechin gallate in niosomal formulation by human intestinal Caco-2 cells. Int J Nanomedicine 9: 2157-2165.

219. Henning SM, Choo JJ, Heber D (2008) Nongallated compared with gallated flavan-3-ols in green and black tea are more bioavailable. J Nutr 138: 1529s-1534s.

220. Chow HH, Hakim IA, Vining DR, Crowell JA, Ranger-Moore J, et al. (2005) Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. Clin Cancer Res 11: 4627-4633.

221. Peters CM, Green RJ, Janle EM, Ferruzzi MG (2010) Formulation with ascorbic acid and sucrose modulates catechin bioavailability from green tea. Food Res Int 43: 95-102.

222. Felnerova D, Viret JF, Gluck R, Moser C (2004) Liposomes and virosomes as delivery systems for antigens, nucleic acids and drugs. Curr Opin Biotechnol 15: 518-529.

223. Torchilin VP (2005) Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 4: 145-160.

224. Xia S, Xu S, Zhang X, Zhong F, Wang Z (2009) Nanoliposomes mediate coenzyme Q10 transport and accumulation across human intestinal Caco-2 cell monolayer. J Agric Food Chem 57: 7989-7996.

225. Luo X, Guan R, Chen X, Tao M, Ma J, et al. (2014) Optimization on condition of epigallocatechin-3-gallate (EGCG) nanoliposomes by response surface methodology and cellular uptake studies in Caco-2 cells. Nanoscale Res Lett 9: 291.

226. Fitzgerald P, Hadgraft J, Kreuter J, Wilson C (1987) A -scintigraphic evaluation of microparticulate ophthalmic delivery systems: liposomes and nanoparticles. Int J Pharm 40: 81–84.

227. Alexander M, Acero Lopez A, Fang Y, Corredig M (2012) Incorporation of phytosterols in soy phospholipids nanoliposomes: encapsulation efficiency and stability. LWT-Food Sci Technol 47: 427–436.

228. Lambert JD, Sang S, Hong J, Kwon SJ, Lee MJ, et al. (2006) Peracetylation as a means of enhancing in vitro bioactivity and bioavailability of epigallocatechin-3-gallate. Drug Metab Dispos 34: 2111-2116.

229. Matsuo Y, MacLeod RA, Uphoff CC, Drexler HG, Nishizaki C, et al. (1997) Two acute monocytic leukemia (AML-M5a) cell lines (MOLM-13 and MOLM-14) with interclonal phenotypic heterogeneity showing MLL-AF9 fusion resulting from an occult chromosome insertion, ins(11;9)(q23;p22p23). Leukemia 11: 1469-1477.

230. Lange B, Valtieri M, Santoli D, Caracciolo D, Mavilio F, et al. (1987) Growth factor requirements of childhood acute leukemia: establishment of GM-CSF-dependent cell lines. Blood 70: 192-199.

231. Iida S, Saito M, Okazaki T, Seto M, Yamamoto K, et al. (1992) Phenotypic and genotypic characterization of 14 leukemia and lymphoma cell lines with 11q23 translocations. Leuk Res 16: 1155-1163.

232. Taketani T, Taki T, Sugita K, Furuichi Y, Ishii E, et al. (2004) FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood 103: 1085-1088.

233. Furukawa Y, Vu HA, Akutsu M, Odgerel T, Izumi T, et al. (2007) Divergent cytotoxic effects of PKC412 in combination with conventional antileukemic agents in FLT3 mutation-positive versus -negative leukemia cell lines. Leukemia 21: 1005-1014.

234. Tsuchiya T, Hagihara M, Shimakura Y, Ueda Y, Gansuvd B, et al. (2002) The generation of immunocompetent dendritic cells from CD34+ acute myeloid or lymphoid leukemia cells. Int J Hematol 75: 55-62.

235. Kano Y, Ohnuma T, Okano T, Holland JF (1988) Effects of vincristine in combination with methotrexate and other antitumor agents in human acute lymphoblastic leukemia cells in culture. Cancer Res 48: 351-356.

236. Kano Y, Akutsu M, Tsunoda S, Mori K, Suzuki K, et al. (1998) In vitro schedule-dependent interaction between paclitaxel and SN-38 (the active metabolite of irinotecan) in human carcinoma cell lines. Cancer Chemother Pharmacol 42: 91-98.

237. Armstrong SA, Kung AL, Mabon ME, Silverman LB, Stam RW, et al. (2003) Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. Cancer Cell 3: 173-183.

238. Ozeki K, Kiyoi H, Hirose Y, Iwai M, Ninomiya M, et al. (2004) Biologic and clinical significance of the FLT3 transcript level in acute myeloid leukemia. Blood 103: 1901-1908.

239. Choudhary C, Schwable J, Brandts C, Tickenbrock L, Sargin B, et al. (2005) AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations. Blood 106: 265-273.

240. Gilliland DG, Griffin JD (2002) The roles of FLT3 in hematopoiesis and leukemia. Blood 100: 1532-1542.

241. Kang HJ, Lee JW, Kho SH, Kim MJ, Seo YJ, et al. (2010) High transcript level of FLT3 associated with high risk of relapse in pediatric acute myeloid leukemia. J Korean Med Sci 25: 841-845.

242. Buchner J (2010) Bacterial Hsp90--desperately seeking clients. Mol Microbiol 76: 540-544.

243. da Rocha Dias S, Friedlos F, Light Y, Springer C, Workman P, et al. (2005) Activated B-RAF is an Hsp90 client protein that is targeted by the anticancer drug 17-allylamino-17-demethoxygeldanamycin. Cancer Res 65: 10686-10691.

244. Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, et al. (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Biol Chem 276: 3702-3708.

245. Ahn HY, Hadizadeh KR, Seul C, Yun YP, Vetter H, et al. (1999) 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: 1093-1104.

246. Ma YC, Li C, Gao F, Xu Y, Jiang ZB, et al. (2014) Epigallocatechin gallate inhibits the growth of human lung cancer by directly targeting the EGFR signaling pathway. Oncol Rep 31: 1343-1349.

247. Ahsan A, Ramanand SG, Whitehead C, Hiniker SM, Rehemtulla A, et al. (2012) Wild-type EGFR is stabilized by direct interaction with HSP90 in cancer cells and tumors. Neoplasia 14: 670-677.

248. Tachibana H, Koga K, Fujimura Y, Yamada K (2004) A receptor for green tea polyphenol EGCG. Nat Struct Mol Biol 11: 380-381.

249. Kumazoe M, Sugihara K, Tsukamoto S, Huang Y, Tsurudome Y, et al. (2013) 67-kDa laminin receptor increases cGMP to induce cancer-selective apoptosis. J Clin Invest 123: 787-799.

250. Ren X, Guo X, Chen L, Guo M, Peng N, et al. (2014) Attenuated migration by green tea extract (-)-epigallocatechin gallate (EGCG): involvement of 67 kDa laminin receptor internalization in macrophagic cells. Food Funct.

251. Tsukamoto S, Hirotsu K, Kumazoe M, Goto Y, Sugihara K, et al. (2012) Green tea polyphenol EGCG induces lipid-raft clustering and apoptotic cell death by activating protein kinase Cdelta and acid sphingomyelinase through a 67 kDa laminin receptor in multiple myeloma cells. Biochem J 443: 525-534.

252. Wang CT, Chang HH, Hsiao CH, Lee MJ, Ku HC, et al. (2009) The effects of green tea (-)-epigallocatechin-3-gallate on reactive oxygen species in 3T3-L1 preadipocytes and adipocytes depend on the glutathione and 67 kDa laminin receptor pathways. Mol Nutr Food Res 53: 349-360.

253. Shammas MA, Neri P, Koley H, Batchu RB, Bertheau RC, et al. (2006) Specific killing of multiple myeloma cells by (-)-epigallocatechin-3-gallate extracted from green tea: biologic activity and therapeutic implications. Blood 108: 2804-2810.

254. Kumazoe M, Kim Y, Bae J, Takai M, Murata M, et al. (2013) Phosphodiesterase 5 inhibitor acts as a potent agent sensitizing acute myeloid leukemia cells to 67-kDa laminin receptor-dependent apoptosis. FEBS Lett 587: 3052-3057.

255. Kinjo J, Nagao T, Tanaka T, Nonaka G, Okawa M, et al. (2002) Activity-guided fractionation of green tea extract with antiproliferative activity against human stomach cancer cells. Biol Pharm Bull 25: 1238-1240.

256. Valcic S, Burr JA, Timmermann BN, Liebler DC (2000) Antioxidant chemistry of green tea catechins. New oxidation products of (-)-epigallocatechin gallate and (-)-epigallocatechin from their reactions with peroxyl radicals. Chem Res Toxicol 13: 801-810.

257. Ravindranath MH, Saravanan TS, Monteclaro CC, Presser N, Ye X, et al. (2006) Epicatechins Purified from Green Tea (Camellia sinensis) Differentially Suppress Growth of Gender-Dependent Human Cancer Cell Lines. Evid Based Complement Alternat Med 3: 237-247.

258. Nihal M, Ahmad N, Mukhtar H, Wood GS (2005) Anti-proliferative and proapoptotic effects of (-)-epigallocatechin-3-gallate on human melanoma: possible implications for the chemoprevention of melanoma. Int J Cancer 114: 513-521.

259. Burnett AK (2012) New induction and postinduction strategies in acute myeloid leukemia. Curr Opin Hematol 19: 76-81.

260. Preisler H, Davis RB, Kirshner J, Dupre E, Richards F, 3rd, et al. (1987) Comparison of three remission induction regimens and two postinduction strategies for the treatment of acute nonlymphocytic leukemia: a cancer and leukemia group B study. Blood 69: 1441-1449.

261. Wells RJ, Woods WG, Buckley JD, Odom LF, Benjamin D, et al. (1994) Treatment of newly diagnosed children and adolescents with acute myeloid leukemia: a Childrens Cancer Group study. J Clin Oncol 12: 2367-2377.

262. Rees JK, Gray RG, Wheatley K (1996) Dose intensification in acute myeloid leukaemia: greater effectiveness at lower cost. Principal report of the Medical Research Council's AML9 study. MRC Leukaemia in Adults Working Party. Br J Haematol 94: 89-98.

263. Feldman EJ, Gergis U (2012) Management of refractory acute myeloid leukemia: re-induction therapy or straight to transplantation? Curr Hematol Malig Rep 7: 74-77.

264. Ofran Y, Rowe JM (2012) Treatment for relapsed acute myeloid leukemia: what is new? Curr Opin Hematol 19: 89-94.
265. Wunderlich M, Mizukawa B, Chou FS, Sexton C, Shrestha M, et al. (2013) AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. Blood 121: e90-97.
266. Daver N, Cortes J (2012) Molecular targeted therapy in acute myeloid leukemia. Hematology 17 Suppl 1: S59-62.
267. Ohanian M, Cortes J, Kantarjian H, Jabbour E (2012) Tyrosine kinase inhibitors in acute and chronic leukemias. Expert Opin Pharmacother 13: 927-938.
268. Kaspers GJ (2012) Pediatric acute myeloid leukemia. Expert Rev Anticancer Ther 12: 405-413.
269. Pratz KW, Levis MJ (2010) Bench to bedside targeting of FLT3 in acute leukemia. Curr Drug Targets 11: 781-789.
270. Fathi AT, Arowojolu O, Swinnen I, Sato T, Rajkhowa T, et al. (2012) A potential therapeutic target for FLT3-ITD AML: PIM1 kinase. Leuk Res 36: 224-231.
271. Takahashi S, Harigae H, Yokoyama H, Ishikawa I, Abe S, et al. (2006) Synergistic effect of arsenic trioxide and flt3 inhibition on cells with flt3 internal tandem duplication. Int J Hematol 84: 256-261.
272. Bettuzzi S, Brausi M, Rizzi F, Castagnetti G, Peracchia G, et al. (2006) 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: 1234-1240.
273. Tsao AS, Liu D, Martin J, Tang XM, Lee JJ, et al. (2009) Phase II randomized, placebo-controlled trial of green tea extract in patients with high-risk oral premalignant lesions. Cancer Prev Res (Phila) 2: 931-941.
274. Shanafelt TD, Call TG, Zent CS, LaPlant B, Bowen DA, et al. (2009) Phase I trial of daily oral Polyphenon E in patients with asymptomatic Rai stage 0 to II chronic lymphocytic leukemia. J Clin Oncol 27: 3808-3814.
275. Zhao H, Zhu W, Xie P, Li H, Zhang X, et al. (2014) A phase I study of concurrent chemotherapy and thoracic radiotherapy with oral epigallocatechin-3-gallate protection in patients with locally advanced stage III non-small-cell lung cancer. Radiother Oncol 110: 132-136.
276. Meshinchi S, Appelbaum FR (2009) Structural and functional alterations of FLT3 in acute myeloid leukemia. Clin Cancer Res 15: 4263-4269.

FIGURES
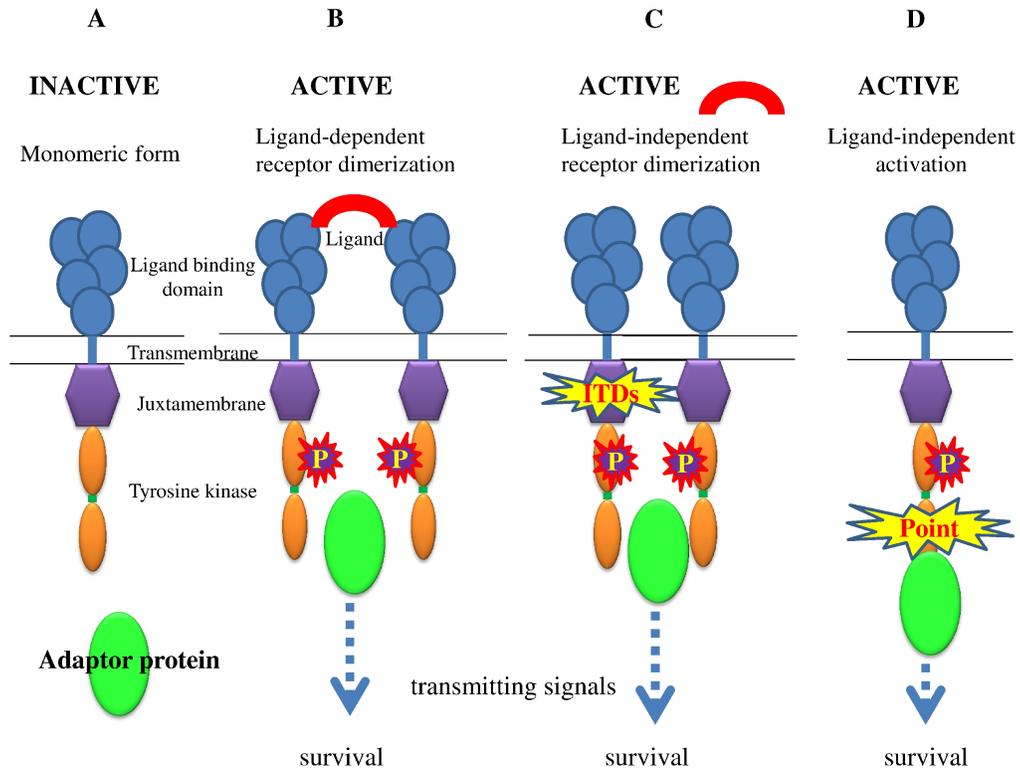
Figure 1. The inactive and active forms of FLT3

(A) The monomeric inactive FLT3 form that containing a ligand-binding extracellular domain, a single transmembrane domain, a cytoplasmic juxtamembrane domain and a kinase domain which split into two by a kinase insert. **(B)** FLT3-WT in dimerization activated form upon binding to its ligand. **(C)** FLT3-ITD with auto-dimerization and phosphorylation without FLT3 ligand binding. **(D)** FLT3-TKD with point mutation in kinase domain that leads to constitutive activation.
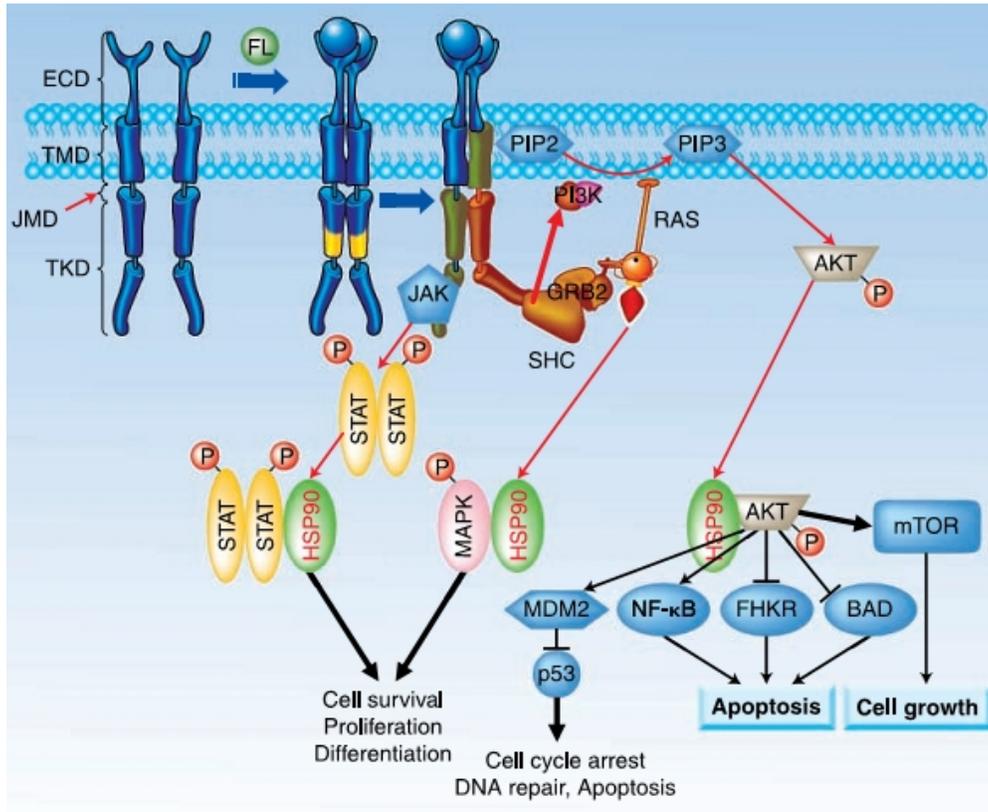
Figure 2. FLT3 signal transduction pathway

The binding of FLT3 ligand (FL) to its receptor leads to receptor dimerization and activation of the intracellular kinase.Tyrosine kinase activation leads to phosphorylation of multiple sites in the intracellular kinase moiety. The activated receptor recruits a number of proteins in the cytoplasm including SHC and GRB2 to form a complex of protein-protein interactions, leading to activation of a number of intracellular mediators including AKT, MAPK, and STAT. Activated mediators interact with Hsp90, which protects them from inactivation and chaperones the active mediators to the nuclear interphase, in which they are released into the nucleus and act to mediate vital cellular functions including cell growth, differentiation, apoptosis, DNA repair and proliferation [276].
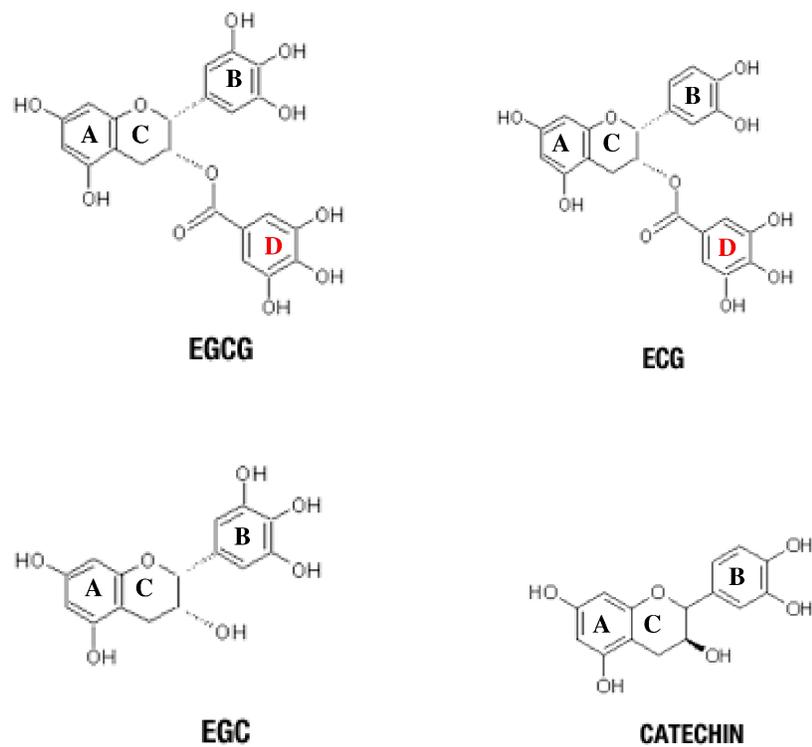
Figure 3. Chemical structure of the green tea catechins used in this study

Chemical structure of the green tea catechins. EGCG, (-)-epigallocatechin-3-gallate; EGC, (-)-epigallocatechin; ECG, (-)-epicatechin-3-gallate; and catechin
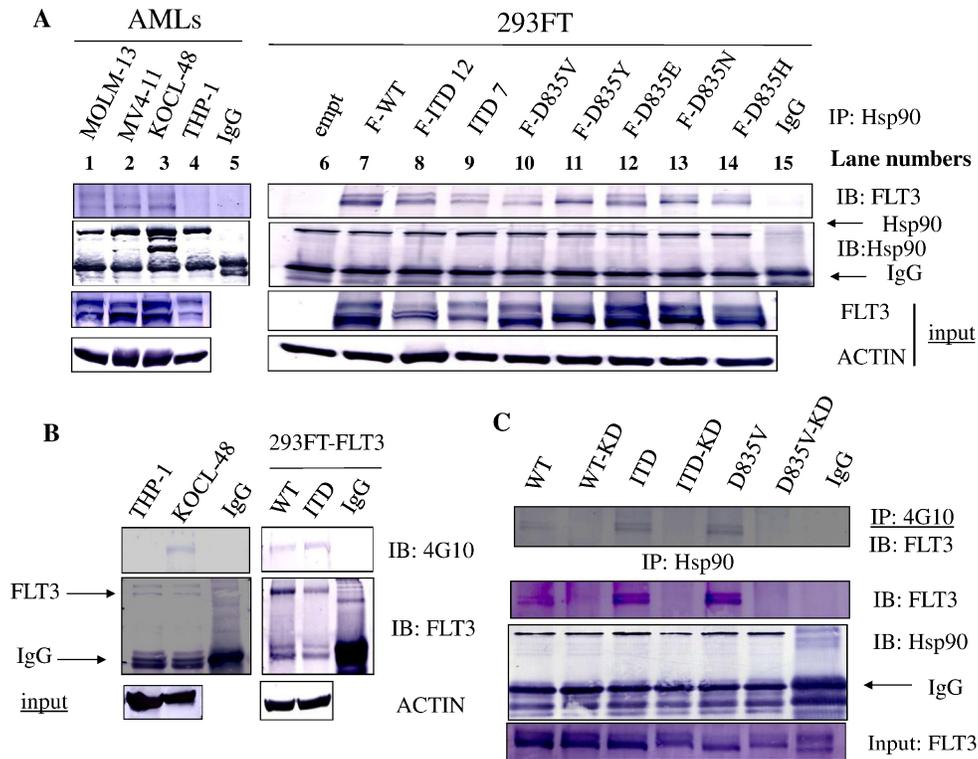
Figure 4. The phosphorylation status of FLT3 is needed for its binding to Hsp90

A.   Total cell lysates of MOLM-13, MV4-11, KOCL-48, THP-1 and 293FT-transient expressing different constructs of FLT3 were immunoprecipitated with Hsp90 antibody and subjected to immunolot analysis with FLT3 and Hsp90 antibody. The levels of -Actin served as the loading control

**B.**   KOCL-48; THP-1; 293FT-FLT3-WT and 293FT-FLT3-ITD cells at a density of 1×10^5 cells/ml were IP with FLT3 antibody and subjected to immunolot analysis with 4G10 and FLT3 antibody. The levels of   -Actin served as the loading control.

C.   293FT-FLT3-WT/ITD/D835V-K644R cells at a density of 1×10^5 cells/ml were IP with 4G10 or Hsp90 antibody and subjected to immunolot analysis with indicated antibody. The levels of   -Actin served as the loading control.
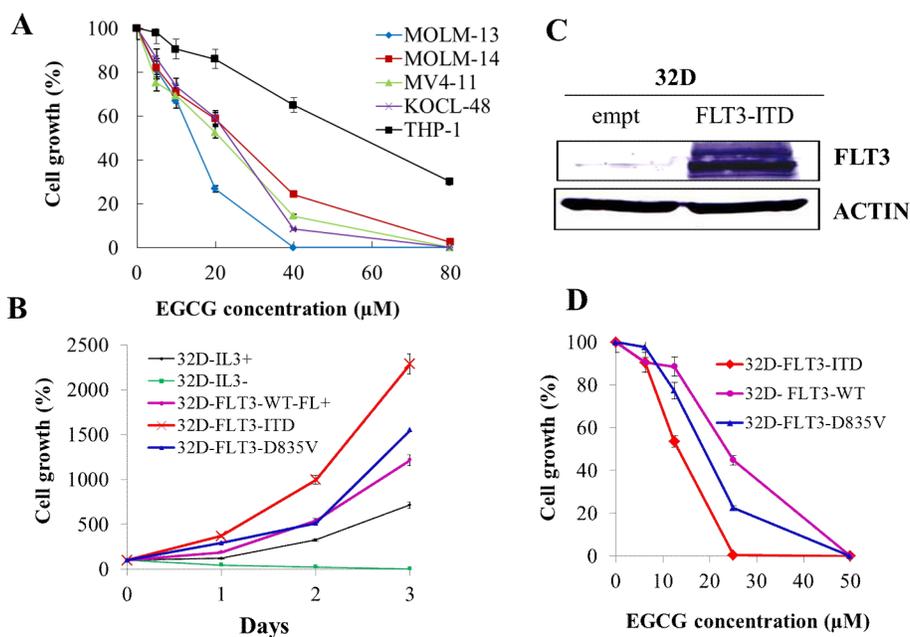
Figure 5. Effect of EGCG on cell proliferation of AMLs cell lines.

A. MOLM-13, MOLM-14, MV4-11, KOCL-48 and THP-1 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of EGCG or DMSO alone as control for 72 h. The number of viable cells was counted after the trypan blue exclusion test. Results were calculated as the percentage of the control values.

B. The number of 32D-tranfectant cells were counted after the trypan blue exclusion test.

C. Total cell lysate of 32D-parental and 32D-FLT3-ITD were subjected to western blot analysis with indicated antibodies.

D. 32D-FLT3-ITD/D835V/WT cells were treated with indicated concentration of EGCG, or DMSO alone as control for 72 h. The number of viable cells was counted after trypan blue exclusion test. Results were calculated as the percentage of the control values.
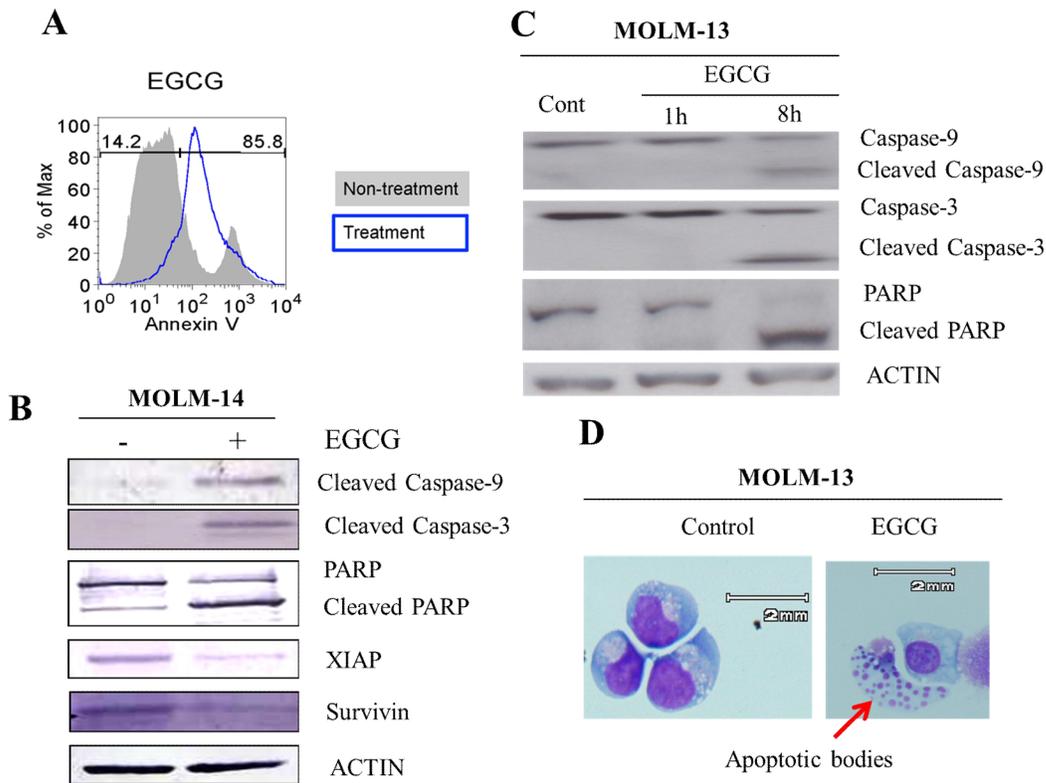
Figure 6. EGCG induced apoptosis in MOLM-14 and MOLM-13 cells.

MOLM-13 and MOLM-14 cells at a density of 1×10^5 cells/ml were treated with 60 µM EGCG or DMSO alone as control for 16 h. MOLM-14 cells were staining with PE-Annexin V and analysed by FACS Calibur. Collected data were analysed by FlowJo software (**A**). Total cell lysates of MOLM-14 cells were subjected to western blot analysis with indicated antibodies (**B**). Panel **C** showed the evidences of apoptosis induced by EGCG treatment in MOLM-13 cells. To assess the nuclei, cells were fixed onto slides and stained with Wright-Giemsa after treated with or without 60 µM EGCG, the morphology of MOLM-13 cells observed under an inverted microscope. The arrow indicated that the nuclei of MOLM-13 cells were fragmented by EGCG treatment (**D**).
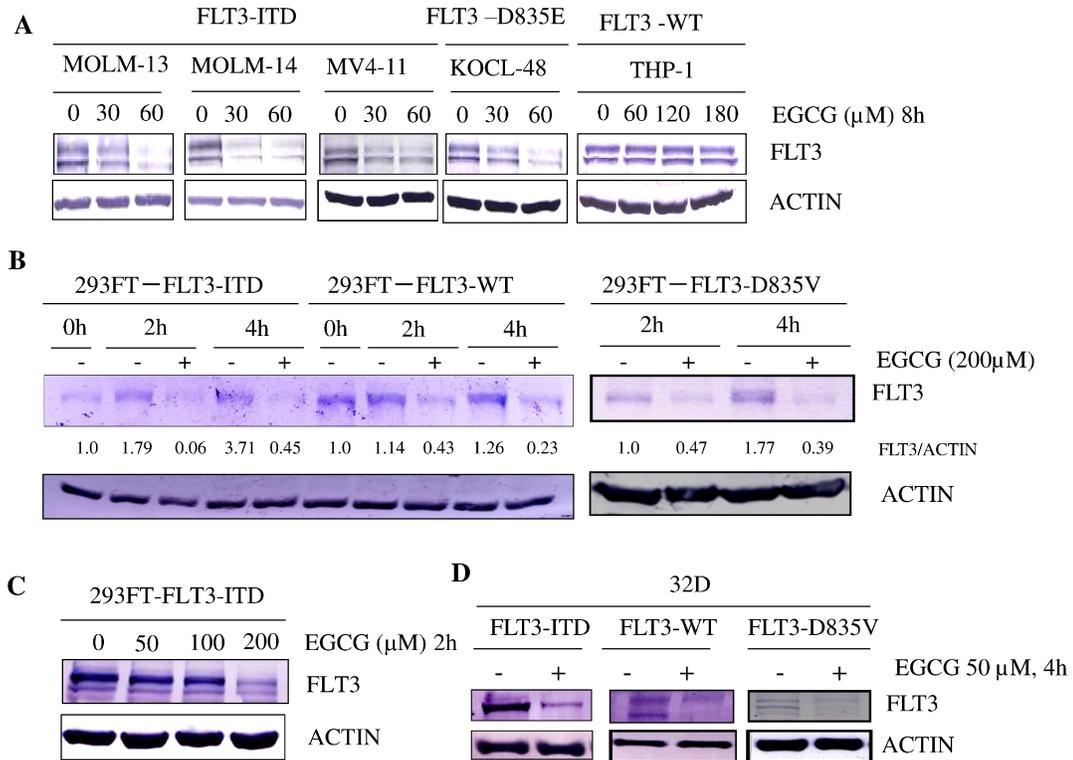
Figure 7. EGCG inhibited expression of FLT3 protein

A. MOLM-13, MOLM-14, MV4-11, KOCL-48 and THP-1 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of EGCG or DMSO alone as control for 8 h. Total cell lysates were subjected to western blot analysis with indicated antibodies.

B and C. After 24 h transfected with FLT3-WT, FLT3-ITD, FLT3-D835V, 293FT cells were treated with indicated concentration of EGCG or DMSO alone as control for indicated hours. Total cell lysates were subjected to western blot analysis with indicated antibodies.

D. 32D-FLT3-ITD/D835V/WT cells at a density of 1×10^5 cells/ml were treated with 50 µM EGCG or DMSO alone as control for 8 h. Total cell lysates were subjected to western blot analysis with indicated antibodies.
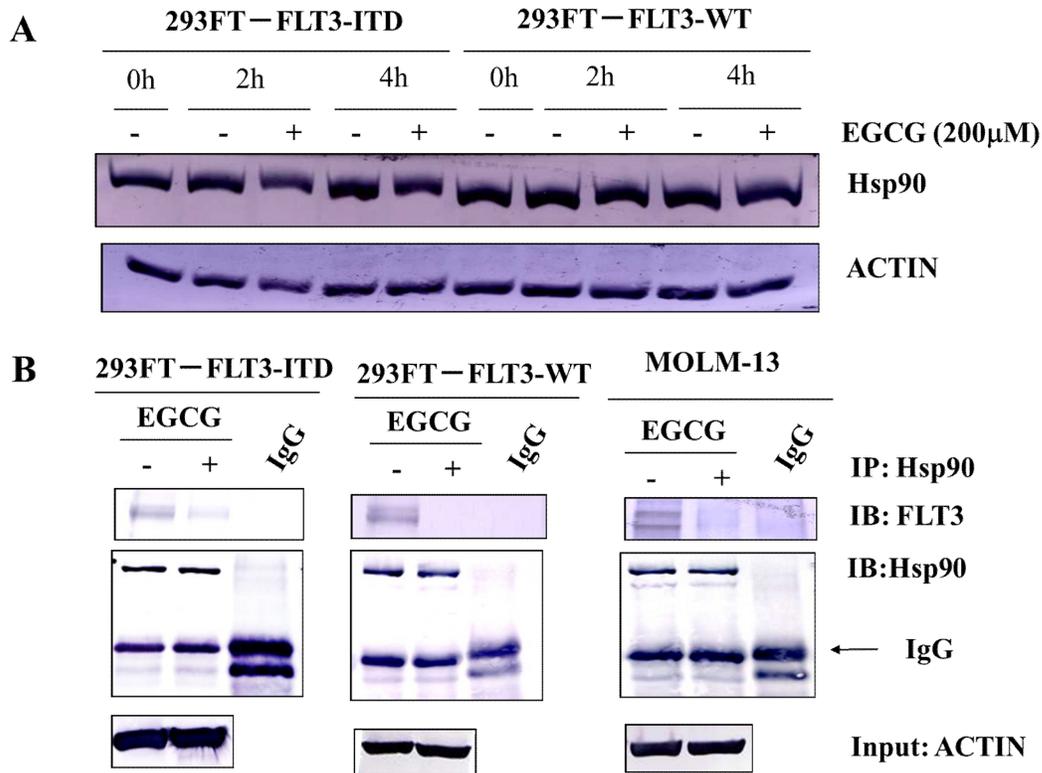
Figure 8. EGCG dissociated the interaction between FLT3 and Hsp90 in 293FT-expressing FLT3 and MOLM-13 cells

A.   After 24 h transfected with FLT3-WT and FLT3-ITD, 293FT cells were treated with 200 µM concentration of EGCG or DMSO alone as control for indicated hours. Total cell lysates were subjected to western blot analysis with indicated antibodies.

B.   293FT-FLT3-WT/ITD and MOLM-13 cells at a density of 1×10^5 cells/ml were treated with 200 µM or 60 µM EGCG or DMSO alone as control for 4 or 8 h, respectively. Total cell lysates were IP with anti-Hsp90. Precipitated protein were subjected to western blot analysis with anti-FLT3 and anti Hsp90. The levels of β-Actin served as the loading control.
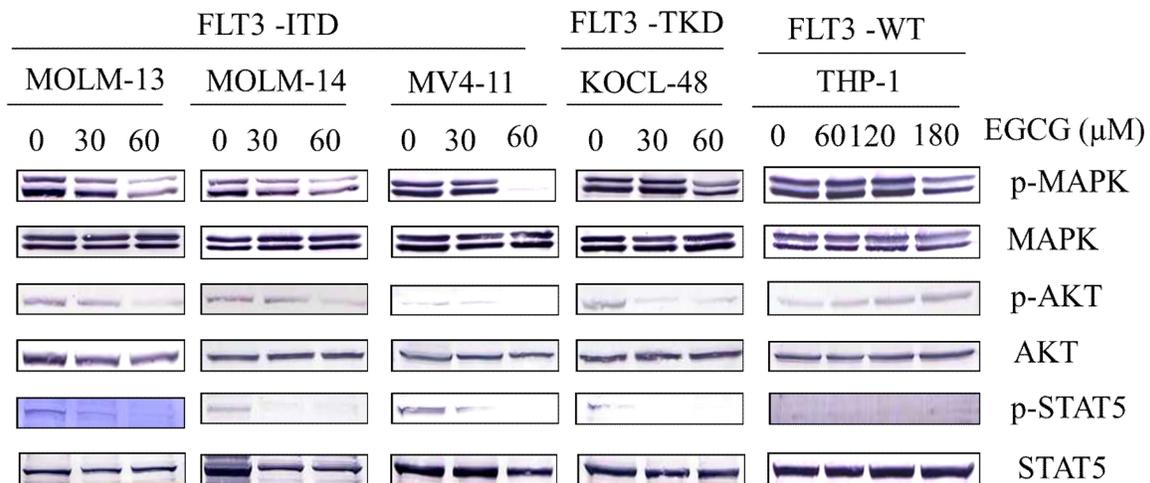
Figure 9. Down-regulation of FLT3 downstream molecules in EGCG-treated AML cells.

MOLM-13, MOLM-14, MV4-11, KOCL-48 and THP-1 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of EGCG or DMSO alone as control for 8 h. Total cell lysates were subjected to western blot analysis with indicated antibodies.
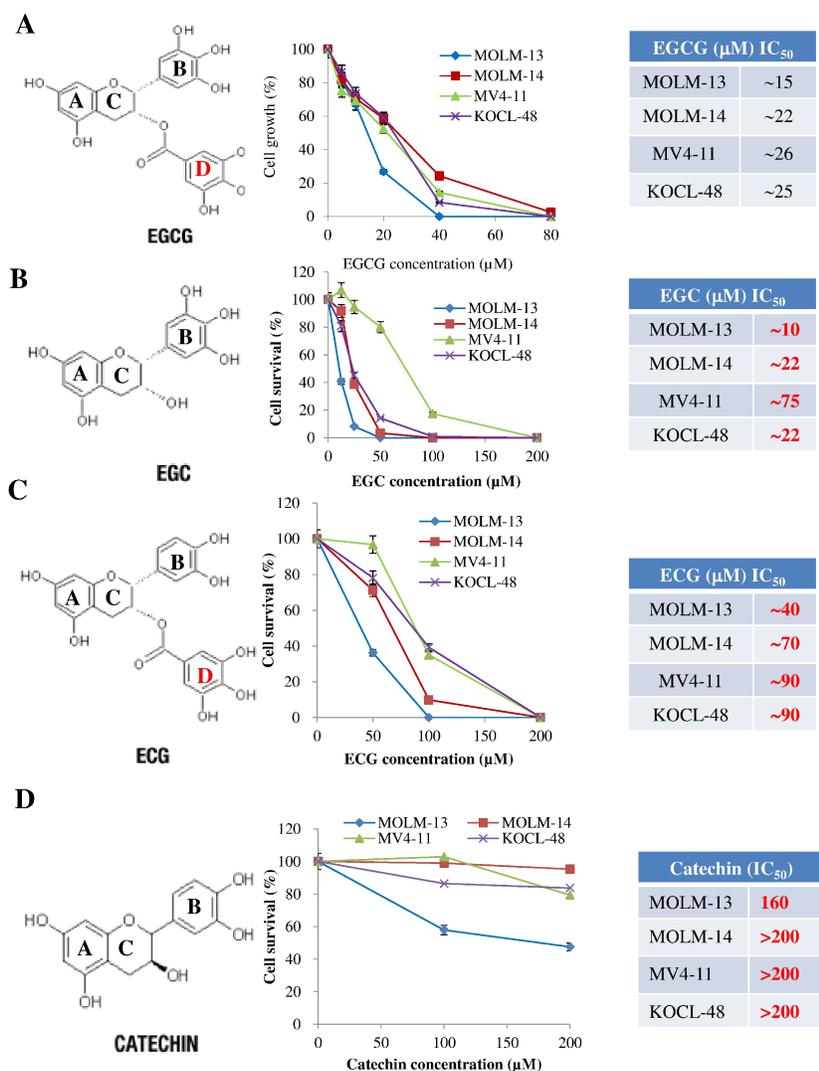
Figure 10. Effect of EGC, ECG and Catechin on cell proliferation of MOLM-13, MOLM-14, MV4-11 and KOCL-48 cell lines.

MOLM-13, MOLM-14, MV4-11, KOCL-48 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of EGCG (**A** = figure 5A, for comparison), EGC (**B**), ECG (**C**), catechin (**D**) or DMSO alone as control for 72 s. The number of viable cells was counted after the trypan blue exclusion test. Results were calculated as the percentage of the control values.
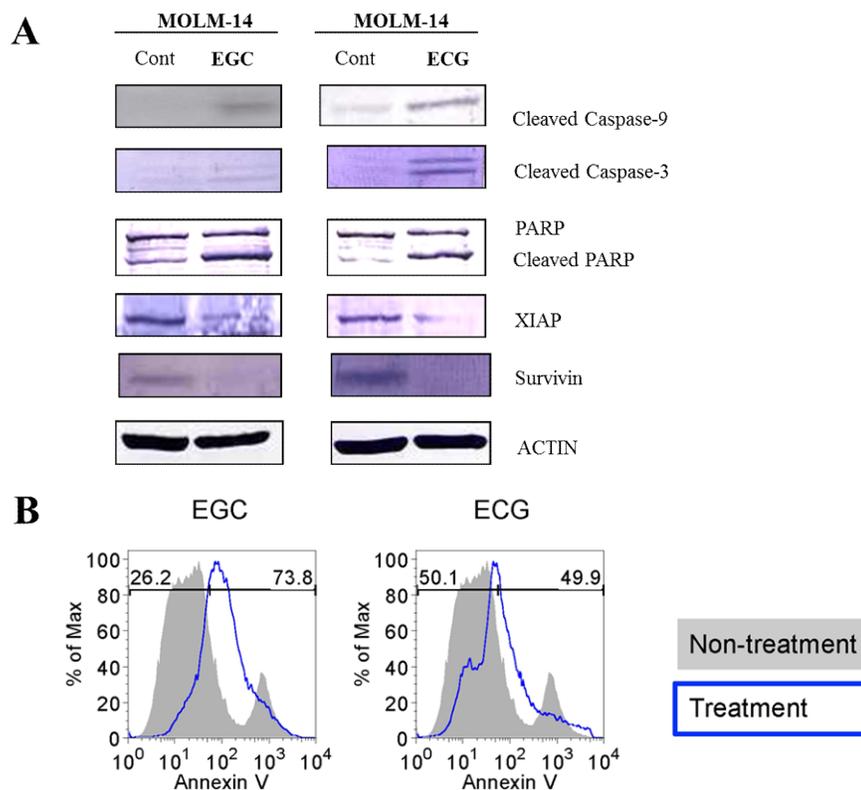
Figure 11. EGC and ECG induced apoptosis in MOLM-14 cells

MOLM-14 cells at a density of 1×10^5 cells/ml were treated with 100 µM EGC, 200 µM ECG or DMSO alone as control for 16 h. Total cell lysates were subjected to western blotting analysis with indicated antibodies (**A**) or staining with PE-Annexin V and analyzed by FACS Calibur. Collected data were analysed by Flowjo software (**B**).
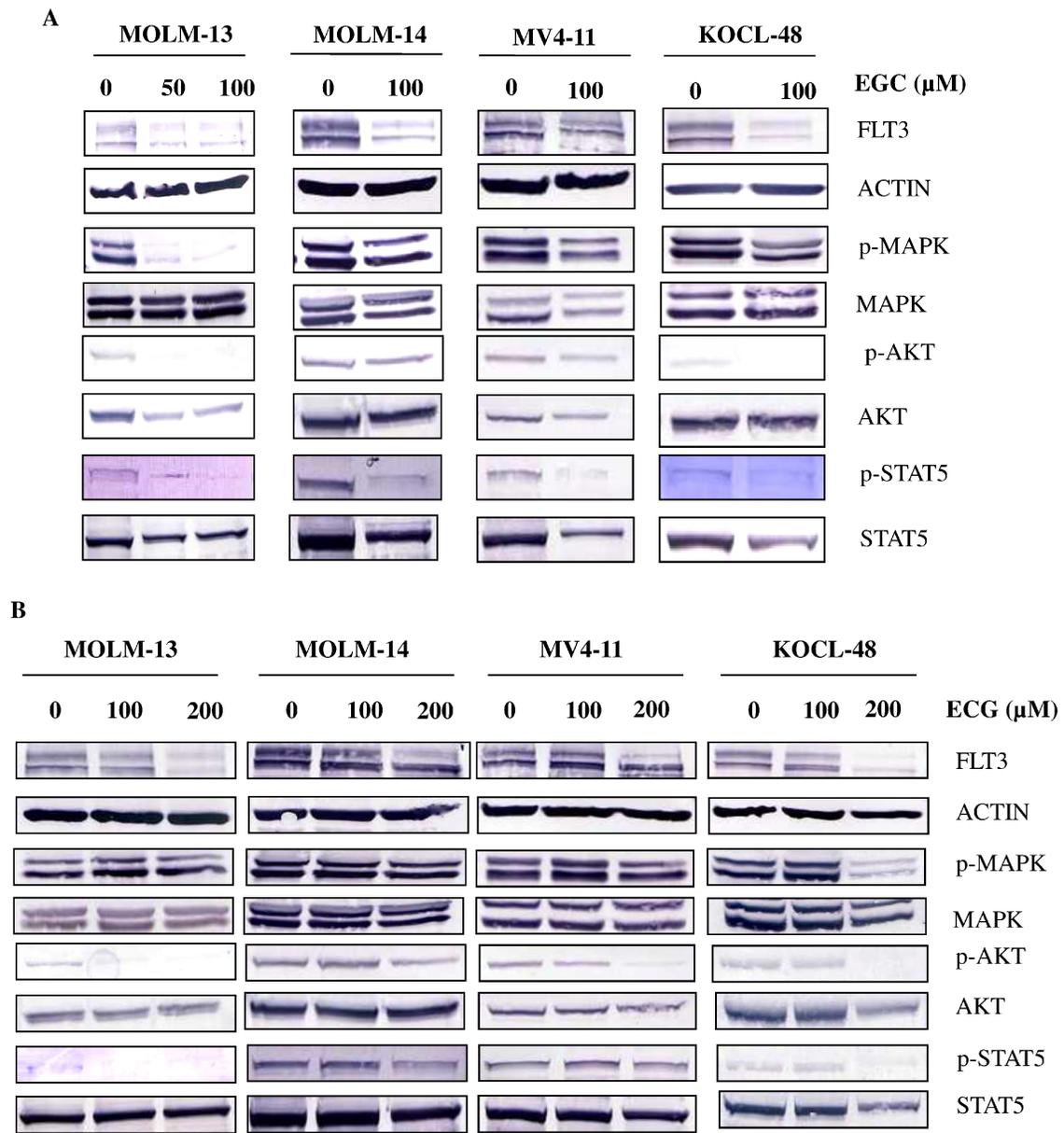
Figure 12. Down-regulation of FLT3 expression and its downstream molecules in EGC- and ECG-treated AML cells.

MOLM-13, MOLM-14, MV4-11 and KOCL-48 cells at a density of 1×10^5 cells/ml were treated with indicated concentration of EGC (**A**) or ECG (**B**) or DMSO alone as control for 8 h. Total cell lysates were subjected to western blot analysis with indicated antibodies.
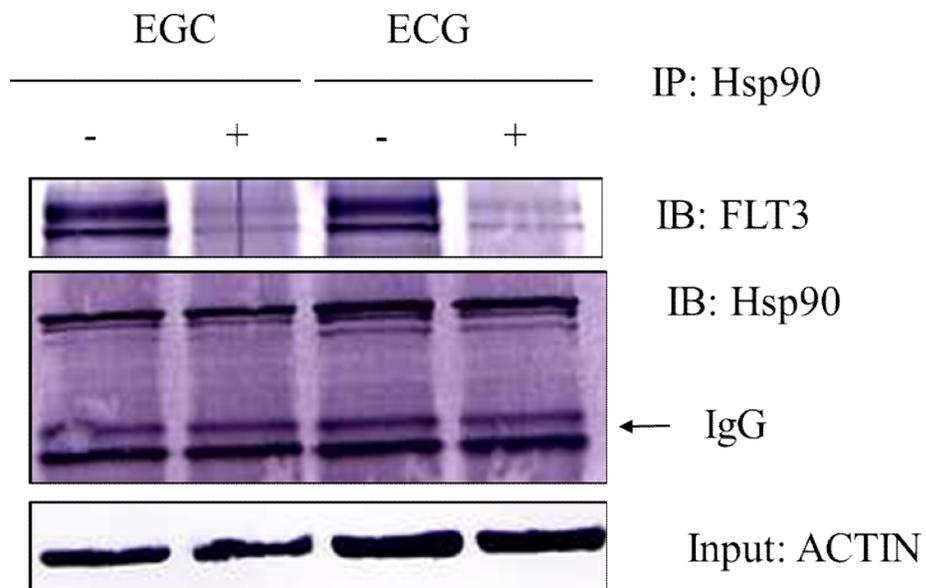
Figure 13. EGC and ECG dissociated the interaction between FLT3 and Hsp90 in

MOLM-13 cells

MOLM-13 cells at a density of 1×10^5 cells/ml were treated with 100 µM EGC, 200 µM ECG or DMSO alone as control for 8 h. Total cell lysates were immunoprecipitated with anti-Hsp90. Precipitated protein were subjected to western blot analysis with indicated antibodies.

A

MOLM-13 MOLM-14 MV4-11 KOCL-48 THP-1

\- + \- + \- + \- + \- +   EGCG (60µM)

FLT3

GAPDH

B
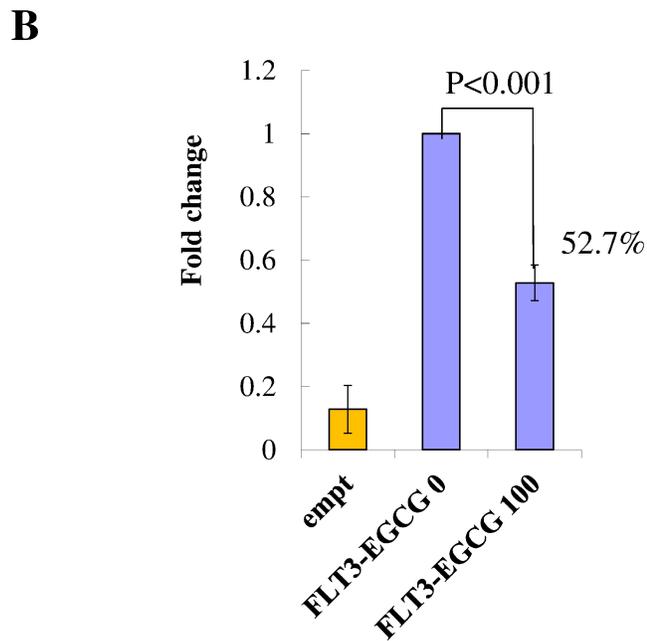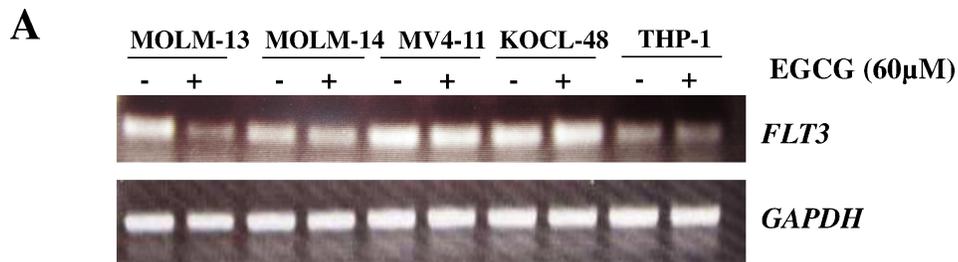
Figure 14. EGCG inhibited FLT3 promoter activity

A.   Semi-quantitative reverse transcription-PCR, MOLM-13, MOLM-14, MV4-11,

KOCL-48 and THP-1 cells at a density of 1×10^5 cells/ml were treated with 60 µM EGCG

for an indicated duration. Total RNA was extracted and cDNA was synthesized to perform

PCR reaction with indicated primers.

B.   The inhibition of EGCG on *FLT3* promoter activity was analyzed by reporter assay (n

= 5, mean ± SD). Luciferase activities of reporter series were tested in a presence or

absence of 100 µM EGCG.
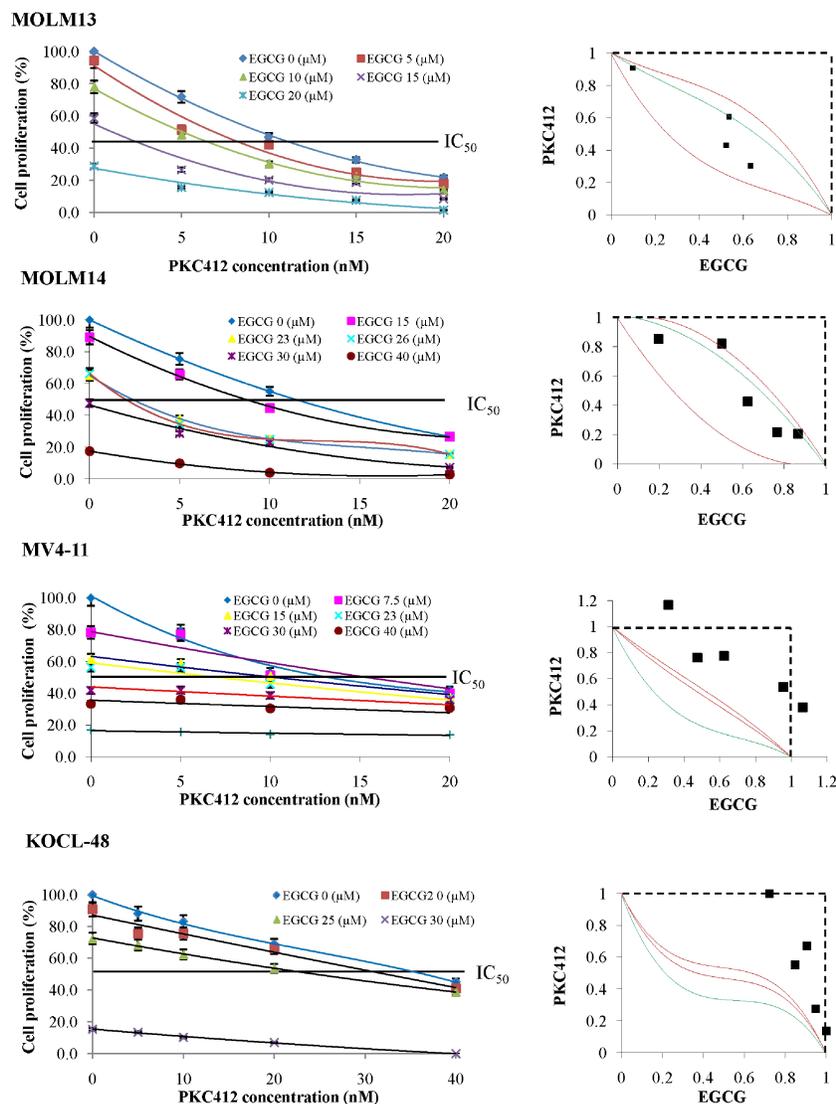
Figure 15. Isobolograms of simultaneous exposure to EGCG and PKC412 in MOLM-13, MOLM-14, MV4-11 and KOCL-48 cell lines.

The isobolograms shown are representative of at least three independent experiments. Each point represents the mean value of at least three independent experiments. The combination of EGCG with PKC412 showed additive effect (MOLM-13 and MOLM-14) and antagonism effect (MV4-11 and KOCL-48).
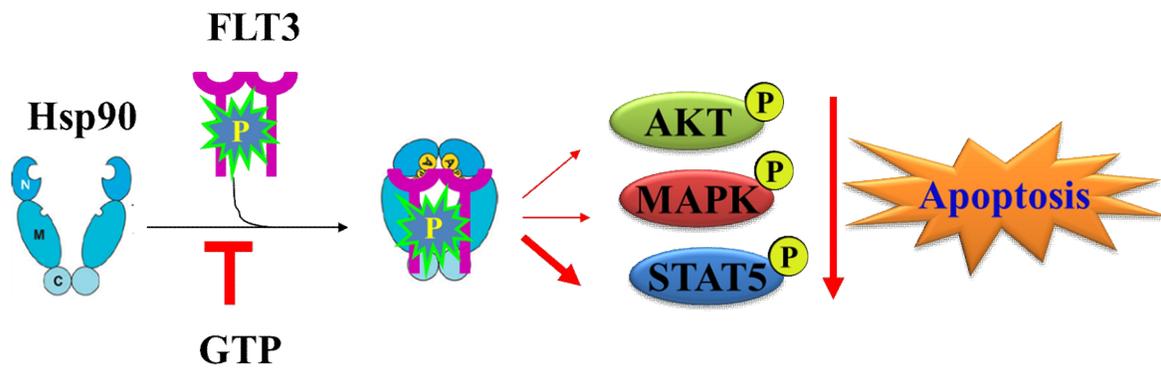
Figure 16. The mechanism of down-regulated FLT3 protein expression by GTP

The phosphorylated FLT3 bind to Hsp90. This association was disrupted by green tea polyphenols (GTP) that lead to down-regulate FLT3 and its downstream signalling molecules, finally induce apoptosis in AML overexpressing FLT3

TABLES

Table 1. Mean values of observed data and predicted minimum and maximum values

of the combination of EGCG and PKC412

Cell lines	n	Observed data	Predicted values for an additive effect		Effect
			Minimum	Maximum	
MOLM-13	4	0.423	0.379	0.766	Additive
MOLM-14	5	0.414	0.121	0.549	Additive
MV4-11	5	0.803	0.205	0.410	Antagonism (<0.01)
KOCL-48	5	0.887	0.467	0.579	Antagonism (<0.01)