SC29EK, a peptide fusion inhibitor with enhanced α-helicity, inhibits replication of human immunodeficiency virus type 1 mutants resistant to enfuvirtide

Running title: Peptide HIV-1 fusion inhibitor

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ABSTRACT

Peptides derived from α-helical domains of HIV type 1 (HIV-1) gp41 inhibit HIV-1 fusion to the cell membrane. Enfuvirtide (T-20) is a peptide-based drug that targets the step of HIV fusion, and as such, it suppresses effectively replication of HIV-1 strains that are either wild-type or multi-drug resistant to reverse transcriptase and/or protease inhibitors. However, HIV-1 variants with T-20 resistance have emerged; therefore, development of new and potent inhibitors is urgently needed. We have developed a novel HIV fusion inhibitor, SC34EK, which is a gp41-derived 34-amino acid peptide with glutamate (E) and lysine (K) substitutions on its solvent-accessible site that stabilize its α-helicity. Importantly, SC34EK effectively inhibits replication of T-20-resistant HIV-1s as well as wild-type HIV-1 (HIV-1WT). In this study, we introduce SC29EK, a 29-amino acid peptide that is a shorter variant of SC34EK. SC29EK blocked replication of T-20-resistant HIV-1s and maintained antiviral activity even at high serum concentrations (up to 50%). Circular dichroism analysis revealed that the α-helicity of SC29EK was well maintained, while that of parental C29, which showed moderate and reduced inhibition of wild-type and T-20-resistant HIV-1s, was lower. Our results show that α-helicity in a peptide-based fusion inhibitor is a key factor for activity and enables the design of short peptide inhibitors with improved pharmacological properties. (210 words)
INTRODUCTION

The envelope proteins of human immunodeficiency virus type 1 (HIV-1), exist as functional trimeric complexes of gp120-gp41 heterodimers, and play an important role in viral entry into host cells. Interactions of gp120 with CD4 molecules expressed on the cell surface cause structural changes that allow further interactions with the co-receptors CXCR4 or CCR5. These interactions also induce a conformational change in gp120 that initiates gp41-mediated membrane fusion that leads to viral entry (4). In the process of fusion, the amino-terminal heptad repeat (N-HR) of gp41 trimer interacts with the carboxyl-terminal heptad repeat (C-HR) of gp41 trimer, to form a six-helix bundle that makes viral and cell membranes accessible (3).

Peptides derived from N-HR or C-HR, such as N36 (3, 18) and enfuvirtide (T-20) (30), suppress the six-helix bundle formation, resulting in inhibition of membrane fusion. T-20 blocks entry of various HIV-1 strains, even those resistant to inhibitors of reverse transcriptase (RT) and/or protease (15, 16). However, T-20-resistant HIV-1 variants, which frequently show mutations in gp41, such as V38A and N43D, have emerged (14, 25, 26, 28, 32). Therefore, novel fusion inhibitors that suppress the replication of T-20-resistant variants are urgently needed.

C34, a C-HR-derived peptide (Fig. 1A), also inhibits fusion in vitro, much more efficiently than T-20 (3, 18, 22). Previously, we remodeled C34 by introduced amino acid substitutions that resulted into highly soluble and active derivatives (24). We replaced amino acids at the solvent-accessible site of the helical bundle with
glutamate (E) and lysine (K), and maintained those at the interactive site, as these are critical for the interaction with N-HR. In an α-helical heptad repeat residues separated by 3 positions (i versus i+4) are located on the same side of the helix, closely positioned in space (Fig. 1B). Hence, we introduced consecutive EK motifs separated by 3 residues (E at positions i, i+1 and K at positions i+4, i+5) of the solvent accessible site of C34, resulting in a repeat of the following type: X-EE-XX-KK (X indicates the original amino acid in HIV-1). A C34 derivative, SC34EK, which has two complete and three incomplete X-EE-XX-KK motifs (Fig. 1), showed enhanced anti-HIV-1 activity compared with the parental C34 (24). A similar result was obtained with T-20EK, the peptide derived by introducing this motif into T-20 (23). Circular dichroism (CD) analysis revealed that the α-helicity of SC34EK and the thermal stability of the N36/SC34EK complex were both enhanced. Interestingly, the antiviral activity of SC35EK, with five complete X-EE-XX-KK motifs, was comparable to that of SC34EK (24), indicating that five complete X-EE-XX-KK motif repeats are not required for strong anti-HIV-1 activity. To address how many complete X-EE-XX-KK motifs are involved in the potent antiviral activity of SC34EK, we synthesized SC29EK and SC22EK (Fig. 1) that contain four and three complete repeats of X-EE-XX-KK, respectively, and evaluated their antiviral activity against T-20 resistant viruses.

MATERIALS AND METHODS

Cells. HeLa CD4/LTR-β-galactosidase cells obtained from M. Emerman through the
AIDS Research and Reference Reagent Program (Germantown, Md) and 293T cells were grown in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin G and 50 µg/ml streptomycin.

**Viruses.** An HIV-1 infectious clone, pNL4-3 (1), was used for the construction and production of HIV-1 clones. Clones with certain resistance mutation(s) were introduced by site-directed mutagenesis (29) into the pNL4-3 construct. Although the vast majority of HIV-1 strains have a glycine (G) at position 36 in gp41, the NL4-3 used in this study has an aspartic acid (D) residue that impaired the fusion kinetics of HIV-1 (13, 17). Therefore, in this study, we first constructed a D36G clone, pNL4-3_{D36G}, and used this as a template for introducing T-20-resistant mutations as described (21, 31). We constructed three T-20-resistant clones, HIV-1_{D36G/V38A}, HIV-1_{D36G/N43D} and HIV-1_{D36G/N43D/S138A} (8), and two C34 resistant clones, HIV-1_{D36G/N126K} and HIV-1_{AV4/D36G/I37K/N126K/L204I} (AV4 indicates a five-amino acid [FNSTW] deletion in the V4 region of gp120) (22). Infectious HIV-1 clones were generated by transfection of plasmid clones into 293T cells.

**Antiviral agents.** Peptide–based fusion inhibitors, including T-20, were synthesized by standard Fmoc-based solid-phase techniques (24). HPLC purification of crude materials on a preparative Cosmosil 5C18 AR-II column using a linear gradient of MeCN containing 0.1% TFA gave the desired peptide samples for biological tests. 2’,3’-Dideoxycytidine (ddC) was purchased from Sigma-Aldrich (St. Louis, Mo.).

**Determination of efficacy of antiviral agents.** The efficacy of antiviral agents was determined using multinuclear activation of galactosidase indicator (MAGI) assays (12,
Briefly, $10^4$ HeLa CD4/LTR-β-galactocidase cells per well were plated in 96-well flat culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI U/well, giving 60 blue cells after 48 hr of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. After incubation for 48 hr after virus inoculation, all of the blue cells stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in each well were counted. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration [EC$_{50}$]).

**Effect of sera on anti-HIV activity.** The effect of FCS concentration on antiviral activity was measured by MAGI assays with several FCS concentrations (5, 10, 20 and 50%). The effect of serum components on antiviral activity was assessed by MAGI assays. Briefly, T-20 or SC29EK were dissolved in phosphate-buffered saline (PBS), FCS or freshly prepared human sera from HIV-seronegative healthy volunteers, at 4 µM, and incubated for 2 hr at 37°C. The mixture was diluted into concentrations of about 1X and 5X the EC$_{50}$ value using a DMEM-based complete media supplemented with 10% FCS, and subjected to MAGI assay.

**Circular dichroism analysis.** N36- and C-HR-derived peptide complexes were incubated at 37°C for 30 min (the final concentration of peptides was 10 µM in PBS). CD spectra were acquired on a spectropolarimeter (Model J-710, Jasco Inc., Tokyo, Japan) at 25°C as the average of eight scans. Thermal stability was assessed by monitoring the change in CD signal at 222 nm as a function of temperature. Thermal unfolding at intervals of 0.5°C was performed after a 15 min equilibration at the desired
temperature and an integration time of 1.0 sec. The midpoint of the thermal unfolding
transition (melting temperature, $T_m$) of each complex was determined from the
maximum of the first derivative, with respect to the reciprocal of the temperature, of the
$[\theta]_{222}$ values.

RESULTS

Antiviral activities of EK introduced peptides. Because W117, W120 and I124,
which are crucial for binding to N-HR (2, 3) are located in the N-terminus of C34, we
deleted the C-terminal region of SC35EK to produce short peptides. SC29EK, which
has four complete X-EE-XX-KK motifs, inhibited HIV-1$_{\text{NL4-3}}$ infection at a level
comparable to SC34EK (Table 1). As observed with SC34EK, SC29EK also
maintained an inhibitory effect toward T-20-resistant clones. Although SC34EK
blocked replication of the C34-resistant clone HIV-1$_{\text{AVA4/D36G/I37K/N126K/L204I}}$, SC29EK
failed to do so. On the other hand, C29, with a five amino acid deletion from the
C-terminus of C34, exerted drastically reduced antiviral activity. SC22EK, consisting
of three X-EE-XX-KK motifs, also showed much reduced antiviral activity compared
with SC29EK and SC34EK. A native peptide corresponding to SC22EK, C22
exhibited no antiviral activity toward HIV-1$_{\text{NL4-3}}$ at concentrations up to 10 µM (data
not shown). Thus, to inhibit the physiological interaction of N-HR and C-HR, a
peptide 22 amino acids in length, without modification, may be insufficient. D36G
substitution enhanced the susceptibility of HIV-1 to T-20 (28), but not to C34 or its
derivatives (Table 1). These results suggest that four X-EE-XX-KK motifs are required to maintain the inhibitory effect of peptides on the membrane fusion of HIV-1 strains resistant to T-20, as well as HIV-1_{NL4-3}.

**α-Helicity of the six-helix bundle.** To elucidate the mechanism by which SC29EK exerts strong anti-HIV activity, we performed CD analysis of the N36/SC29EK complex. The CD spectrum for the N36/SC29EK complex revealed an α-helix conformation with a characteristic double minimum at 208 nm and 222 nm, similar to those of N36/C34 and N36/SC35EK complexes. The N36/C29 complex showed an α-helical conformation, while a complex of N36 with C22 showed decreased α-helical spectra (Fig. 2A), in direct correlation to their decreased antiviral activities. The CD spectra of complexes of N36s containing T-20 resistance-associated mutations with SC29EK, N36_{V38A}/SC29EK and N36_{N43D}/SC29EK, were almost identical to that of N36 with SC29EK, indicating that SC29EK retains binding affinity for the mutated N36s (Fig. 2B). On the other hand, the mutated N36s and C29 complexes showed little α-helical conformation. These results indicate that introducing the X-EE-XX-KK motif increases the binding affinity of SC29EK for the mutated N36s.

The thermal stabilities of these complexes were assessed by monitoring the shift in $[θ]_{222}$ (Fig. 2C). A relatively low melting temperature ($T_m$; 48.5°C, Fig. 2D) and approximately 80% α-helicity at 37°C (Fig. 2C) were observed with the N36/C29 complex, consistent with its moderate antiviral activity (Table 1). The $T_m$ values of N36 and C-HR-derived peptides with a X-EE-XX-KK motif introduced were higher than those of the N36/C34 complex (Fig. 2D), while those of native sequence peptides
without the introduced motif were lower. The relationships between the EC\textsubscript{50} values of C-HR derived peptides and their \( T_m \) values are shown in Fig. 2E. The correlation between EC\textsubscript{50} and \( T_m \) values was weak \((r^2=0.4412)\); however, excluding SC22EK, which showed weak antiviral activity despite its high \( T_m \) value, the strength of this correlation was increased \((r^2=0.8002)\), suggesting that other factors including the solubility and intra-peptide interactions, may be involved in the enhanced antiviral activity of EK-containing peptides.

**Effect of serum on antiviral activity.** Finally, we assessed the anti-HIV activity of SC29EK in the presence of a high concentration of FCS and in fresh human sera. The antiviral activities against HIV-1\textsubscript{NL4-3} in various concentrations of FCS (5, 10, 20 and 50\%) were determined. An RT inhibitor, ddC, was used as a control. The antiviral activity of ddC was decreased in a concentration-dependent manner (Fig. 3A). In the presence of 50\% FCS, the reduction in the EC\textsubscript{50} value of ddC was statistically significant \((p=0.01)\). Similarly, but to a much lesser extent, that of T-20 appeared to be reduced with FCS concentration dependently. Even in the presence of 50\% FCS, the mean EC\textsubscript{50} value was comparable to those in 5\% and 10\% FCS \((p=0.082 \text{ and } 0.075, \text{ respectively})\). However, the effects of SC29EK and SC34EK were less affected by increased FCS concentration.

For further evaluation, freshly isolated human sera from two HIV-seronegative healthy volunteers were prepared. T-20 and SC29EK incubated for 2 hr at 37\(^\circ\)C in fresh human sera, FCS or PBS were diluted using a DMEM-based complete media supplemented with 10\% FCS and subjected to MAGI assay. The final
FCS concentrations of various sera including FCS in these diluted mixtures ranged from 9.75 to 12.2%. Because the antiviral activities of T-20 and SC29EK were not influenced significantly by FCS concentration (Fig. 3A), it is unlikely that the differences in FCS concentration in this experiment had any effect on antiviral activity. Compared with PBS-treated peptides, small changes in the inhibitory effects of both T-20 and SC29EK treated with FCS and human sera were observed (Fig. 3B, C). Taken together, these findings suggest that SC29EK stably exerts its strong anti-HIV-1 activity in vivo in the same manner as does T-20.

**DISCUSSION**

Here, we show that SC29EK inhibits the membrane fusion of T-20-resistant HIV-1 strains, suggesting that four X-EE-XX-KK motifs are sufficient to inhibit the fusion of T-20-resistant variants. As revealed by the EC$_{50}$ and $T_m$ values (Table 1 and Fig. 2), resistance-associated mutations in the N-HR region, such as V38A and N43D, seem to decrease the binding affinity of C-HR-derived peptides for N-HR. Therefore, HIV-1 strains with V38A or N43D show resistance to T-20. However, the anti-HIV-1 activity of SC29EK was less affected by these mutations, because at the physiological temperature for HIV-1 replication, SC29EK showed a stable interaction with N36 peptides containing mutations conferring resistance to T-20. The anti-HIV-1 activity of SC29EK was decreased against the C34-resistant clone HIV-1$_{\Delta V4/D36G/I37K/N126K/L204I}$, while SC34EK maintained its activity. One of the primary mutations underlying C34...
resistance, I37K, is located close to but outside of the putative binding site of SC29EK.

Previously, we reported that an N126K substitution in C-HR enhances the intra-gp41 binding of N-HR and C-HR (22); therefore, we hypothesized that the activity of SC29EK might be decreased by competing with C-HR with the N126K mutation. However, SC29EK also inhibits entry of HIV-1_{D36G/N126K}. Although no structural analysis of the mutated six-helix bundle was performed, it is possible that mutations conferring C34 resistance might induce some structural changes at or adjacent to the SC29EK binding site, because a peptide shortened by a further seven amino acids, SC22EK, suppressed the entry of the C34-resistant clone.

C34 itself did not have an α-helical spectrum, while SC29EK did (data not shown). SC29EK may achieve its strong antiviral activity by forming an α-helix as a result of E/K substitutions on the solvent-accessible site (Figure 1). From CD analysis, HIV-1 builds up resistance to T-20 by introducing certain mutations in N-HR, such as V38A and N43D, which reduce the binding affinity between N-HR and C-HR.

SC29EK can efficiently inhibit fusion of these mutant HIV-1 strains, suggesting that the ability of SC29EK to bind to mutated N-HR, and its weak affinity for C-HR, are maintained. On the other hand, the mutations D36G, N126K and S138A increase viral fusion activity (13) by enhancement of the binding affinity of C-HR for N-HR (22, 31). SC29EK effectively suppresses the replication of viruses that have these mutations, such as HIV-1_{D36G}, HIV-1_{D36G/N43D/S138A} and HIV-1_{D36G/N126K}. This indicates that the binding capacity of SC29EK is stronger than that of mutated C-HR containing the N126K or S138A mutations. Therefore, the monomeric α-helical form may inhibit the
interactions of N-HR and C-HR, mutations in which affect their binding affinity, and
thus the formation of the six-helix bundle.

Although SC22EK has enhanced $\alpha$-helicity and a high $T_m$ value, it has less antiviral activity compared with SC29EK. In the interaction between N-HR- and C-HR-derived peptides, while the cavity-forming region (from L54 to Q66) of the C-terminus of N-HR, the “pocket”, and the cavity-binding region (side chains of W117, W120 and I124) of the N-terminus of C-HR, the “knob”, play an important role (2, 3, 10), another region of C-HR may also be required. A constrained fourteen-residue peptide (C14linkmid), which corresponds to the “knob” region, shows chemical cross-linking and contains amino acid substitutions (27), is about 15,000-fold less active compared with SC29EK, which contains additional regions to “knob” region. These findings also suggest that the “knob” region of C-HR is important, but not sufficient for the formation of a stable complex. Another possible explanation of the weak activity of C14linkmid is that, because during fusion, not only binding of N-HR and C-HR, but also dynamic structural changes are easily anticipated, it would be difficult to maintain tight binding to the target N-HR due to its rigid constrained form. To maintain the binding of C-HR to N-HR in spite of such drastic conformational changes during fusion, there may be some unknown interaction, besides the interaction between “pocket” and “knob”, necessary for membrane fusion. At present, we cannot conclude whether i) the length of peptide itself is crucial, ii) some other domain has a role, or iii) a combination of both is important. Further experiments will be needed to clarify the mechanism of inhibition. Such information will be valuable to generate effective short
peptide inhibitors or small molecules. To generate effective small molecule inhibitors, if the second possibility is correct, a combination of two agents, one of which interacts with the pocket and the other of which interacts with an unidentified domain, should provide enhanced efficacy. To date, only a limited number of small molecular compounds that inhibit the six-helix bundle formation with marginal activities, have been reported (5, 9, 11), although in peptide based inhibitors, several effective peptides have been developed, including T1249 (7), SC34EK (24), T2635 (6) and T-20EK (23).

The $T_m$ value for the N36/SC29EK complex was higher than that of the N36/C29 complex, suggesting that EK substitutions reinforced the binding affinity to N-HR through enhanced $\alpha$-helicity. It has been considered that the enhanced $\alpha$-helical structure is maintained by intra-helical salt bridges formed by EK introduction (19). We recently revealed that an electrostatic interaction formed by EK alignment is involved in enhanced $\alpha$-helicity (Nishikawa et al. in printing), indicating that the strong $\alpha$-helical stability of SC29EK is probably provided by an identical mechanism to that of SC34EK. Similar peptides with substitutions of glutamate and arginine, to increase $\alpha$-helicity, have been reported (6). These peptides also increase helix stability and have antiviral activity against T-20-resistant HIV-1. Moreover, these peptides were relatively stable in an in vivo model. It is possible that enhanced binding affinity confers non-specific binding to other $\alpha$-helical regions of cellular proteins, for example, human serum albumin, which contains 31 $\alpha$-helical regions (20). However, this effect will be minimal, because the antiviral activity of SC29EK was highly stable in higher concentrations of FCS and was less affected by human sera.
In this study, we demonstrated that a 29-amino acid short peptide, SC29EK, suppresses replication of T-20-resistant variants. SC29EK maintained its activity in high concentrations of sera, indicating that SC29EK is a candidate short peptide fusion inhibitor. (main text: 2969 words)

Acknowledgments

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References


Figure legends

Fig. 1. Schematic diagram of HIV-1 gp41 and sequences of C-HR-derived peptides. (A) FP: fusion peptide; N-HR: N-terminal heptad repeat; C-HR: C-terminal heptad repeat; TM: transmembrane domain. Residues at each position in helical turns were denoted in italics. *The Z indicates an artificial amino acid, norleucine, instead of methionine, to avoid oxidation of the side chain of methionine. †Possible electrostatic interactions are indicated by lines and correlating amino acids, glutamate (E) and lysine (K), are represented blue and magenta, respectively. (B) One heptad helical turn was depicted.
Fig. 2. Analysis of N36 and various C-HR-derived peptides complexes by CD spectroscopy. CD spectra for N36/C-HR-derived peptides complexes (A) and mutated N36/C29 or SC29EK complexes (B). (C) Temperature-dependent transitions of the dissociation degree of N36 and various C-HR-derived peptides complexes. (D) \( T_m \) values of complexes of various N-HR peptides and C-HR peptides. NA: not available. (E) Relation between EC\(_{50}\) of C-HR-derived peptides and \( T_m \) values of N36 and various C-HR-derived peptide complexes. The strength of the correlation between EC\(_{50}\) and \( T_m \) values is increased \( (r^2=0.8002) \) when data for SC22EK are excluded.

Fig. 3. Effect of serum components on antiviral activity. Antiviral activities in various serum concentrations, 5, 10, 20 and 50%, were determined by MAGI assay. (A) Black dots indicate EC\(_{50}\) (nM) values, each of which was determined three times independently \( (*p=0.01 \) by the Student’s \( t \) test). Antiviral activities of T-20 (B) and SC29EK (C) in human HIV-seronegative sera (hs-1 and hs-2) were assessed by counting the number of blue cells. Bars indicate percentages of blue cell counts in PBS, FCS and human sera compared with that in no antiviral agents (control as 100%). Error bars represent the SD of each mean.
Table 1. Antiviral activity of HIV-1 gp41-derived peptides against T-20-resistant mutants

<table>
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<th>Viruses</th>
<th>EC$_{50}$a(nM)</th>
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<tr>
<td></td>
<td>T-20</td>
</tr>
<tr>
<td>HIV-1$_{NL4-3}$</td>
<td>15 ± 1$^b$ (6.3)$^c$</td>
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<tr>
<td>HIV-1$_{D36G}$</td>
<td>2.4 ±0.6</td>
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<td>HIV-1$_{D36G/V36A}$</td>
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<td>HIV-1$_{D36G/N41D/S138A}$</td>
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<td>HIV-1$_{D36G/N126K}$</td>
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<tr>
<td>HIV-1$_{AV4/D36G/I37K/N126K/L204I}^d$</td>
<td>390 ± 155 (163)</td>
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</table>

$^a$ Antiviral activity shown as EC$_{50}$ (50% effective concentration) was determined by MAGI assay.

$^b$ Each EC$_{50}$ value represents a mean ± SD obtained from at least three independent experiments.

$^c$ Parentheses indicate fold decrease in EC$_{50}$ value compared with the EC$_{50}$ value in D36G.

$^d$ ΔV4 indicates a five amino acid deletion (FNSTW) in the V4 region of gp120.
Figure 1

A

T-20

YTSLIHSLIIESQNQQEKNEQELLELDELKWASLWNWF

C34

WMEWDRERINNYTSLIHSLIIESQNQQEKNEQELLE

SC34EK


SC35EK


SC29EK

W^E^E^W^D^K^I^E^E^Y^T^K^K^I^E^E^L^I^K^K^S^E^E^Q^Q^K^N

SC22EK

W^E^E^W^D^K^I^E^E^Y^T^K^K^I^E^E^L^I^K^K

B

E^i

K^i^+^4

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**Figure 2**

**A**

\[ [\theta] (10^4 \text{deg cm}^2 \text{dmol}^{-1}) \]

![Graph A](image)

**B**

![Graph B](image)

**C**

Fraction of unfolded peptide

Temperature (ºC)

**D**

<table>
<thead>
<tr>
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<td>N43D/SC29EK</td>
<td>57</td>
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</table>

**E**

\[ r^2 = 0.4412 \]

![Graph E](image)
Figure 3

A

EC_{50} (nM)

ddC

T-20

SC29EK

SC34EK

FCS concentration (%)

B

T-20

PBS

FCS

hs1

hs2

100 nM

20 nM

C

SC29EK

PBS

FCS

hs1

hs2

12 nM

2.4 nM

Blue cell counts (%)

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