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FLT3 tyrosine kinase as a target molecule for selective antileukemia therapy

Abstract Fusion gene products such as PML-RARα and BCR-ABL generated by leukemia-specific chromosomal translocations have been identified as target molecules for the treatment of leukemia. Here we describe one possibility for extending the frontier of mechanism-based medicine for acute myeloid leukemia (AML). FLT3, a receptor tyrosine kinase (RTK) preferentially expressed in hematopoietic progenitor cells, frequently has a gain-of-function mutation in AML. To search for FLT3-targeted compounds, we screened the growth-inhibitory effects of several tyrosine kinase inhibitors (TKIs) on mutant *FLT3*-transformed 32D cells. Herbimycin A at a concentration of 0.1 μ*M* markedly inhibited the growth of the transfectants but at that concentration was ineffective in parental 32D cells. It suppressed the constitutive tyrosine phosphorylation of the mutant FLT3, but not the phosphorylation of the ligand-stimulated wild-type FLT3. In mice transplanted with transformed 32D cells, the administration of herbimycin A completely prevented leukemia progression. Recent studies have indicated that herbimycin A binds directly with HSP90, a molecular chaperone, and destabilizes HSP90-associated proteins. Another HSP90 inhibitor, radicicol, also induced apoptosis selectively in transformed 32D cells. HSP90 is a promising target for the treatment of AML with mutant FLT3.

Keywords Acute myeloid leukemia · FLT3 · Molecule-targeted therapy · HSP90 · Herbimycin A

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Introduction

The molecular understanding of leukemogenesis has presented a new paradigm for the treatment of cancer [4]. In leukemia, fusion-gene products such as PML-RARα and BCR-ABL, generated by chromosomal translocations are molecular targets for all-*trans*-retinoic acid and a tyrosine kinase inhibitor, respectively. These oncoprotein-directed compounds have led to a breakthrough in the treatment of leukemia [2, 6]. The next issue is how to extend the frontier of mechanism-based therapy. Although the targeting of various kinases including Ras, Raf, PKC, STAT, and CDK has been under investigation [2, 4, 13], a more selective approach to oncoproteins is needed.

Receptor tyrosine kinase (RTK) plays important roles in regulating the proliferation, differentiation, and survival of hematopoietic cells [8]. Mutations of RTK class III, which includes platelet-derived growth factor receptor (PDGFR), FMS, c-KIT, and FLT3, are found in human leukemia and myelodysplastic syndrome (MDS) [3, 5, 19, 30]. We have shown the clinical and biological significance of *FLT3* gene mutations [7, 9, 10, 18, 34]. The *FLT3* gene is most frequently mutated among class III RTKs. We therefore investigated the possibility of antileukemia therapy targeting mutant FLT3.

Mutations of *FLT3* gene in acute myeloid leukemia

FLT3, which is a class III RTK, has an extracellular region composed of five immunoglobulin-like domains, a single transmembrane region, a juxtamembrane (JM)

domain, a tyrosine kinase region spaced by a kinase insert region, and a C-terminal domain [15, 22]. FLT3 is preferentially expressed in early hematopoietic progenitors, the central nervous system, placenta, and liver [21]. FLT3 is activated by its ligand (FL), which is expressed as a membrane-bound or soluble form by bone marrow stroma cells [14]. FL stimulation facilitates and/or stabilizes dimer formation of the FLT3 molecule, which is critical for subsequent phosphorylation [23]. The phosphorylated FLT3 mediates signals through physical association with and/or further phosphorylation of other signal proteins [1]. Although the function of FLT3 has not been fully elucidated, the kinase is thought to play important roles in the proliferation, differentiation, and survival of hematopoietic stem cells and progenitor cells [20, 27].

In 1996, a unique mutation of the *FLT3* gene was identified in clinical samples from acute myeloid leukemia (AML) patients [19]. In this mutation, the JM domain-coding sequence is tandemly duplicated as a direct tail-to-head succession. The location and length of the internal tandem duplication (ITD) varies among samples, and a nucleotide sequence was frequently inserted between the ITD [19, 34]. Importantly, the ITD sequence is always readable in frame. Thus the JM domain is elongated in mutant FLT3 but the downstream domains are unaffected. The mutant FLT3 products are constitutively dimerized and phosphorylated at their tyrosine residues [10]. Although the molecular mechanism by which the elongated JM domain causes autophosphorylation is still under investigation, the JM domain likely regulates the dimer formation (Kiyoi et al., manuscript in preparation). In clinical samples, the *FLT3* gene with the ITD (FLT3/ITD) has been identified in 20% of de novo AML cases and in 5% of MDS cases [9, 11, 34]. The presence of FLT3/ITD is related to leukocytosis and progression/relapse of AML, but not significantly to the FAB classification [9, 11, 18].

Recently, we have identified missense mutations at D835, which is located in the activation loop within the kinase domain [33]. This mutation is found in over 5% of AML cases, but rarely in other hematologic malignancies. The *FLT3* mutation is so far the most frequent gene alteration reported to be involved in AML, and it is suggested that FLT3 signaling plays a causal role in the development of AML.

In vitro screening assay for mutant FLT3-selective agents

How should one search for agents that selectively suppress the function of mutant FLT3? As described previously [7, 35], transfection of mutant *FLT3* cDNA abrogates the dependency of the murine myeloid cell line 32D on interleukin-3 (IL-3). We used transformed and parental 32D cells to search for FLT3-targeted compounds. Several tyrosine kinase inhibitors (TKIs) were evaluated for growth-inhibitory effects using mutant *FLT3*-transfected 32D (MtFLT3/32D) and parental 32D cells. Herbimycin A suppressed the proliferation of MtFLT3/32D cells more significantly than 32D cells stimulated with IL-3 at the low concentrations of 0.1 to 0.3 μM [35]. Treatment with herbimycin A selectively induced apoptosis in MtFLT3/32D cells (Fig. 1). The viable cell number after a 3-day culture was reduced by 50% (IC_{50}) at 0.06 and 0.2 μM in MtFLT3/32D and 32D cells, respectively. Other TKIs (CGP52411, genistein, tyrphostin A9, and erbstatin) did not exhibit selective cytotoxicity. STI571, a TKI known to be specific for c-ABL, did not inhibit the growth of either cell line.

Inhibition of autophosphorylation

To study whether herbimycin A at the above concentrations suppresses the phosphorylation of FLT3, *FLT3*-cDNA was transfected into Cos7 cells and the phosphorylation status of FLT3 was analyzed. Phosphorylation of FLT3 was augmented by the addition of FL, which was only slightly inhibited by the addition of herbimycin A. In contrast, mutant FLT3 was phosphorylated in an FL-independent manner. Herbimycin A markedly suppressed the phosphorylation of mutant FLT3 and did not significantly reduce the expressed level of FLT3 [35]. These results indicate that herbimycin A inhibits the autophosphorylation of mutant FLT3 more significantly than the ligand-dependent phosphorylation of wild-type FLT3.

The addition of 0.1 μM herbimycin A induced apoptosis of MtFLT3/32D cells but not parental 32D

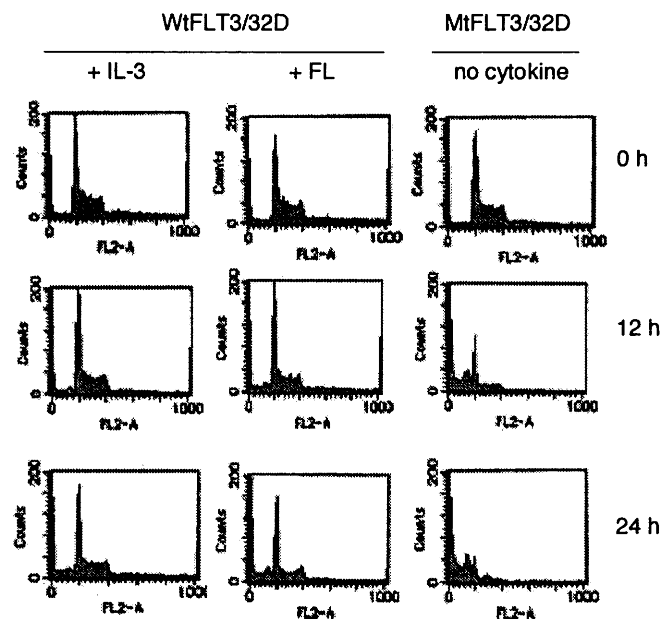

Fig. 1 Induction of apoptosis by treatment with herbimycin A. 32D cells transfected with wild-type *FLT3* cDNA (WtFLT3/32D) and with mutant *FLT3* cDNA (MtFLT3/32D) were treated with 0.3 μM herbimycin A for 0, 12, and 24 h. The DNA histograms demonstrated that a sub-G_1 fraction was selectively increased in MtFLT3/32D cells

cells, suggesting the possibility of in vivo therapy with herbimycin A.

In vivo effectiveness of herbimycin A

MtFLT3/32D cells (1×10^6) were subcutaneously inoculated into the right side of the back of C3H/HeJ mice ($n=10$). Five mice received herbimycin A 2.5 μg i.p. 3 days per week for 4 weeks. All of the five control mice developed subcutaneous tumors and died of leukemia within 6 weeks after the inoculation of MtFLT3/32D. However, none of the treated mice developed tumors and all survived for >3 months. These results indicate that herbimycin A is also effective in vivo.

HSP 90-inhibitors for mutant *FLT3*-transformed 32D cells

Recently, benzoquinone ansamycins including herbimycin A have been reported to inhibit the function of HSP90, a chaperoning protein, and to destabilize HSP90-associated proteins [29]. We investigated whether another HSP90 inhibitor, radicicol [25], inhibited the growth of the MtFLT3/32D cells. Radicicol had more selective cytotoxic activity than herbimycin A at one-log lower concentration (Fig. 2). This suggests that the function of HSP90 is selectively associated with the mutant FLT3.

Discussion

We have shown that comparing MtFLT3/32D and parent 32D cells is useful for screening compounds targeting mutant FLT3. Since the specific inhibitor of FLT3 kinase is unknown, herbimycin A, CGP 52411, genistein, tyrphostin A9, erbstatin, and STI571 were evaluated in this study. Erbstatin, genistein, and CGP 52411 were isolated or synthesized by screening for the inhibition of EGFR kinase (reviewed in reference 12). Tyrphostin A9 is an inhibitor of the kinase activity of PDGFR. STI571 specifically inhibits c-Abl, Bcr-Abl, PDGFR, and c-KIT [2]. However, these compounds did not show selective inhibition of MtFLT3/32D cells. Only herbimycin A had inhibitory activity. Herbimycin A was originally isolated from the culture broth of a strain of *Streptomyces* on the basis of its ability to revert rat kidney cells transformed by v-*Src* to normal morphology [31]. Further study revealed that it inhibited the kinase activity of Src, Yes, Fps, Ros, and Abl [32]. Herbimycin A has also been reported to downregulate RTK selectively [17].

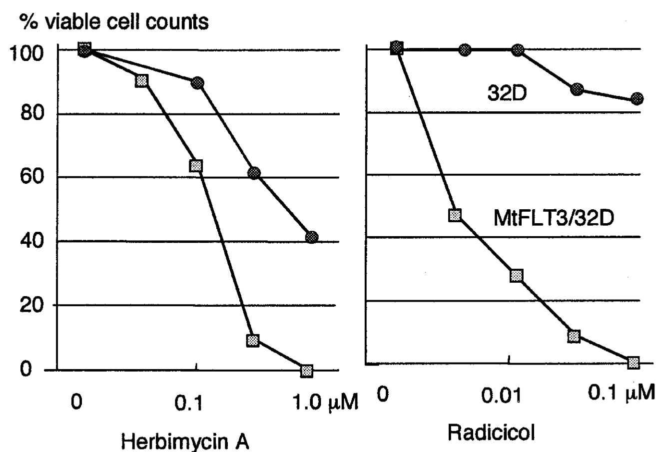

Fig. 2 Cytotoxic effects of herbimycin A and radicicol on MtFLT3/32D cells. Various concentrations of herbimycin A or radicicol were added to the cultures of IL-3-dependent 32D cells and MtFLT3/32D cells. Viable cells were counted by the trypan blue dye exclusion assay after 48 h of culture (● 32D, □ MtFLT3/32D)

Recent studies have shown that benzoquinone ansamycins including herbimycin A and geldanamycin bind with the ATPase active site of HSP90 and inhibit its chaperoning function [29]. HSP90 is an abundant heat-shock protein required for de novo folding of certain proteins such as steroid receptor, c-Src, Raf-1, and Cdk [16, 24, 28]. Although the mechanism of selectivity remains unclear, the target molecules appear to be intrinsically labile or need to maintain a competent conformation to bind with a cofactor or ligand [16]. Additionally, HSP90 is likely to target misfolded proteins generated by mutations or various stress treatments. HSP90 is involved in refolding these proteins in cooperation with HSP70 [16].

The mechanism by which the kinase activity of mutant FLT3 was inhibited more by herbimycin A than that of wild-type FLT3 remains to be elucidated. One possibility is that the noncanonical FLT3 molecule with an elongated or missense mutated sequence is unstable and requires chaperoning by HSP90 in leukemia cells. Radicicol reportedly dissociates BCR-ABL from the HSP90 complex and destabilizes it, and this causes differentiation and apoptosis in the chronic myeloid leukemia cell line K562 [26]. Of interest is whether fusion products in general require HSP90 chaperoning and are sensitive to HSP90 inhibitors.

In determining which molecules are suitable treatment targets, the targeting of activated FLT3, which is located at the most upstream end of the signals, is a more rational approach than targeting downstream cascades. Accordingly, the development of an FLT-specific inhibitor is necessary. Until a compound specific to FLT3 is identified, HSP90 inhibitors are promising for the treatment of AML with mutant FLT3. In the USA, a phase I trial using an analogue of geldanamycin, 17-*N*-allylamino-17-demethoxy geldanamycin, has been started for patients with malignancies (http://cancernet.nci.nih.gov/). HSP90 inhibitors may extend the frontier of mechanism-based medicine for AML.

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