Iranian Journal of Basic Medical Sciences

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IJBMS

Differences in growth promotion, drug response and intracellular protein trafficking of FLT3 mutants

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ARTICLE INFO

Article type:
Original article

Article history:
Received: Dec 30, 2013
Accepted: May 6, 2014

Keywords:
Activating mutation
Drug response
FLT3
Protein trafficking

ABSTRACT

Objective(s): Mutant forms FMS-like tyrosine kinase-3 (FLT3), are reported in 25% of childhood acute lymphoid leukemia (ALL) and 30% of acute myeloid leukemia (AML) patients. In this study, drug response, growth promoting, and protein trafficking of FLT3 wild-type was compared with two active mutants (Internal Tandem Duplication (ITD)) and D835Y.

Materials and Methods: FLT3 was expressed on factor-dependent cells (FDC-P1) using retroviral transduction. The inhibitory effects of CEP701, imatinib, dasatinib, PKC412 and sunitinib were studied on cell proliferation and FLT3 tyrosine phosphorylation. Total expression and proportion of intracellular and surface FLT3 was also determined.

Results: FDC-P1 cells became factor-independent after expression of human FLT3 mutants (ITD and D835Y). FDC-P1 cells expressing FLT3-ITD grow 3 to 4 times faster than those expressing FLT3-D835Y. FD-FLT3-ITD cells were three times more resistant to sunitinib than the FD-FLT3-WT cells. The Geo means for surface FLT3 expression in FD-FLT3-ITD and –D835Y were 65 and 70% less than the FD-FLT3-WT cells. About 40% of expressed FLT3 was detected as intracellular in FD-FLT3-D835Y cell compared to 4 and 4.5% in FD-FLT3-WT and –ITD cells.

Conclusion: Retention of D835Y FLT3 mutant protein may cause altered signaling, endoplasmic reticulum stress and activation of apoptotic signaling pathways leading to lower proliferation rate in FD-FLT3-D835Y than the FLT3-WT and ITD mutant., these may also also contribute, along with the preferential affinity, to the increased sensitivity of D835Y of CEP701 and PKC412. Studying these genetic variations can help determining the prognosis and designing a therapeutic plan for the patients with FLT3 mutations.

► *Please cite this paper as:*
Mashkani B, Griffith R, Ashman L. Differences in growth promotion, drug response and intracellular protein trafficking of FLT3 mutants. Iran J Basic Med Sci 2014; 17:867-873.

Introduction

FMS-like tyrosine kinase-3 (FLT3), a member of type 3 receptor tyrosine kinase family (RTK), is normally expressed by primitive hematopoietic stem/progenitor cells and is lost during differentiation (1). Under physiological conditions, FLT3 signaling has an important role in hematopoietic stem cell differentiation (2), B-cell lineage commitment (3) and dendritic cell expansion (4).

Overexpression of FLT3 mRNA has been reported in patients with acute myeloid leukemia (AML), acute lymphoid leukaemia (ALL) and blast crisis of chronic myeloid leukemia (CML) (5). Here aberrant signaling of FLT3 results from overexpression of the wild-type form, activating mutations in its juxtamembrane (JM) or in its tyrosine kinase (TK) domain. Segmental duplications occur in the form of repetition from 3 to over 400 base pairs (6) in the JM domain (exons 14 and 15) (7, 8); in the region containing amino acids residues Y591-Y597 (9). Other major mutation site in FLT3 is the conserved aspartate residue (D835) in the activation loop which may be replaced by valine (10), histidine (11), asparagine, glutamate, or tyrosine (12). Mutant forms of FLT3 have been reported in 25% of childhood ALL (10) and 30% of AML patients (13, 14), in myelodysplastic syndrome (MDS) and chronic myelomonocytic leukemia (CMML) (15). They have a confirmed role in transformation of MDS to AML (16), which results in a higher rate of AML relapse and poor response to stem cell transplantation therapy (17). FLT3 signaling is likely involved in autoimmune diseases (18) such as rheumatoid arthritis (RA) (19) and diabetes (20). Therefore, FLT3 and its downstream signaling pathways have been considered as therapeutic targets in some of these disorders.

FLT3 is not sensitive to imatinib (21) due to the

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presence of a phenylalanine (Phe) in the position 691 of FLT3, as the gate-keeper residue, instead of a threonine in imatinib sensitive kinases such as stem cell factor receptor (c-KIT), macrophage colony stimulating factor receptor (FMS) and platelet-derived growth factor receptor (PDGFR) (22). AG1295 and AG1296 were the first compounds to be identified as FLT3 inhibitors. These non-specific tyrosine kinase inhibitors (TKIs) have been shown to inhibit autophosphorylation of FLT3 and induce apoptosis in cells transfected with FLT3-IDT mutant (23, 24). Some other inhibitors have also been discovered including CEP701 (lestaurtinib), PKC412 (midostaurin) and SU11248 (sunitinib) that were active in nM concentrations and have been shown to kill cell lines derived from leukemias. Bis(1H-2-indolyl)-1-methanone derivatives such as D-64406 and D-65476 inhibited proliferation of growth factor-dependent BA/F3 cells transfected to express the oncogenic fusion protein TEL-FLT3 with IC_{50} value of 200 to 300 nM in the absence of the IL-3, but more than 1000 nM in the presence of the IL-3 (25).

Despite all efforts, no selective inhibitor has yet been developed for FLT3 to be used as a drug. Thus, a better understanding of downstream signaling pathways could provide new insights into the molecular mechanisms and alternative drug targets. Although mutations in both JM and TK domains are believed to render the kinase domain constitutively active, the activation dynamics may be different. Additionally, as reported for mutants of another type 3 RTK, KIT (26), constitutive activation of the FLT3 kinase domain may affect its protein trafficking. This can affect the downstream signaling pathways, and also drug response (27) in the cells harboring such FLT3 mutations. Identification of these differences may be useful in development of therapeutic strategy. In this study, growth promoting, drug response to CEP701, PKC412 and sunitinib as well as protein trafficking of FLT3 wild-type were compared with two different constitutively active mutants (FLT3-ITD and –D835Y).

Materials and Methods

The factor-dependent murine early myeloid cell line FDC-P1 was routinely maintained in DMEM 10% FCS supplemented with murine granulocyte-macrophage colony stimulating factor (GM-CSF) as previously described (28). The DNA constructs for FLT3 (WT, ITD, and D835Y) were supplied by Dr. Hitoshi Kiyoi (Nagoya University School of Medicine, Nagoya, Japan) and subcloned into MSCV-IRES-GFP vector. The vector was prepared by removing FMS from MSCV-FMS-IRES-GFP supplied by Dr AB Lyons, (Hanson Institute, Adelaide), with the consent of the originator Dr M Roussel, (St. Jude Children's Research Hospital, Memphis; TN.). MSCV-FLT3-IRES-GFP was introduced into Phoenix (Clontech, Mountain View, CA) cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and packaged as ecotropic retroviral particles. The transfected cells were incubated for 36 hr and the supernatant was collected and used for transduction of the FDC-P1 parent cells. DNA was introduced into FDC-P1 cells by retrovirus-mediated gene transfer as previously described (29) with some modifications (centrifugation of transfection mix at 2000 g for 2 hr) to improve the infection rate as described for dendritic cells (DCs) (30). FDC-P1 cells expressing FLT3-WT (FD-FLT3-WT) were selected using FLT3L as growth factor, while those expressing constitutively active mutant FLT3 (FD-FLT3-ITD, and FD-FLT3-D835Y cells) selected in growth factor free medium. Then FD-FLT3 cells were sorted (FACS Aria, BD Biosciences, CA) for expression of GFP to obtain a homogenous population. FLT3 expression on sorted cells was confirmed by flow cytometry using FLk2/FLT3 (sc-19635) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and sheep anti-mouse IgG conjugated with PE (Chemicon, Billerica, MA) as secondary antibody.

Cell proliferation of FDC-P1 parent, FD-EV and FD-FLT3 cell lines in growth factor-free media. All the cell lines were cultured for 48 hr and then the number of live cells in each well was determined by MTS. MTS solution (Promega, Madison, WI), 20 µl, was added to each well, incubated for 2 hr and the absorbance at 590nm was measured using a FLUOstar OPTIMA plate reader (BMG LABTECH, Offenburg, Germany). Results were analyzed using GraphPad Prism 4.3 for Windows, (GraphPad Software, San Diego, CA).

For cell proliferation inhibition assay, serial dilutions of inhibitors were prepared in 96-well tissue culture plates in DMEM medium (GIBCO, Invitrogen) containing FLT3L and 10% FCS (JRH). FD-FLT3 (WT, ITD, and D835Y) ($4x10^4$) cells were added to each well and the plates were incubated at 37˚C in a humidified 5% CO_2 in air atmosphere for 48 hr. The number of viable cells was assessed as described for cell proliferation assay using MTS. Results were analyzed using GraphPad Prism 4.3 and each EC_{50} value is calculated as the average of three independent experiments ± standard deviation and with four replicates in each experiment.

In the phosphorylation inhibition assay, FD-FLT3-D835Y cell were pre-incubated with different concentrations (25, 5, 1, 0.2 and 0.04 nM) of CEP701 for 30 min before pulsing with FLT3L for 5 min. Finally, the cells were washed once with cold PBS and lysed with ice-cold 1% NP40 in TSE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA pH 8.0) with complete protease inhibitor cocktail (Roche, Basel, Switzerland), 5 mM sodium fluoride, 5 mM tetra sodium pyrophosphate, 5 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride (Sigma). Protein concentration in the cell lysates was determined by a MicroBCA kit (Pierce, Rockford, IL).

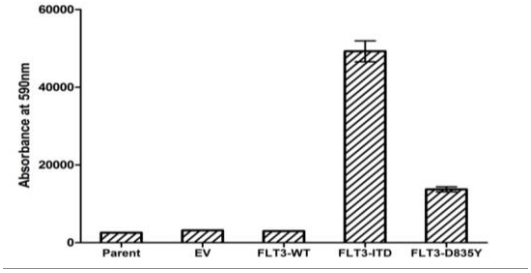

Figure 1. Cell proliferation of FDC-P1 parent, FD-EV and FD-FLT3 cell lines in growth factor-free media. Results were analyzed using GraphPad Prism 4.3 for Windows. The data presented as mean ± SD values of four replicates for each point

FLT3 protein was immunoprecipitated from the cell lysate containing 500 µg total protein using 2 µg of anti-FLT3 SF-1.340 (Santa Cruz Biotech) with protein-G Sepharose beads (Invitrogen) (30 µl of 50% slurry). The immunoprecipitated FLT3 resolved on 6% SDS/polyacrylamide gels along with protein markers (Fermentas or Bio Rad). Proteins on the gels were transferred to Hybond nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA).

The membranes were blocked with 2% BSA (Sigma) for 30 min and one of them probed for phosphotyrosine using a cocktail of 4G10 (Upstate, Temecula, CA) and pY20 (BD Biosciences, San Jose, CA) antibodies. The membrane was washed three times with TBST (10 mM Tris pH8, 150 mM NaCl and 0.1% Tween-20 (all from sigma)) and incubated with secondary anti-mouse antibody conjugated with HRP (GE Healthcare) for 45 min. The membranes were washed three times in TBST buffer and incubated with ECL-Plus reagent (GE Healthcare) for 1 min, and the fluorescent product was measured by a Typhoon 9410. Then the membrane was stripped and probed with FLT3 (sc-479 antibody, Santa Cruz Biotechnology) as described for phosphotyrosine.

To evaluate proportions of intra- and cell surface FLT3 receptor, FD-FLT3 cells were fixed in 5% formaldehyde for 10 min and then divided into two parts and one of them was permeabilised using 100% methanol (15 min).

Both samples of the fixed and the fixed-permeabilised cells were immunostained for FLT3. Briefly, the samples ($2x10^6$ cells) of negative control (FDC-P1 parent cells), the FDC-P1 cells transduced to express FLT3 WT, FLT3-ITD, and FLT3-D835Y were washed once with PBA (PBS with 0.5% BSA and 0.05% sodium azide) (all chemicals from Sigma). Then cells were resuspended in 100 µl of normal rabbit serum containing 2 µg of FLT3 antibody (FC1.340 (Santa Cruz Biotechnology), vortexed and incubated on ice for 60 min, then washed three times with 2 ml PBA. The cells were resuspended in 100 µl PBA containing 1 µg secondary antibody (anti-mouse IgG conjugated with PE (Chemicon, Billerica, MA); and incubated on ice for 30 min. Finally, the cells were washed three times with 2 ml PBA and fixed with cold FACS-Fix solution (0.5% paraformaldehyde in PBS) and analyzed using a FACSCalibur Flow Cytometer (BD Biosciences). Data for intensity of PE fluorescence (representing FLT3 expression) and also GFP (as the reporter gene) were analyzed using CellQuest Software (BD Biosciences).

The Geo Mean of the fixed and fixed permeablized cells was considered as the representative of surface and total FLT3 expression, respectively. The FD-FLT3-WT, -ITD and D835Y cells, the Geo Mean values for FLT3 were normalized to the GFP in each cell type and the intracellular FLT3 was calculated by deduction of surface from total FLT3.

Results

Proliferation/survival of FD-FLT3 (WT, ITD, D835Y)

To compare the effects of FLT3 (WT, ITD and D835Y mutants) expression on factor-dependent cell growth and survival, FDC-P1 parent cells were compared to those transduced with MSCV-IRES-GFP empty vector (EV), or MSCV-FLT3-IRES-GFP (WT, ITD and D835Y). As shown in the Figure 1, parental, EV and FLT3-WT FDC-P1 cells did not proliferate in the growth factor (GF)-free medium while expression of FLT3 mutants (ITD and D835Y) enabled proliferation. After 48 hr, the number of live cells was ~7 and ~21 times more for the FD-FLT3-D835Y and FLT3-ITD expressing cells, respectively. Thus while both mutants conferred factor dependent growth, the FLT3-ITD appeared more efficient with >3 fold more cells than the D835Y mutant.

Drug response of the FD-FLT3 (WT, ITD, D835Y)

In order to assess the effects of FLT3 mutations on its susceptibility to inhibitors, cell proliferation assays were repeated on the FDC-P1 cells in the presence of CEP701, dasatinib, imatinib, PKC412 and sunitinib. The experiments showed that CEP701, PKC412 and sunitinib could inhibit the growth of FD-FLT3-WT cells (Table 1). However, dasatinib and imatinib were not effective at high nM concentrations (EC_{50} >100 and 2000 nM, respectively). Sunitinib with an EC_{50} value of 7.9 nM appeared to be the most potent among the five compounds tested in these experiments. The EC_{50} value of CEP701 and PKC412 were 12.2 and 47.1 nM, respectively.

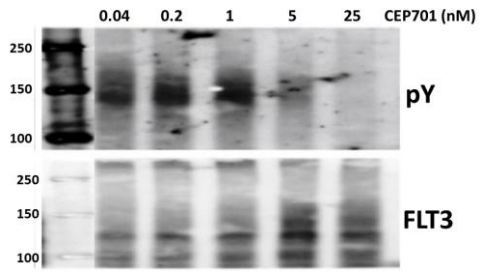

Figure 2. Inhibition phosphorylation by different concentrations of CEP701 prevents FLT3–D835Y protein degradation. Immunoprecipitated FLT3 was probed for phosphotyrosine and then the membrane was stripped and probed for FLT3. The expected places of 130 to 160 kD bands of FLT3 are indicated by arrows on the left side

Table 1. Comparison of EC_{50} values of the small molecules for inhibition of cell growth of FD-FLT3-WT, -ITD and D835Y cells. Each EC_{50} value is calculated as the average of three independent experiments ± standard deviation and with four replicates in each experiment

FD-FLT3-	CEP701 (nM)	Dasatinib (nM)	Imatinib (nM)	PKC412 (nM)	Sunitinib (nM)
WT	12.2±1.2	>100	>2000	47.1±1.2	7.9±0.9
ITD	14.3±1.6	>100	>2000	66±12	27.3±3.6
D835Y	0.9±0.1	>100	>2000	2±0.2	8.6±1.3

Comparing the response of FD-FLT3-ITD against FD-FLT3-WT cells indicated similar sensitivities to the different compounds, apart from sunitinib which was three times less effective against the FLT3-ITD mutant than the FLT3-WT (Table 1). It was observed that the FD-FLT3-D835Y cells were 15 times more sensitive to CEP701 and 30 times more sensitive to PKC412 compared to the FLT3-WT and ITD mutant cells, respectively (Table 1). While the ITD mutant appeared resistant to sunitinib, the response of the FLT3-D835 mutant was almost equivalent to WT cells.

Phosphorylation inhibition of FLT3-D835Y kinase

To better understand the sensitivity of FLT3-D835Y to CEP701, the phosphorylation status of FLT3-D835Y was investigated by Western blot analysis. In Figure 2, blotting FLT3 immunoprecipitates against phosphotyrosine indicated that FLT3-D835Y is present as multiple bands, which possibly caused by different levels of posttranslational modification including phosphorylation and glycosylation. However, it's fully glycosylated 160 kDa form is missing, which indicates that very small proportion of FLT3 can be mature to be detectable by flowcytometry (unpublished data), but not by Western blotting. Figure 2 also shows that the treatment of FD-FLT3-D835Y cells with different concentrations of kinase inhibitor, and CEP701, resulted in diminished FLT3 phosphorylation and increased intensity of FLT3-reactive bands. There was a dose dependent inhibition of phosphorylation of FLT3-D835Y mutant; in addition, CEP701 was able to completely inhibit receptor phosphorylation at the concentration of 25 nM.

Intracellular and cell surface expression of FLT3

To investigate the differences in the growth-promoting activities of the FLT3-ITD and D835Y constructs, their relative expression levels were evaluated. The system used to express each protein involved retroviral transduction of a polycistronic mRNA with each FLT3 cDNA followed by an IRES

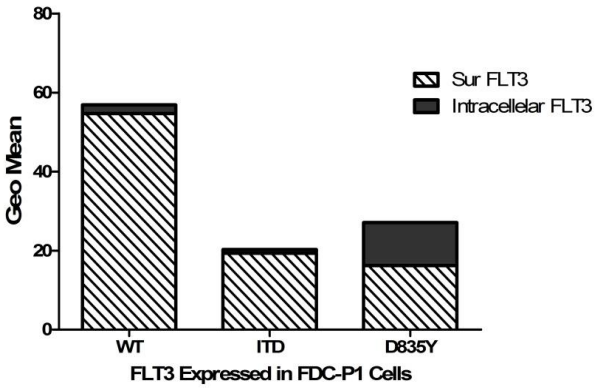

Figure 3. Statistical analysis of surface and intracellular FLT3 expression in FD-FLT3 cells. The FLT3 expressions were normalized to the GFP in each cell type and intracellular FLT3 was calculated through deduction of surface from total FLT3

(Internal Ribosome Entry Site) and the cDNA for GFP (Green Fluorescent Protein). Since both proteins are derived from the same transcript, GFP expression can therefore be considered as proxy for FLT3 mRNA expression levels. Examination of GFP levels as geometric means (Geo Means) showed these to be almost equal in the FD-FLT3-WT, -ITD and D835Y cells with values of 85, 72 and 70, respectively (Table 2).

The surface and total (both surface and intracellular) expression of FLT3 was then determined comparing the values from fixed and fixed-permeabilised cells as described in the methods section. Analysis of FLT3 expression levels across the cell lines showed that the surface expression of FLT3-WT was almost three times higher than the activating mutants (FLT3-ITD, D835Y) (Figure 3). The signal for PE (representing FLT3) was similar for fixed and fixed-permeabilised samples for cells expressing FLT3-WT or the FLT3–ITD mutant suggesting that all expressed FLT3 could reach the cell surface. In contrast, the surface expression of FLT3 in D835Y mutants was less than those in ITD. Total FLT3 signal was increased about 30% in the D835Y after permeabilisation indicating a high percentage of FLT3 protein trapped inside the cells.

Table 2. Statistical analysis of FLT3 expression in FD-FLT3 cells. The geometric Mean (Geo Mean) of the PE florescence in fixed (F) and fixed-permeabilised (FP) cells represents the surface and total FLT3, respectively. GFP fluorescence was also measured in the cells without fixing or permeabilisation

FD-FLT3	Geo Mean		
Cell type	GFP	Total FLT3	Surface FLT3
WT	85	48.4	46.5
ITD	72	14.6	13.9
D835Y	70	18.9	11.4

Discussion

To investigate the possible differences in growth promoting properties and drug responses, these FLT3 mutants were expressed on murine factor dependent FDC-P1 cells. The FDC-P1 cell line needs either mouse IL-3 or GM-CSF for proliferation in DMEM medium (31) which, however, can be abrogated by expression of oncogenic tyrosine kinases via induction of c-myc (32), or wild type tyrosine kinases in the presence of the specific ligand such as CSF-1 (33). This makes FDC-P1 cells a suitable host for expression and studying these growth factor receptors and oncogenic kinases.

In this study, we demonstrated that FDC-P1 cells became factor-independent by expression of human FLT3-ITD and -D835Y mutants (Figure 1). Both these mutations render the kinase domain constitutively active via different mechanisms. JM domain mutation (ITD) relieves the autoinhibitory function of this domain or disrupts the steric hindrance preventing receptor dimerization in the absence of FLT3L (6). Similar to the KIT D816 mutants (34), replacement of D835, on the other hand, may destabilize the inactive conformation via disruption of the H-bonds in the activation loop. Comparing the homology models of FLT3-D835Y with FLT3-WT structure (1rjb) showed that several H-bonds between side-chain of Asp835 with Met837 and Ser838 in the activation loop would be eliminated if Asp835 replaced with a Val or Tyr (unpublished data) which may contribute to the destabilization of inactive structure.

In these FD-FLT cells, the transcription of a polycystronic mRNA for both GFP and FLT3 is controlled by a single MSCV promoter. Therefore, it can be assumed that the expression of FLT3 and GFP are equal in the mRNA level. This was confirmed by the similarity of GFP expression in FD-FLT3-WT, -ITD and –D835 mutants (Table 2). However, the Geo Means for total FLT3 expression in the FD-FLT3-WT cells are about 70 and 62% higher than FD-FLT3-ITD and –D835Y, respectively. This indicates that a significant proportion of the mutant FLT3 protein disappears during posttranslational stages. Constitutive activity of FLT3 mutants results in its autophosphorylation, which are subjected to degradation via ubiquitin-binding (35) and also being detected as misfolded protein (36). Misfolded proteins are retained in the ER due to interaction with calnexin and calreticulin and may be subjected to degradation (37). Therefore, it can be concluded that the majority of the expressed mutant FLT3 was subjected to degradation as misfolded protein.

It was reported that phosphorylation process affects maturation of the RTKs including FLT3 (36). Treatment of FD-FLT3-D835Y cells with CEP701 reduced FLT3 phosphorylation dose-dependently and confirmed that phosphorylation inhibition is involved in the preservation of FLT3 protein (Figure 3). The minimum molecular weight of FLT3 is 130 kD which can be increased up to 160 in mature protein. It was shown in Figure 3 that the mature 160-kD FLT3 form is missing in the Western blot analysis; but, inhibition of phosphorylation may protect the FLT3 from degradation in the early stages. Therefore, FLT3 protein underwent more maturation and appeared with increased molecular weight bands in blotting for FLT3. However, this needs more investigation as it seems that phosphorylation may have also adversely affected interaction of the FLT3 with the antibody.

This study also revealed that 40% of detected FLT3 was intracellular in FD-FLT3-D835Y cell compared to 4 and 4.5% in FD-FLT3- ITD cells. This difference may result from the dynamics of the kinase domain activation between JM domain and activation loop mutants. In other words, it can be speculated that the inactive conformation of FLT3-ITD protein is more stable than FLT3-D835Y. Therefore, it is more probable for FLT3-ITD to remain unphosphorylated to reach cell membrane for being activated via dimerization. It differs with FLT3-D835Y where high proportion of expressed protein may adopt the FLT3 active conformation upon translation via a dimerization-independent mechanism as described for the kinase domain mutants of epidermal growth factor receptor (EGFR) (38). As mentioned earlier, phosphorylated FLT3-D835Y proteins are subjected to degradation.

FLT3-D835Y mutant protein was retained intracellularly; however, despite the report of FLT3-ITD retention in the ER of COS-7 cells (36), our data did not show a significant retention of intracellular FLT3-ITD protein in the FD-FLT3-ITD cells (Figure 2). This suggests that the protein degradation capacity of the FDC-P1 may be higher than COS-7 cells; not allowing intracellular accumulation of constitutively active forms of FLT3-ITD. These degradation products, then, appear as smear in western blot analysis of the cell lysate (unpublished data). Again, since greater proportion of FLT3-D835Y (40%) (compared to ITD mutant) adopts active conformation, gets phosphorylated upon translation, and retained in the ER as misfolded protein, which exceeds degradation capacity, it can be detected as intracellular FLT3.

FD-FLT3-ITD cells were three times more resistant to sunitinib than the FD-FLT3-WT cells. Since FLT3-ITD is a constitutively active mutant (24), the higher EC_{50} value is consistent with the finding in KIT that sunitinib prefers the inactive conformation of the kinase domain (39). FLT3-ITD expression also increased the EC_{50} value of CEP701, PKC412 and sunitinib by 15, 30 and 3.5 times compared to the FLT3-D835 mutants. This is consistent with the frequent reports of drug resistant blast cells expressing FLT3-ITD in the patients (40).

It was shown in this study that the number of viable FDC-P1 cells expressing FLT3-ITD was 3 to 4 times faster than those expressing FLT3-D835Y. As a result, it can be speculated that D835Y mutation may have some adverse effects on cell proliferation/survival. This may explain the finding that the JM domain mutations are the most common genetic alteration in AML (41). Lower proliferation/survival capacity and high sensitivity to kinase inhibitors may result from intracellular retention of FLT3-D835Y mutant protein because it was shown that protein retention can cause ER stress, altered signaling pathways and activation of apoptotic signaling pathways (36, 42-44). A combination of preferential affinity of CEP701 and PKC412 to the kinase domain compared to the JM domain mutants (29) and damaging effects of ER stress may contribute to the high sensitivity of D835Y to kinase inhibitors compared to the FLT3-WT and ITD mutant. Therefore, studying these genetic variations can be applied to the determination of prognosis as well as designing of a therapeutic plan for the patients with FLT3 mutations.

Conclusion

This study demonstrates that even though both types of juxtamembrane and kinase domain mutations render the FLT3 kinase constitutively active, but they have different sub-cellular effects and drug responses. Therefore, identifying the mutation type is required for designing a personalized treatment strategy for the patients.

Acknowledgment

This work was funded in part by a research grant to LKA from The Anthony Rothe Memorial Trust.

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