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# A Novel Fusion Protein Diphtheria Toxin–Stem Cell Factor (DT-SCF)—Purification and Characterization

**Sirisha Potala · Rama Shanker Verma**

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**Abstract** Fusion toxins are an emerging class of targeted therapeutics for the treatment of cancer. Diphtheria toxin-stem cell factor (DT-SCF) is one such novel fusion toxin designed to target malignancies expressing c-kit. Since, c-kit overexpression has been reported on many types of cancers, it appeared to be a reasonably good molecule to target. In the present study, we report construction, expression, purification, and characterization of DT-SCF. DT-SCF gene coding for 1–387 amino acids of diphtheria toxin, His-Ala linker, 2–141 amino acids of SCF was cloned into expression vector with C terminal His tag. The induced DT-SCF protein was exclusively expressed in insoluble fraction. Purification of DT-SCF was achieved by inclusion body isolation and metal affinity chromatography under denaturing and reducing conditions. Purified DT-SCF was renatured partially on-column by gradually reducing denaturant concentration followed by complete refolding through rapid dilution technique. Cell viability assay provided the evidence that DT-SCF is a potent cytotoxic agent selective to cells expressing c-kit. The novelty of this study lies in employing SCF as a ligand in construction of fusion toxin to target wide range of malignancies expressing c-kit. Efficacy of DT-SCF fusion toxin was demonstrated over a range of malignancies such as chronic myeloid leukemia (K562), acute lymphoblastic leukemia (MOLT4), pancreatic carcinoma (PANC-1), and cervical carcinoma (HeLa 229). This is the first study reporting specificity and efficacy of DT-SCF against tumor cells expressing c-kit. There was significant correlation ($P$=0.007) between c-kit expression on cells and their sensitivity to DT-SCF fusion toxin.

**Keywords** Diphtheria toxin · SCF · Immunotoxins · Protein expression · Refolding · Cell cycle analysis · Cytotoxicity

**Abbreviations**

| | |
|---|---|
| DT | diphtheria toxin |
| SCF | stem cell factor |

S. Potala · R. S. Verma (✉)
Department of Biotechnology, Stem Cell and Molecular Biology Laboratory,
Indian Institute of Technology Madras, #201 Bhupat and Jyoti Mehta School of Biosciences,
Chennai 600036, India
e-mail: vermars@iitm.ac.in

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|---|---|
| CTAB | cetyl trimethylammonium bromide |
| IPTG | isopropyl-β-D-thiogalacto-pyranoside |
| TMB | tetramethyl benzidine |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide |
| TBS | tris buffer saline |
| TBST | tris buffer saline tween-20 |

## Introduction

The main stay of cancer treatment till date includes surgery, chemotherapy, and radiation therapy. These conventional therapies lack targeted approach leading to unwanted effects on normal cells, i.e., treatment-related toxicities [1, 2]. In contrast, fusion toxins are a novel class of targeted therapeutics designed to specifically target tumor cells. Fusion toxins are chimeric proteins generated by genetically fusing coding sequences of modified toxin and tumor cell selective ligand. Toxin sequences that are devoid of native receptor binding domain were employed for the construction of fusion toxins to direct the cytotoxic action selectively to tumor cells. The identification of appropriate tumor cell selective ligand is based on the fact that cancer cells highly overexpress certain antigens or growth factor receptors on their surface when compared to normal cells. Therefore, the most widely used tumor cell selective ligands are antibodies or growth factors whose receptors are highly over expressed on tumor cells. Ligands used in the construction of fusion toxins play the most crucial role in achieving selective targeting. The uniqueness of receptor expression therefore dictates specificity and hence toxicity [3].

Numerous toxins of plant, bacterial, fungal, and viral origins were tried for construction of immunotoxins. Among these, diphtheria toxin (DT) is most widely used as it confers the following advantages: (a) highly potent, a single molecule is lethal to the host cell; (b) well studied with crystal structure available [4, 5]; (c) mechanism of action is well known [6]; and (d) DT fusion proteins are known to exhibit enhanced cytotoxicity than other toxins such as ricin, gelonin, or pseudomonas exotoxin [7, 8]. In the present study, the native receptor binding domain of DT was replaced with stem cell factor (SCF) to develop a potent and selective fusion toxin targeted to malignancies expressing c-kit.

Stem cell factor also referred as kit ligand or mast cell growth factor is essential for the development and function of mast cells, germ cells, melanocytes, and intestinal pacemaker cells [9, 10]. Biologically, SCF exists as soluble and membrane bound protein. SCF exhibits its activities by binding to tyrosine kinase proto-oncogene c-kit receptor [11]. C-kit receptor overexpression has been reported in several cancers like ovarian [12], pancreatic [13], stomach [14], liver [15], small cell lung carcinoma [16], and hematological malignancies. Although SCF is not unique in expression to tumor cells, the relatively high overexpression allows a significantly safe therapeutic window to specifically target tumor cells, leaving behind the normal cells with transient expression unharmed. Use of SCF inhibitors such as ST1571 and tyrosine kinase inhibitors like imatanib mesylate (Gleevac) were implicated in treatment of various cancers overexpressing tyrosine kinase receptors [17]. Production of SCF and overexpression of its receptor c-kit by cancer cells leads to autocrine loop of ligand receptor interaction, thus generating proliferation signals. Furthermore, mutations in c-kit lead to autophosphorylation and constitutive activation thus rendering aggressive proliferation of tumors.

Considering the crucial role of SCF and its receptor in tumor growth and progression, we chose SCF as a tumor cell selective ligand for construction of fusion toxin. In this novel fusion protein SCF, the tumor cell selective ligand is targeted to c-kit receptors over

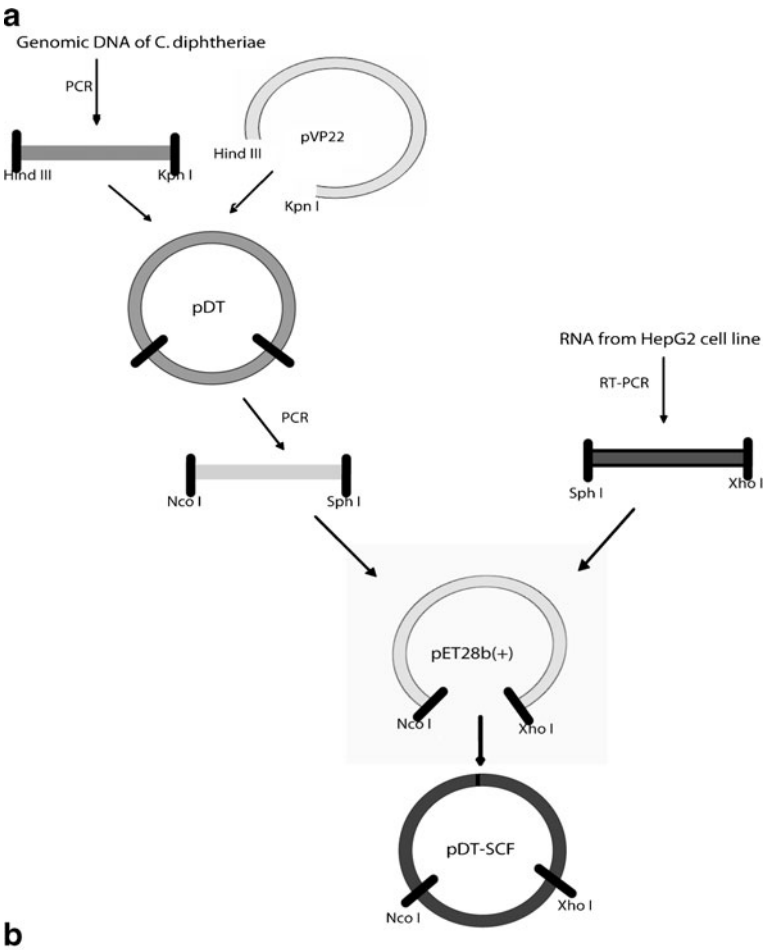
expressed on various malignant cells and modified diphtheria toxin mediates cytotoxic activity. Most fusion toxins developed till date were designed to target specific malignancies, like DT-IL2 for treatment of CTCL (cutaneous T cell lymphoma) [18], IL4-PE for treatment of gliomas [19], DT-GMCSF for treatment of AML [20], etc. In contrast, DT-SCF will have wider application window for the treatment of wider range of malignancies such as cancers of ovary, pancreas, liver, stomach, lung, and hematological malignancies. Present study reports (a) optimization of expression, as SCF expression was not easily accomplished in *Escherichia coli*; (b) easy, efficient, fast, and economical refolding method in contrast to conventional refolding procedure (rapid dilution) which was used for other fusion toxins [21]; (c) characterization of purified DT-SCF fusion protein; (d) evidence of potency and specificity of DT-SCF fusion protein over a range of malignancies; and (e) correlation between receptor expression (c-kit) and sensitivity to fusion toxin (DT-SCF).

## Materials and Methods

*Construction of Expression Vectors* Construction strategy of recombinant vector carrying DT-SCF is depicted in Fig. 1a. Genomic DNA of *Corynebacterium diphtheriae* was extracted according to conventional protocol described in current protocols in molecular biology by Ausubel. The coding sequence for diphtheria toxin was polymerase chain reaction (PCR) amplified from genomic DNA and cloned into a vector to yield pDT. The plasmid pDT was used as template to PCR amplify 1–387 amino acid fragment of diphtheria toxin; NcoI and SphI restriction sites were introduced through primers. First strand cDNA was synthesized from total RNA of HepG2 cell line, extracted using guanidine hydrochloride method using reverse transcriptase enzyme and oligo d(T) primer. The resulting cDNA was used as template to PCR amplify SCF fragment; SphI and XhoI sites were introduced through primers. To clone DT-SCF sequence with C terminal His tag into expression vector, three piece ligation was performed. DT (1–387) fragment was digested with NcoI and SphI, SCF fragment was digested with SphI and XhoI, and pET-28b was digested with NcoI and XhoI. All digested fragments were purified and ligated to yield pDT-SCF.

*Expression of Fusion Toxin* To optimize expression, we carried out a series of experiments with alteration in temperature (15 °C–42 °C), induction time (0.5–16 h), inducer concentration (0.1–1 mM), medium compositions (LB, 2xYT with and without glucose) in different *E. coli* host strains (RIL, RossettagamiII (DE3), BL21 (DE3)). Only successful conditions have been described below. The generated recombinant plasmid pDT-SCF carrying sequence for DT-SCF was transformed into, BL21 (DE3) *E. coli* cells. The transformants were expanded in 2xYT media (supplemented with kanamycin 50 μg/ml and 1% glucose) by incubation at 37 °C, 250 rpm for 6 h (OD~1). This initial culture was subcultured at a ratio of 1:10 and was continued to grow at 37°C, 250 rpm until the $OD_{600}$ reached 0.7. At this OD, uninduced sample was collected and protein expression was induced with 0.4 mM IPTG and cells were cultured for additional 60 min. Finally, cells were harvested by centrifugation, and expression of DT-SCF in total cell protein from uninduced and induced samples was analyzed by SDS-PAGE.

*Inclusion Body Isolation* One gram cell pellet was resuspended in 10 ml of inclusion body buffer pH 8 (TrisCl 50 mM, EDTA 10 mM, 8% sucrose, and 5% Triton X 100) lysed by sonication and centrifuged at 12,000 g for 45 min at 4 °C. The resulting pellet was again resuspended in inclusion body buffer, sonicated, and centrifuged for additional two times.


a
Genomic DNA of C. diphtheriae
PCR
Hind III
Kpn I
pVP22
Hind III
Kpn I
pDT
PCR
Nco I
Sph I
RNA from HepG2 cell line
RT-PCR
Sph I
Xho I
pET28b(+)
Nco I
Xho I
pDT-SCF
Nco I
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117
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45
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mSCF
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175
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58
46
30
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17
mHis
M
kDa
117
66
45

◀ **Fig. 1** Cloning, expression, purification, and immunoblot of DT-SCF fusion protein. **a** Schematic representation of the construction strategy used in assembling DT-SCF plasmid: The 1.6 kb insert containing 1–387 amino acids of diphtheria toxin, His-Ala, 2–141 amino acids of SCF was cloned into pET-28b expression vector between NcoI and XhoI sites resulting in pDT-SCF. DNA fragment coding for DT was derived from PCR amplification from pDT which carries genomic DNA of *C. diphtheriae*. SCF fragment was obtained by RT-PCR from RNA of HepG2 cell line. **b** SDS-PAGE analysis of expression and purification of DT-SCF: *UNI* total cell protein of uninduced pDT-SCF, *IND* total cell protein of induced pDT-SCF, *IB* inclusion body of pDT-SCF, *M* protein molecular weight marker, *DT-SCF* purified DT-SCF protein. **c** Western blot analysis of purified DT-SCF fusion protein: *M* prestained protein molecular weight marker, *mSCF* purified DT-SCF detected by anti-SCF antibody, *mHis* purified DT-SCF detected by anti-His antibody

Isolated inclusion bodies were washed by brief sonication and centrifugation for three times to remove Triton X 100 completely. Finally, inclusion bodies were solubilized in denaturation buffer pH 8 (100 mM $NaH_2PO_4.H_2O$, 10 mM TrisCl, and 8 M urea) with 10 mM 2-mercaptoethanol at 37 °C for half an hour. The volume of denaturation buffer added was adjusted to a protein concentration of 10–20 mg/ml.

*Affinity Purification and Refolding of Fusion Toxin* Soluble inclusion body fraction obtained was filtered through 0.45 μm filter and loaded on a Ni-NTA column pre-equilibrated with denaturation buffer. The loaded column was washed with denaturation buffer to remove unbound proteins. Further, to achieve partial refolding on-column, urea was gradually reduced in wash buffer (100 mM $NaH_2PO_4.H_2O$, 10 mM TrisCl pH 8). The recombinant protein of interest was finally eluted with 200 mM imidazole containing buffer. The eluted protein was dialyzed against 10 mM Tris pH 8 and 100 mM urea for 16 h with frequent buffer changes to remove imidazole completely. The partially refolded purified protein was subjected to further refolding by rapid dilution into refolding buffer containing 50 mM TrisCl, 50 mM NaCl, 5 mM reduced glutathione, 1 mM oxidized glutathione, and 0.5 M arginine HCl. To enhance refolding efficiency, low protein concentration of 50–100 μg/ml was maintained. Refolding was continued at 4 °C for 72 h. Refolded proteins were extensively dialyzed against PBS and concentrated by lyophilization.

*SDS-PAGE and Western Blot Analysis* Protein containing samples were subjected to reducing 12% SDS-PAGE and visualized by Commassie Brilliant Blue staining. Western blot analysis was carried out according to protocol recommended by Bio-Rad (USA). Following electrophoresis on 12% reducing SDS-PAGE, proteins were transferred to nitrocellulose membrane. The membrane was incubated with primary antibody anti-hSCF or anti-His, washed, and incubated with secondary antibody HRP conjugated IgG. After further washing, antigen-primary antibody-secondary antibody complexes were detected by tetramethyl benzidine (TMB) /$H_2O_2$ chemiluminescence system.

*Structural Characterization of Fusion Toxin* Fluorescence and circular dichroism spectra were performed for DT-SCF protein under denatured (urea) and refolded conditions to elucidate secondary structure and refolding. The fluorescence spectra were determined at ambient temperature on Jasco spectrofluorimeter (MD, USA). Samples were excited at 280 nm, and emission was recorded between 300 and 500 nm. Circular dichroism (CD) was performed using a Jasco spectropolarimeter (MD, USA) in the far UV region (180–250 nm).

*Cell Lines* Cell lines HepG2, K562, HuT78, MOLT4, PANC-1, and HeLa 229 were procured from NCCS, Pune, India. All cell lines were maintained in RPMI 1640 or DMEM

supplemented with 10% FBS, 2 mM glutamine, 100 IU penicillin, 100 μg/ml streptomycin at 37 °C in 5% $CO_2$ atm.

*Immunofluorescence Staining* Approximately $0.2\times10^6$ cells/well were used, they are washed with PBS, fixed with 4% paraformaldehyde (20 min, R.T.), blocked with 5% BSA (60 min, R.T.), and incubated with c-kit antibody (0.5 μg, 16 h, 4 °C). Following primary antibody binding, cells were washed with PBS for three times and stained with FITC-conjugated secondary antibody (1:20, 60 min, R.T.). Excess unbound antibody was removed by repetitive washing with PBS for three times. Nuclei were stained with propidium iodide. Stained cells were visualized under a fluorescence microscope (Nikon Eclipse Ti, Japan). Random fields were recorded at a magnification of 10×.

*Evaluation of c-kit Expression by Flow Cytometry* Expression of c-kit on various cell lines was qualitatively evaluated by immunofluorescence staining as described earlier. Quantitative expression of c-kit on cell lines was evaluated by flow cytometry to correlate sensitivity and receptor expression on various cell lines used in this study. One million cells were pelleted and washed in PBS/1% FBS. Cells were resuspended in 100 μl of PBS/1% FBS, 0.5 μg of c-kit antibody was added and incubated for 60 min. Excess unbound antibody was removed by washing with PBS/1% FBS. FITC-conjugated secondary antibody was added and incubated in dark for additional 60 min. Excess antibody was removed by washing; finally, cells were resuspended in 0.5 ml of PBS and analyzed by flow cytometry, and unstained cells were used as control. Same procedure was repeated for all cell lines.

*Cell Cycle Analysis* Cells (K562 and HuT78) were seeded at a density of $10^6$ cells/well/ml complete medium into six well plates. Purified DT-SCF protein was added and incubated for 24 h; untreated cells were employed as control. Following treatment, cells were collected by centrifugation, washed twice with PBS, and fixed with ice cold absolute ethanol overnight at −20 °C. The fixed cells were then washed with PBS, stained with propidium iodide, and analyzed using flow cytometry (BD FACS Calibur, CA, USA).

*Cytotoxicity Studies* Cells were plated at $10^4$ cells/well in 100 μl of RPMI medium in a 96-well plate and incubated overnight at 37 °C. Increasing concentrations of DT-SCF ranging from 0.001–1 μM were added to the wells and incubated for 72 h. At the end of incubation, 10 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt) was added to each well and kept in dark for 4 h. Reaction was stopped by addition of 20% (*w*/*v*) SDS in 50% (*v*/*v*) DMF and formazen crystals were solubilized by incubating the plate for additional 2 h at 37 °C. Absorbance were recorded on micro plate reader at 570 nm using medium with MTT as blank and PBS-treated cells as control.

## Results

Restriction enzyme analysis and DNA sequencing confirmed that the hybrid gene had been cloned in frame and codes for DT-SCF as a single protein. Recombinant protein expression remained relative constant to changes in host strain, inducer concentration, induction time and temperature. However, (a) fresh transformants had higher expression than late transformants; (b) 2xYT medium showed better expression than other medium compositions and including

1% glucose in the medium, made a significant impact on expression; and (c) minimizing the number of generations had a definite impact on expression (4 h culture served as better inoculum than overnight culture). Successful expression of protein encoded by pDT-SCF was achieved in *E. coli* BL21 (DE3) as host strain. Expression was induced with 0.4 mM IPTG at an OD~0.7 at 37°C and continued for 1 h. The DT-SCF protein was exclusively expressed in the insoluble fraction as inclusion bodies. Inclusion bodies were purified from total cell protein, solubilized in 8 M urea, and purified on Ni-NTA column. Refolding of DT-SCF was achieved in two steps, (a) on-column by gradually reduction of denaturant concentration (8 to 2 M) and (b) followed by rapid dilution into refolding buffer containing redox system and L-arginine.

Coomassie Brilliant Blue stained SDS-PAGE showed a protein at ~58 kDa, the expected size of DT-SCF. Inclusion body protein yield was ~40% of total cell protein, and final purified renatured DT-SCF used for activity assays was ~85% pure (Fig. 1b). Western blot analysis confirmed the reactivity of DT-SCF with both anti-SCF and anti-His antibodies (Fig. 1c). Unfolding/refolding of DT-SCF was studied by circular dichrosim spectra, fluorescence spectra, and also by activity assays. Fluorescence spectra exhibited increase in fluorescence intensity upon denaturation with various concentrations of urea (Fig. 2a). The far UV CD signals arising from peptide bonds reflected substantial secondary structures (Fig. 2b).

C-kit receptor expression was qualitatively characterized on cell lines by immunofluorescence staining using c-kit primary antibody and anti-mouse IgG FITC secondary antibody and nuclei were stained with propidium iodide. Cell lines K562, MOLT4, PANC-1, and HeLa 229 were found to be c-kit positive whereas HuT78 cell line was found to be c-kit negative (Fig. 3). Flow cytometric analysis quantitated the c-kit receptor on these cell lines. PANC-1, a pancreatic carcinoma cell line exhibited maximum c-kit receptor expression with mean fluorescence intensity (MFI) of 70.66 among all the cell lines used in this study. MOLT4, an acute lymphoblastic leukemia cell line, has MFI of 46.6, HeLa 229 a cervical carcinoma cell line has MFI of 43.33, and K562, a chronic myeloid leukemia cell line has MFI of 34.74, lowest expression of all the cell lines used in this study.

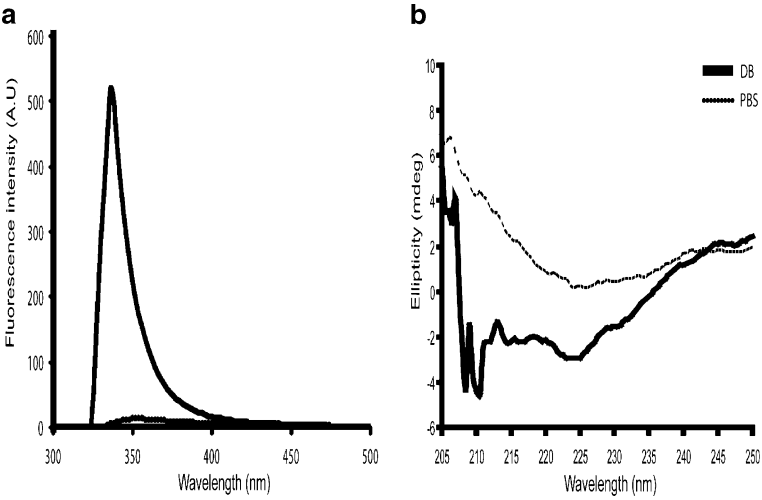


**Fig. 2** Structural characterization of DT-SCF fusion protein. **a** Purified DT-SCF fusion toxin was structurally characterized by recording fluorescence under refolded (*PBS*) and denatured (*urea*) conditions. **b** CD spectra of DT-SCF fusion toxin was recorded under refolded (*PBS*) and denatured (*urea*) conditions

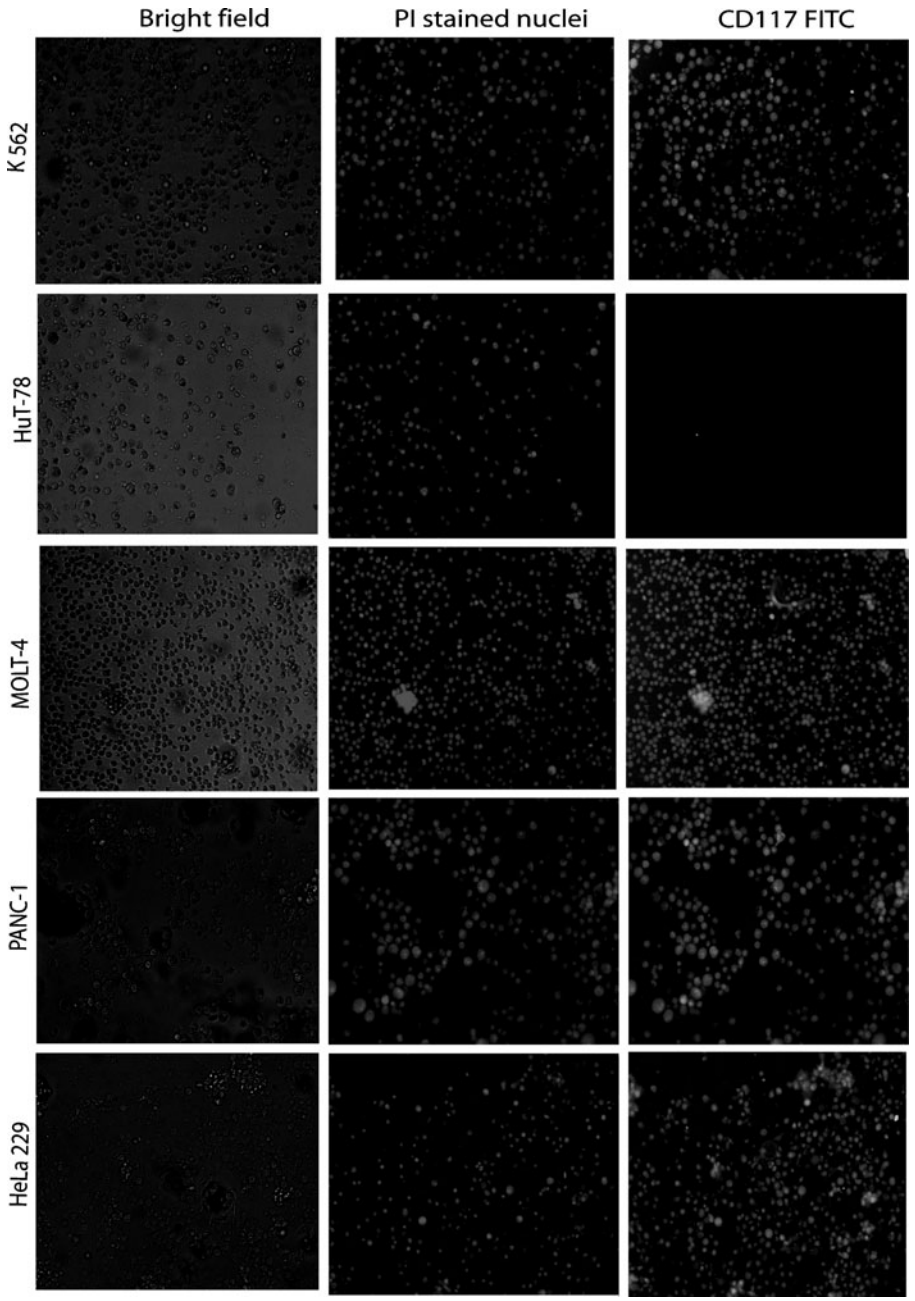


**Fig. 3** C-kit expression on cell lines analyzed by immunofluorescence. Qualitative analysis of c-kit expression on various cell lines by immunofluorescence using c-kit primary antibody and FITC-conjugated secondary antibody. *Left panel* represents images in bright field, *middle panel* represents images of PI stained nuclei, and *right panel* represents images of c-kit FITC surface expression

Cell cycle analysis revealed K562 cells treated with PBS and only DT protein showed 4% and 6.2% apoptotic cells, respectively, whereas cells exposed to DT-SCF protein showed increase in number of apoptotic cells to 16%. Cell cycle analysis of HuT78 cells exposed to DT-SCF and PBS showed similar result, with number of apoptotic cells around 4% in both cases. In addition, DT-SCF protein-treated K562 cells exhibited 50% of cells in S phase whereas PBS-treated cells and DT protein-treated cells showed 14% and 24%, respectively, in S phase (Fig. 4a). However, no such S phase synchronization was observed in HuT78 cells treated with DT-SCF fusion protein. Increasing concentrations of DT-SCF ranging from $10^{-9}$ to $10^{-6}$M were tested for cytotoxicity after 72 h incubation on different cell lines. The purified renatured DT-SCF exhibited potent cytotoxicity to PANC-1, MOLT4, HeLa 229, and K562 cell lines expressing c-kit but not to HuT78 cell line lacking any expression of c-kit establishing the fact that DT-SCF mediates its activity by binding to c-kit receptor. $IC_{50}$ of DT-SCF fusion protein for different cell lines are PANC-1—0.4 μM, MOLT4—0.77 μM, HeLa 229—0.81 μM, and K562—0.88 μM as determined by MTT assay (Fig. 4b). Normal cell including mast cell, germ cells, and melanocytes transiently express c-kit and would therefore require careful evaluation in an animal model to access whether use of DT-SCF affect the survival of normal cells expressing c-kit. Apart from DT-SCF, the cytotoxicity of only DT protein was also tested under similar conditions. The only DT protein coding for 1–387 amino acid residues of diphtheria toxin without any receptor binding domain was used as negative control in these studies. As predicted, the only DT protein exhibited no significant activity on any of the above cell lines (c-kit positive and c-kit negative) suggesting that the activity of DT-SCF is mediated through receptor ligand interaction of DT-SCF and c-kit. Linear regression analysis was performed to correlate c-kit density and $IC_{50}$ of DT-SCF fusion protein. Significant correlation ($P$=0.007) was obtained between c-kit density and $IC_{50}$ of DT-SCF fusion protein (Fig. 4c). Cell lines with relatively more receptor density are more sensitive to DT-SCF fusion protein. PANC-1 having greater receptor density showed least $IC_{50}$ of 0.4 μM; similarly, K562 having least receptor density of all the cell lines had greater $IC_{50}$ of 0.88 μM.

## Discussion

Cancer relapse is often observed in most of the patients after first round of standard treatment regimen, which includes chemotherapy and radiation therapy. These relapses are usually resistant to further treatment with same standard therapies. Fusion toxins provide a promising alternative to standard therapy resistant phenotypes as they act by entirely different mechanism to that of standard therapies. This is the first paper reporting SCF as a potential ligand for construction of fusion toxins. Recently, several studies revealed the high overexpression of c-kit on numerous cancers [12–16]. Moreover, SCF by itself can mediate the internalization of fusion protein which is crucial for cytotoxicity of DT-mediated fusion toxins [22]. Normal cells like mast cells, germ cells, and melanocytes show negligible expression of the c-kit and would therefore be unaffected by DT-SCF. This may be due to the fact that the cell surface receptor expression is inversely related to sensitivity of fusion toxin needed, suggesting the large therapeutic window of DT-SCF [23, 24].

In this present study in pDT-SCF, the linker His-Ala between DT and SCF was introduced by SphI restriction site. To facilitate the transport of fusion toxin, SCF and His tag were placed at C terminal of diphtheria toxin, as N terminus of DT enters into cytosol for cytotoxicity. Furthermore, it was reported earlier that N terminal His tagged diphtheria fusion toxins exhibited tenfold lower activity than their C terminal equivalents [25]. The

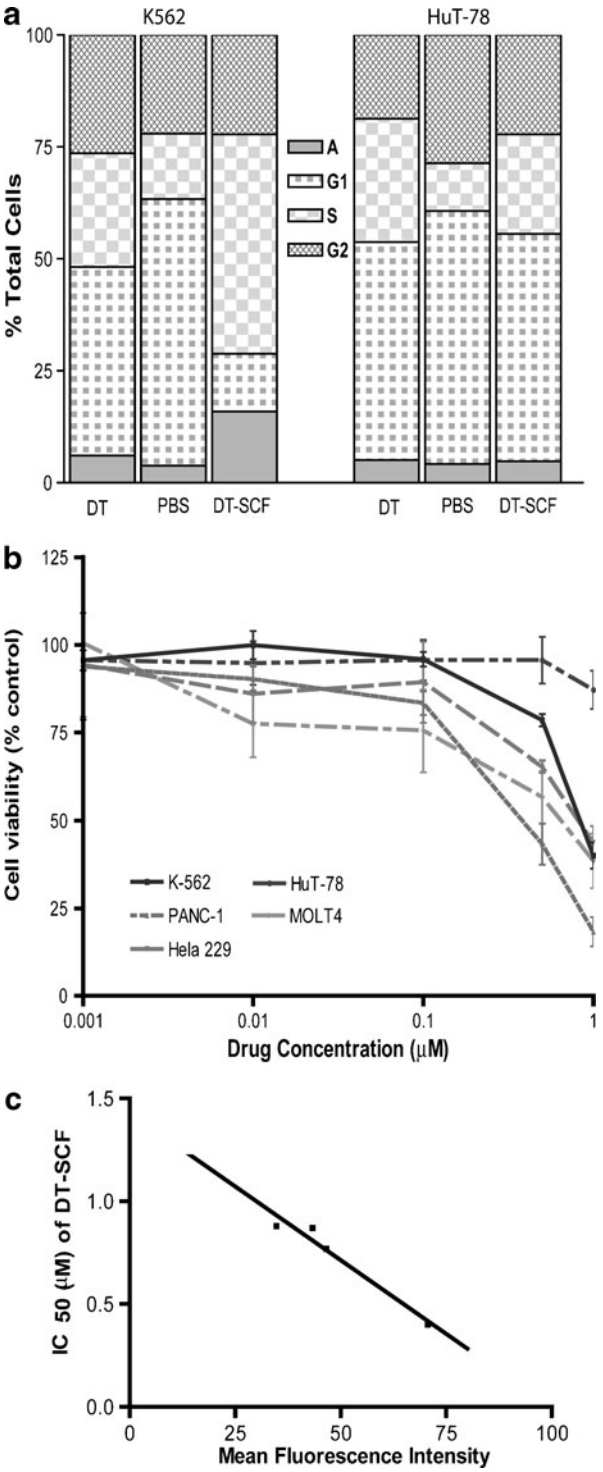


**Fig. 4** Activity assays of DT-SCF fusion protein. **a** Cell cycle analysis of K562 and HuT78 cells following exposure to DT-SCF or DT or PBS and analyzed by flow cytometry after staining nuclei with PI. The graph shows percentage cells in each phase—apoptosis, G1, S, and G2. **b** Different cell lines were exposed to various concentrations of DT-SCF fusion toxin, following 72 h treatment cell viabilities were determined by MTT assay. Mean of percentage cell viability of control (PBS treated) were represented against fusion toxin concentration. **c** Correlation of mean fluorescence intensity and $IC_{50}$, linear regression performed with GraphPad Prism yielded $R^2$=0.986, $P$=0.007

plasmid carrying the insert coding for DT-SCF was successfully expressed in BL21 (DE3). Bacterial cytosol being reducing in nature cannot form disulfide bonds which are indispensable for DT mode of action [3]. Therefore, we tried Rossettagami II (DE3) as host strain. This strain carries *trxB*/*gor* mutations for enhanced disulfide bond formation in the cytosol, in addition to supplementation of rare codons. However, predicted results were not achieved in this strain. It has been reported earlier that SCF is poorly expressed in *E. coli* due to suboptimal codon usage and supplementing *E. coli* favored codons improves expression [26]. However, in our study with *E. coli* strains RIL and Rossettagami II (DE3) carrying a plasmid supplementing rare codons showed no significant change in expression. Other experimental variations like employing fresh transformants, lowering the number of generations, and including 1% glucose in medium significantly improved expression suggesting the probable toxicity of DT-SCF to host bacteria. Similar observation was also reported in case of DTGM fusion toxin [21].

Induced DT-SCF protein was purified from inclusion bodies by metal affinity chromatography. During chromatography, concentration of urea was reduced on-column as first step of refolding. This is easy, economical, and fast in contrast to the most widely used dialysis technique. Refolded DT-SCF protein was characterized by fluorescence and circular dichroism spectra. Upon denaturation, the observed increase in fluorescence intensity may be due to the following: (a) the fluorescence is accountable to Tyr rather than Trp; (b) fluorescence is quenched in refolded state by surrounding residues or by polar solvents which quench fluorescence of indole nucleus; (c) tyrosine fluorescence in quenched by nearby charged carboxyl groups or uncharged amino acids; (d) hydrogen-bonded phenols are nonfluorescent, hyroxyl of Try could be hydrogen bonded to peptide bonds; and (e) it indicates the position of Trp and Tyr residues to periphery in refolded state.

In our study, cell cycle analysis showed a slight increase in number of apoptotic cells (from 4% to 16% as described in results) upon treatment with DT-SCF in K562 cells but not in HuT78 cells, supporting apoptosis as one of the death pathways being activated by fusion toxins. Recently, Frankel et al. reported that diphtheria toxin fusion proteins can activate multiple death pathways including apoptosis and necroptosis [27, 28]. In the cell cycle analysis, we have observed synchronization of DT-SCF treated cells in S phase. Similar observation of synchronization pattern has been reported earlier with other biological molecules and DNA synthesis inhibitors [29]. In our study, the S phase synchronization of DT-SCF treated K562 cells is a consequence of DNA synthesis inhibition which in turn is due to protein synthesis inhibition by DT fragment of fusion toxin.

Cell lines used to evaluate the efficacy of DT-SCF fusion protein were analyzed for c-kit expression by immunofluorescence and flow cytometry. The protein DT-SCF was found to be cytotoxic to K562, PANC-1, MOLT4, and HeLa 229 cell lines expressing c-kit but was nontoxic to HuT78 cell line which was devoid of c-kit expression. Furthermore, the protein encoding only DT (1–387) was found to be nontoxic to both the cell lines. In summary, the cytotoxicity results demonstrate that (a) DT-SCF is a potent antitumor agent and (b) cytotoxicity of DT-SCF is mediated through ligand receptor interaction, i.e., binding of SCF fragment in DT-SCF to c-kit expressed on tumor cells and is therefore selectively toxic to cells expressing c-kit. Significant correlation was observed between c-kit expression and $IC_{50}$ of DT-SCF once again demonstrating that c-kit expression is an important factor conferring specificity and sensitivity to DT-SCF. Expression of c-kit in biopsies can be used as a prognostic marker for treatment with DT-SCF.

In conclusion, we report for the first time, the construction, expression, purification, and characterization of a novel fusion toxin DT-SCF. The fusion protein DT-SCF demonstrated potent cytotoxic activity specific to cells expressing c-kit. Results of

above studies suggested that DT-SCF can be a promising alternative for the treatment of c-kit positive malignancies and insist the need for further development. It will be important to determine whether DT-SCF exhibits same potency and specificity to c-kit expressing malignancies in vivo.

## References


1. Benedict, W. F., Baker, M. S., Haroun, L., Choi, E., & Ames, B. N. (1977). *Cancer Res, 37*, 2209–2213.
2. Rieger, K. E., Hong, W. J., Tusher, V. G., Tang, J., Tibshirani, R., & Chu, G. (2004). *Proc Natl Acad Sci U S A, 101*, 6635–6640.
3. Potala, S., Sahoo, S. K., & Verma, R. S. (2008). *Drug Discov Today, 13*, 807.
4. Bennett, M. J., & Eisenberg, D. (1994). *Protein Sci, 3*, 1464–1475.
5. Choe, S., Bennett, M. J., Fujii, G., Curmi, P. M., Kantardjieff, K. A., Collier, R. J., et al. (1992). *Nature, 357*, 216–222.
6. Van Ness, B. G., Howard, J. B., & Bodley, J. W. (1980). *J Biol Chem, 255*, 10717–10720.
7. Burbage, C., Tagge, E. P., Harris, B., Hall, P., Fu, T., Willingham, M. C., et al. (1997). *Leuk Res, 21*, 681–690.
8. Kreitman, R. J., & Pastan, I. (1997). *Blood, 90*, 252–259.
9. Galli, S. J., Tsai, M., & Wershil, B. K. (1993). *Am J Pathol, 142*, 965–974.
10. McNiece, I. K., & Briddell, R. A. (1995). *J Leukoc Biol, 58*, 14–22.
11. Anderson, D. M., et al. (1990). *Cell, 63*, 235–243.
12. Raspollini, M. R., Amunni, G., Villanucci, A., Baroni, G., Taddei, A., & Taddei, G. L. (2004). *Ann Oncol, 15*, 594–597.
13. Yasuda, A., et al. (2006). *Mol Cancer, 5*, 46.
14. Hirota, S., et al. (1998). *Science, 279*, 577–580.
15. Chung, C. Y., Yeh, K. T., Hsu, N. C., Chang, J. H., Lin, J. T., Horng, H. C., et al. (2005). *Cancer Lett, 217*, 231–236.
16. Naeem, M., Dahiya, M., Clark, J. I., Creech, S. D., & Alkan, S. (2002). *Hum Pathol, 33*, 1182–1187.
17. Smith, M. A., Court, E. L., & Smith, J. G. (2001). *Blood Rev, 15*, 191–197.
18. Duvic, M., Cather, J., Maize, J., & Frankel, A. E. (1998). *Am J Hematol, 58*, 87–90.
19. Kawakami, M., Kawakami, K., & Puri, R. K. (2003). *J Neurooncol, 65*, 15–25.
20. Frankel, A. E., Powell, B. L., Hall, P. D., Case, L. D., & Kreitman, R. J. (2002). *Clin Cancer Res, 8*, 1004–1013.
21. Frankel, A. E., et al. (1999). *Protein Expr Purif, 16*, 190–201.
22. Shimizu, Y., Ashman, L. K., Du, Z., & Schwartz, L. B. (1996). *J Immunol, 156*, 3443–3449.
23. Alexander, R. L., Ramage, J., Kucera, G. L., Caligiuri, M. A., & Frankel, A. E. (2001). *Leuk Res, 25*, 875–881.
24. Ramage, J. G., Vallera, D. A., Black, J. H., Aplan, P. D., Kees, U. R., & Frankel, A. E. (2003). *Leuk Res, 27*, 79–84.
25. Frankel, A. E., et al. (2000). *Leukemia, 14*, 576–585.
26. Lu, H., Zang, Y., Ze, Y., Zhu, J., Chen, T., Han, J., et al. (2005). *Protein Expr Purif, 43*, 126–132.
27. Thorburn, J., Frankel, A. E., & Thorburn, A. (2003). *Clin Cancer Res, 9*, 861–865.
28. Horita, H., Frankel, A. E., & Thorburn, A. (2008). *PLoS One, 3*, e3909.
29. Caraglia, M., et al. (2009). *Curr Cancer Drug Targets, 9*, 690–704.