学位論文
Doctoral Thesis

A small molecule compound that reduces Nef-mediated enhancement of HIV-1 infectivity
（NefのHIV-1感染性増強効果を減弱する低分子化合物）

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2. Abstract

Purpose: Nef, a multifunctional HIV-1 accessory protein, has been shown to enhance the infectivity of progeny viruses. However, the underlying molecular mechanism is still unclear. In this study, to find a clue to the molecular mechanism, we investigated whether a small molecule Nef-binding compound 2c, which we recently identified, interfered with the viral infectivity enhancement by Nef.

Methods: HIV-1 viruses were produced by transfecting proviral plasmids into 293 cells and their infectivity was assessed using TZM-bl cells as a target. The compound 2c was added to either the producer or target cells. The viral replication was assessed by using primary macrophages. The pull-down assay with glutathione S-transferase (GST) fusion proteins was also performed to further verify a direct binding of 2c to Nef.

Results: When added to viral producer 293 cells, 2c did not affect the efficiency of viral production itself, but significantly reduced the infectivity of produced viruses to TZM-bl cells. Such inhibitory activity was observed with the wild-type viruses but not with Nef-defective (ΔNef) viruses, the latter of which had significantly reduced intrinsic infectivity. Consistent with the fact that Nef was dispensable for the infectivity of vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped HIV-1, 2c did not show its inhibitory activity on the pseudotyped viruses. These results suggested that 2c reduced HIV-1 infectivity in a Nef-dependent manner. In fact, 2c also reduced the replication of the wild-type viruses in monocyte-derived macrophages but not of ΔNef viruses. Apart from 2c, a cellular tyrosine kinase, hematopoietic cell kinase (Hck) has been shown to bind Nef through its Src homology 3 (SH3) domain. The pull-down analysis with Nef-GST fusion proteins and various Hck proteins supported the model that both Hck SH3 domain and 2c directly bind Nef and their binding sites overlap.

Conclusions: Our results suggest that the direct 2c-Nef binding inhibits the interaction of Nef with unidentified cellular proteins and thereby reduces Nef-mediated infectivity enhancement.

3. List of Publications

1. **Nopporn Chutiwitoonchai**, Masateru Hiyoshi, Philip Mwimanzi, Takamasa Ueno, Akio Adachi, Hirotaka Ode, Hironori Sato, Oliver T. Fackler, Seiji Okada, Shinya Suzu. The Identification of a Small Molecule Compound That Reduces HIV-1 Nef-Mediated Viral Infectivity Enhancement. PLoS One, 6, (11): e27696, Nov, 2011.

2. Chihara T, Hashimoto M, Osman A, Hiyoshi-Yoshidomi Y, Suzu I, **Chutiwitoonchai N**, Hiyoshi M, Okada S, Suzu S. HIV-1 Proteins Preferentially Activate Anti-Inflammatory M2-type Macrophages. J Immunol, 188, (8): 3620-3627, Apr, 2012.

3. Hiyoshi M, Takahashi-Makise N, Yoshidomi Y, **Chutiwitoonchai N**, Chihara T, Okada M, Nakamura N, Okada S, Suzu S. HIV-1 Nef Perturbs the Function, Structure, and Signaling of the Golgi Through the Src Kinase Hck. J Cell Physiol, 227, (3): 1090-1097, Mar, 2012.

4. Chihara T, Suzu S, Hassan R, **Chutiwitoonchai N**, Hiyoshi M, Motoyoshi K, Kimura F, Okada S. IL-34 and M-CSF Share the Receptor Fms but are not Identical in Biological Activity and Signal Activation. Cell Death Differ, 17, (12): 1917-1927, Dec, 2010.

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5. List of Abbreviations

ABCA1	ATP-binding cassette transporter
APC	Antigen presenting cell
AxxA	PxxP motif-disrupted
CTL	Cytotoxic T lymphocyte
ΔNef	Nef-defective
GST	Glutathione S-transferase
HAART	Highly active anti-retroviral therapy
Hck	Hematopoietic cell kinase
HckN	Kinase domain deleted Hck
ICAM-1	Intracellular adhesion molecule-1
M-CSF	Macrophage-colony stimulating factor
LTR	Long terminal repeat
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
Nef	Negative factor
PACS-2	Phosphofurin acidic cluster sorting protein-2
PAK2	p21-activated kinase 2
PI3K	Phosphatidylinositide 3 kinase
PKC	Protein kinase C
PxxP	Proline-rich
RT	Reverse transcriptase
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TGN	Trans-Golgi network
VSV-G	Vesicular stomatitis virus glycoprotein
WT	Wild-type

6. Background and Objective of Study

Acquired immune deficiency syndrome (AIDS), an immunodeficiency disease resulted from human immunodeficiency virus type 1 (HIV-1) infection which was firstly discovered in 1983(Barre-Sinoussi et al. 1983; Gallo et al. 1983), is remaining the critical social and public health problems worldwide. The pathogenesis is occurred from severe opportunistic infections or cancer resulting from the depletion of host immune systems especially through killing of the $CD4^+$ T cells. Although the complete cure from the virus infection is presently impossible, the highly active anti-retroviral therapy (HAART) is successfully established to suppress the viral replication and prolong the patient life thus effectively decreases the global mortality from the disease (http://www.unaids.org). HAART is based on the multi-antiviral drug treatment by targeting viral structural proteins especially protease and reverse transcriptase (RT) using the combination of viral protease inhibitor and nucleoside/nucleotide reverse transcriptase inhibitors, or non-reverse transcriptase inhibitors. However due to the high rate mutation of viruses, various mutant are produced and some of them have the ability to resist against those antiviral drugs. Finally, these mutant viruses are selected and become the dominated population persisted in the infected patients resulting in the failure of HAART. Therefore, identification and development of novel antiviral drugs are still required. Instead of targeting at the viral structural proteins, the viral accessory proteins, which are (Nef, Vif, Vpu, Vpr) of which essential for facilitating the viral replication and pathogenesis are the alternative effective targets for the drug development. Among them, Nef is early expressed and required to optimize the high viral load at the initial stage of infection. Therefore targeting Nef protein may be helpful to reduce the acceleration of viral replication or viral set-point resulting in lower viral load and slower disease progression.

Nef is a 27-35 kDa protein without enzymatic activity, N-terminal myristoylated membrane binds, localizes mainly in the paranuclear region including plasma membrane. The evidences from *in vivo* studies showed that Nef is the pathogenic factor for AIDS development. Using the rhesus macaque monkey models demonstrated that infection with the Nef-deleted simian immunodeficiency virus (SIV) dramatically reduced viral load and disease progression (Kestler et al. 1991). In the transgenic mice models, expression of the *nef* gene in the mice $CD4^+$ T cells caused the development of severe AIDS-like pathologies similar to the expression of complete HIV-1 genome (Hanna et al. 1998; Hanna et al. 1998). In addition, the Nef-deleted viruses were frequency isolated from the asymptomatic patients or long term non-progressors (LTNP) who had low viral loads and stable $CD4^+$ T cell counts (Deacon et al. 1995; Kirchhoff et al. 1995; Salvi et al. 1998). *In vitro* studies of Nef have been extensively done

revealing various underlying mechanisms to promote viral replication and pathogenesis. However, the most critical function of Nef responsible for the major development of immunopathogenesis is controversial and needed to be elucidated for better understanding of Nef pathogenesis and designment of effective Nef domain for antiviral drug development.

The overall function of Nef seems to be facilitate in both evasion of the infected cells from host immune response and promotion of viral replication. The well-characterized Nef function to escape from recognition and killing by cytotoxic T lymphocytes (CTL) is downregulation of the antigen (endogenous viral antigen) presenting molecule, major histocompatibility complex class I (MHC I) on the infected cell surfaces (Hung et al. 2007; Atkins et al. 2008; Noviello et al. 2008; Schaefer et al. 2008; Wonderlich et al. 2008). Nef downregulates MHC I by both internalization of the cell surface MHC I and disruption of the transportation of newly synthesized MHC I from trans-Golgi network (TGN) to the cell surface. By interaction with the phosphofurin acidic cluster sorting protein-2 (PACS-2), Nef is translocated from the cell surface to TGN where it can mediate Src family kinase (SFK) to phosphorylate the ZAP70/Syk. The phosphorylated ZAP70/Syk then binds with phosphatidylinositide 3 kinase (PI3K) leading to PI3K activation and signaling of which finally accelerates the rate of MHC I endocytosis (Hung et al. 2007; Atkins et al. 2008). In the TGN, Nef is able to directly binds with the MHC I (at the cytoplasmic tail) and adaptor protein-1 (AP-1) of which subsequently redirects MHC I to the lysosomes for degradation (Noviello et al. 2008; Schaefer et al. 2008; Wonderlich et al. 2008). Nef also downregulates the CD8 on the cell surface of CTL which is required for facilitating the T cell receptor (TCR) of CTL to recognize the viral antigen presentated by MHC I (Stove et al. 2005). Through this mechanism, Nef recruited the adaptor protein 2 (AP-2) to the cell surface to trigger the clathrin-mediated endocytosis of CD8 (Stove et al. 2005; Leonard et al. 2011). Not only the CTL-mediated immune response, Nef disrupts the viral antigen (exogenous) presentation of antigen presenting cells (APC) to $CD4^+$ T cells by downregulaiton of their cell surface MHC class II (MHC II) (Schindler et al. 2003) or CD4 (Garcia and Miller 1991), respectively. Nef accumulates and re-localizes MHC II to the lysosomes for degradation through the unclear mechanism but independent of clathrin, dynamin, and other cytoskeleton proteins (Schindler et al. 2003). CD4 downregulation by Nef is another well-known Nef function. The molecular mechanism is similar to CD8 downregulation (Leonard et al. 2011), by direct binding of Nef to the CD4 (at the cytoplasmic tail) and adaptor protein 2 (AP-2) of which then triggers the formation of clathrin coated pit vesicles leading to endocytosis of the surface CD4. Subsequently, Nef exchanges binding to β-COP leading to targeting of CD4 to lysosomes for degradation (Lindwasser et al. 2008; Schaefer et al. 2008; Leonard et al. 2011). In

addition to the downregulations of MHC I, MHC II, CD8, and CD4, Nef downregulate the macrophage-colony stimulating factor (M-CSF) receptor (or Fms) which is important for survival and maintenance of anti-inflammatory stage in macrophages was reported (Hiyoshi et al. 2008). The mechanism is occurred by activation of a Src family kinase Hck by Nef resulting in the aberration of the Golgi apparatus (Golgi cisternal unstacking) where the *N*-glycosylation and trafficking of Fms occurred (Hassan et al. 2009; Hiyoshi et al. 2012). In addition to the protection of the infected cells from host immune responses, Nef prevents the infected T cells by downregulation of a co-stimulatory receptor CD28 of TCR signaling to minimize the TCR activation by APC of which the hyperactivation leads to apoptosis of the infected T cells (Swigut et al. 2001). By depletion of these immunological molecules by Nef, the infected cells can escape from the effective host immune systems.

In addition to facilitate evasion from the host immune responses, Nef promotes viral replication by increasing the capacity of infected cells to produce viruses and enhancing the ability of progeny viruses to infect into the target cells or as well-known as “infectivity”. To increase virus production in T cells, Nef modulate variety of intracellular signaling cascades 97%-similar to the activation by TCR (Simmons et al. 2001). The activation throughout an unclear molecular signaling suck as activation of Lck and protein kinase C (PKC) finally increase the activity of viral long terminal repeat (LTR) promoter to upregulate the expression of viral proteins thus enhancing viral production (Wang et al. 2000; Simmons et al. 2001; Witte et al. 2008). This mechanism also allows viral production in the non-permissive cells such as resting T cells (Sawai et al. 1994; Stevenson 2003). In addition, Nef prevent T cell activation from the APC of which the activation leads to hyperactivation-induced apoptosis by impairment of the immunological synapse or their interaction. The molecular mechanism is occurred by disruption of the TCR clustering through the modification of actin cytoskeleton triggered by the activation of p21-activated kinase 2 (PAK2) by Nef (Nunn and Marsh 1996; Arora et al. 2000). In addition, Nef associates several cellular molecules involving with actin rearrangement to impair immunological synapse including Vav (Fackler et al. 1999; Rauch et al. 2008), CDC42 and Rac1 (Rauch et al. 2008), Raf-1 kinase (Hodge et al. 1998), and PKC (Smith et al. 1996). Not only beneficial for the evading from immune response, Nef downregulates CD4 to enhance the viral release by preventing the tethering between cell surface CD4 and viral particle Env (Arganaraz et al. 2003). In addition, the viral Env can fully function because no blocking from the CD4 molecule (Arganaraz et al. 2003). Another strategy to increase viral production is to protect the infected cells from the apoptosis. Nef inhibits the activation of apoptosis signal regulating kinase 1 (ASK1), a key signaling intermediated in the Fas and TNF-α death signaling pathways (Geleziunas et al. 2001). Nef also

destabilizes p53, a tumor suppressor playing role in induction of apoptosis (Greenway et al. 2002), or associates with PAK and PI3K to inactivate the pro-apoptotic Bad signaling (Wolf et al. 2001). Since the super-infection is spontaneously occurred especially at the chronic phase of infection leading to the death of infected cells, Nef downregulates the cell surface receptors for infection including CD4 (as mentioned before) and its co-receptors, CXCR4 (Venzke et al. 2006) and CCR5 (Michel et al. 2005). All of these Nef functions are proposed to promote viral replication by boosting the production of viruses from infected cells.

As mention earlier, Nef also enhances the viral infectivity to promote viral replication. This function of Nef is maintained by the selective pressure among different group of primate lentiviruses suggesting the critical role of viral infectivity to the viral replication (Munch et al. 2007). Nef was found to enhance viral infectivity in the single-round infection assay and greater increasing in replication assay in the non-stimulated or resting cells while did not affect viral replication in the stimulated, fully proliferated, or chronically infected cells (Kimpton and Emerman 1992; Chowers et al. 1994; Miller et al. 1994; Spina et al. 1994). In addition, studying in rhesus macaques demonstrated that infection with the SIV clone having Nef-mediated viral infectivity enhancement ability, resulted in the higher viral loads comparing with the infection of SIV clone lacking this function of Nef (Iafrate et al. 2000; Brenner et al. 2006). The precise underlying molecular mechanism of this Nef-enhanced viral infectivity is not well understood but at least Nef is required in the virus producer cells to achieve the higher infectivity of progeny viruses. In consistency, expression of Nef in the target cells failed to rescue infectivity of the Nef-defective viruses (Aiken and Trono 1995; Pandori et al. 1996). The virion incorporation of Nef was reported did not involved with the viral infectivity enhancement since there were no correlation between these two observations (Miller et al. 1997; Welker et al. 1998; Fackler et al. 2006) and only small amounts of Nef were incorporated into the virion (~10 molecules/virion) (Pandori et al. 1996; Welker et al. 1996; Welker et al. 1998). Instead, Nef increased the virion incorporation of cellular membrane proteins were reported to associate with viral infectivity enhancement, such as human leukocyte antigen (HLA) (Cantin et al. 1997; Martin et al. 2005), co-stimulatory molecule CD80 and CD86 (Giguere et al. 2004), and intracellular adhesion molecule-1 (ICAM-1) (Bounou et al. 2002; Beausejour and Tremblay 2004) (Figure 1A). Incorporations of these cellular molecules may facilitate the binding of viral particles with the target cells, for example the binding of incorporated ICAM-1 to the LFA-1 on target cells (Bounou et al. 2002; Beausejour and Tremblay 2004). Another cellular component that was reported to be incorporated into virions and associated with viral infectivity enhancement is the lipid rafts (Campbell et al. 2002; Guyader et al.

2002). Lipid rafts or detergent-resistant membranes are microdomains in the plasma membrane enriched with sphingolipids and cholesterol of which essential for the budding of viruses from producer cells (Nguyen and Hildreth 2000; Campbell et al. 2002; Guyader et al. 2002). Nef was reported to increases viral budding from lipid rafts including increasing of the ganglioside content, a major component of lipid rafts in the viral envelops, which were correlated with the enhancement of viral infectivity (Zheng et al. 2001). Indeed, gene expression profile of the T cells infected with Nef expressing viruses showed up-regulation of multiple genes involved in the cholesterol biosynthesis such as a regulatory enzyme, sterol-responsive element binding factor 2 (SREBF-2) and a rate-limiting step enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (van 't Wout et al. 2005) (Figure 1B). In addition, the expression of low-density lipoprotein receptor (LDLR) of which plays role in the uptake of extracellular low-density lipoprotein was also upregulated (van 't Wout et al. 2005). Moreover, Nef was reported to increases the transportation of newly synthesized cholesterol from endoplasmic reticulum to the lipid rafts in plasma membranes where the viral budding occurred (Figure 1C) (Zheng et al. 2003). The direct binding of Nef and cholesterol has been demonstrated *in vitro* and *in vivo* indicating the L202, Y206, Y207, and K208 localized at the C-terminus of Nef are important for the binding (Zheng et al. 2003). Nef also facilitates the preservation of intracellular cholesterol for the infectious virus production by competing the function of cholesterol efflux protein, ATP-binding cassette transporter (ABCA1) (Figure 1D) (Cui et al. 2012). Through this mechanism, Nef was proposed to inhibit the accessibility of ABCA1 to the cell surface, to induce the ABCA1 degradation in proteasome, or to re-localizes ABCA1 from the non-raft region where it functions to the lipid raft region for recruitment of the cholesterol (Cui et al. 2012). Regarding to these mentioned abilities of Nef to incorporate the essential cellular components into virions, the viral infectivity of progeny viruses is increased. In contrast, Nef depletes or downregulates the CD4 as mentioned earlier also participates in the enhancement of viral infectivity by inhibiting the blocking of CD4 molecules to the Env of viral particles (Figure 1E) (Arganaraz et al. 2003). Moreover, Pizzato and coworkers (2007) proposed the presence of a CD4-liked cellular molecule that is also able to block the function of virion Env and Nef downregulated this CD4-liked molecule by endocytosis through the dynamin 2 – clathrin association pathway (Figure 1F). This hypothesis was based on the finding that the viral infectivity was inhibited when producing the viruses with the expression of dominant-negative dynamin 2 or clathrin, or producing in the dynamin 2 or clathrin knock-down cells (Pizzato et al. 2007). Altogether represent the role of Nef to modify the components of cellular plasma membrane to facilitate the production of high infectivity viruses.

Figure 1. The proposed molecular mechanisms of Nef to enhance viral infectivity. *(A)* In the virus producer cells, Nef promotes virion incorporation of a cellular molecule, e.g., ICAM-1 to facilitate binding of virions with the target cells. *(B)* Nef upregulates lipid biosynthesis genes, e.g., CYP51 to increase lipid productions such as cholesterol, a component of lipid rafts essential for viral budding and enhancement of infectivity. *(C)* Nef facilitates transportation of newly synthesized cholesterol from the endoplasmic reticulum to lipid raft domain on plasma membrane surface. *(D)* Nef re-localizes the cholesterol efflux protein, ABCA1 from the cell surface to proteasome for degradation, to preserve the intracellular cholesterol for viral production. *(E)* Nef downregulate CD4 to increase the efficiency of viral Env function. *(F)* With the same advantage on downregulation of CD4, Nef also downregulates an unidentified CD4-liked molecule through the dynamin 2 – clathrin mediated pathway. *(G)* In the target cells, with an unidentified modification of viral particle by Nef during viral production, the efficiency of viral reverse transcription is increased such as by association with trafficking to the appropriated environment for reverse transcription or recruitment of cellular molecules involved with the reverse transcription processes. *(H)* Nef-modified virus promotes the penetration of viral core through the cortical actin barrier by association with actin rearrangement. *(I)* By the same mechanism, the viral core is trafficked away from proteasome to protect from the degradation.

Other possibilities of Nef to enhance viral infectivity were reported to be occurred in the target cells during the early step of infection. The efficiency of viral RT of the viruses produced in the

presence of Nef was higher than that of in the absence of Nef (Aiken and Trono 1995; Chowers et al. 1995; Schwartz et al. 1995). This observation is not due to increasing of the RT activity by Nef, since no difference between the RT activity of viruses produced in the presence or absence of Nef (Aiken and Trono 1995; Chowers et al. 1995; Khan et al. 2001). Rather increasing the efficiency of reverse transcription in target cells may be resulted from the Nef-mediated virion modification that can overcome an unidentified specific blocking of the reverse transcription processes in the target cells. Such an increasing of the RT efficiency were suggested by trafficking of the reverse transcription complex by actin remodeling to an appropriated cellular environment for RT activation (such as an adequate nucleotide concentrations) (Figure 1G) (Bukrinskaya et al. 1998), or recruitment of a cellular molecule involved with the reverse transcription processes (Warrilow et al. 2009). In addition, enhancement of viral infectivity was proposed to be occurred in viral entry step during penetration of viral core through the cell membrane barrier. Since the cortical actin network lying beneath the cell membrane acts as a barrier to obstruct infection of several viruses including HIV-1 (Yoder et al. 2008), the Nef-modified virions was reported to promote the movement of viral core through this barrier relating to actin rearrangement (Figure 1H) (Campbell et al. 2004). This hypothesis was proposed from the finding that inhibition of the cortical actin network and stress fibers by inhibitors CytD, LatB, or Jas could restore infectivity of the Nef-defective viruses to the comparable level of the Nef-modified viruses (Campbell et al. 2004). In addition, Nef does not affect viral infectivity of the pseudotyped HIV with the vesicular stomatitis virus glycoprotein (VSV-G) of which enters the cell by endocytosis, rather enhances infectivity of viruses entering the cell by plasma membrane fusion (Aiken 1997; Luo et al. 1998; Chazal et al. 2001), further supports the effect of Nef-modified viruses against the cortical actin barrier. Another proposed mechanism of Nef to promote viral infectivity is modification of viral core to avoid the proteasomal degradation after entry into the target cells such as associating with actin to traffic away from the proteasome (Figure 1I). An indirect experiments support that treating the target cells with the proteasome inhibitors could enhance the viral infection during the early stage of infection (Schwartz and Klotman 1998; Wei et al. 2005). In addition, treating with the proteasome inhibitors, MG132 or lactacystin could rescue infectivity of the Nef-defective viruses (Qi and Aiken 2007). This underlying mechanism was proposed to be occurred by reducing the recognition of Nef-modified viruses from the ubiquitylation-induced proteasomal degradation (Qi and Aiken 2007). All of these results emphasize again the role of Nef during viral production processes to modify the progeny viruses to overcome the natural viral restriction mechanisms in the target cells.

According to all of these literature reviews about the multi-function of Nef on facilitating evasion of the infected cells from the host immune responses and promoting the viral replication, suggest that targeting the Nef protein by inhibitor may be helpful to reduce or delay the pathogenesis of AIDS. Targeting Nef by short peptides or single domain antibodies have been developed and reported (Hiipakka et al. 2001; Bouchet et al. 2011; Breuer et al. 2011; Bouchet et al. 2012). Most of these inhibitors were designed based on targeting at the hydrophobic region of Nef (F90, W113, and I114; referenced from the NL43 strain virus) including the proline-rich (PxxP) motif (residues 71-79; referenced from the NL43 strain virus) of which together forming the tertiary structure to the deep pocket shape. Recently, Bouchet and colleagues (2011) were successful to develop the single-domain antibody that can inhibit the functions of Nef on downregulation of CD4 and enhancement of viral infectivity. Later, the improved single-domain antibody by fusion with the Src homology 3 (SH3) domain of Hck (the high affinity binding partner of Nef PxxP motif), was constructed and showed the additional inhibitory effect on the Nef-downregulated MHC I (Bouchet et al. 2011; Bouchet et al. 2012). However, the inhibitory effects of these antibodies were based on the enforcing intracellular expression in the virus producer cells where the Nef protein functions, the direct evidence such as by direct addition to the cells has not been demonstrated yet. In addition, since these short peptides or antibodies were designed by mimicking the cellular SH3 domain of Hck protein, the possibility that these inhibitors may interrupt the physiological functions of the involved cellular partner proteins may be occurred. Alternatively to the short peptides or single domain antibodies, Benzi and coworkers (2007) succeeded to discover the first two chemical compound inhibitors for the Nef protein, named D1 (2-hydroxy-5-[(4-tert-butylphenoxy)carbonyl amino]benzoic acid) and DLC27 (3-[((4-tert-butyl phenoxy)acetyl)amino]benzoic acid) (Figure 2A). This identification was based on the *in silico* screening of the SH3 binding surface of Nef (PxxP motif, residues 71-79 and the hydrophobic region, F90, W113, and I114; referenced from the NL43 strain virus) against the compound library from the National Cancer Institue Diversity Library (Betzi et al. 2007). The candidate compounds were then validated with the mammalian two-hybrid assay based on inhibition of the Nef-SH3 Hck binding (Betzi et al. 2007). Throughout these processes, D1 showed 77% blocking effect at the concentration of 32 μM (Betzi et al. 2007). In addition, D1 also partially reduced the Nef-mediated MHC I downregulation (35% inhibition at 25 μM concentration) (Betzi et al. 2007). This Nef function was reported to associate with the SH3 binding surface of Nef (Hung et al. 2007). DLC27 was then identified by the *in silico* screening of D1-liked compound against the Chembridge EXPRESS-Pick database following with the mammalian two-hybrid assay (Betzi et al. 2007). DLC27 showed >75% blocking effect at the

concentration of 20 μM (Betzi et al. 2007). The molecular docking model of Nef and D1, or DLC27 suggested that lacking of the phenolic group in DLC27 improved the affinity binding with the SH3 binding surface of Nef (Betzi et al. 2007). Later Emert-Sedlak and colleagues (2009) were also successful to identify the chemical compound inhibitors for Nef based on the inhibition of Hck activation by Nef. By using the high-throughput *in vitro* kinase assay screening with the chemical compound libraries, the 3-(5,6-diphenylfuro[2,3-d]pyrimidin-4-ylamino)propan-1-ol (diphenylfuro pyrimidine or DFP-4AP) (Figure 2B) was identified (Emert-Sedlak et al. 2009). At the micromolar concentration levels, DFP-4AP inhibited Nef-activated Hck (IC_{50} value of 4 μM) and partially inhibited viral replication in the Nef-dependent viral replication cell lines, U87MG (Emert-Sedlak et al. 2009). In addition, the more potent analogs of DFP-4AP, DFP-4APF and DFP-4AB (Figure 2B) were synthesized and showed the higher inhibitory efficiency (Emert-Sedlak et al. 2009). However, these inhibitors exactly target at the Hck molecule, on its ATP-binding sites instead of the Nef molecule to inhibit Hck activation by Nef (Emert-Sedlak et al. 2009). Recently, our group also succeeded to find the chemical compound inhibitor; 2,4-dihydroxy-5-(1-methoxy-2- methyl propyl)benzene-1,3-dialdehyde (2c) (Figure 2C) with the abilities to inhibit the function of Nef on Hck activation (Hassan et al. 2009) and MHC I downregulation (Dikeakos et al. 2010). 2c was originally identified by Oneyama and colleagues (2003) as a non-kinase inhibitor of Src-signal transduction. It is an analog of UCS15A, an antibiotic produced by *Streptomyces sp*. of which firstly identified by screening from the yeast two-hybrid assay (Sharma et al. 2001). 2c and UCS15A disrupted the SH3-mediated protein-protein interaction including; c-Src (SH3)-Sam68(PxxP), Fyn(SH3)-Sam68(PxxP), Grb2(SH3)-Sam68(PxxP), and Grb2(SH3)-Sos1(PxxP) (Sharma et al. 2001; Oneyama et al. 2002; Oneyama et al. 2003). 2c showed more potent activity than UCS15A by at the same concentration, 20 μM, 2c inhibited 72% of Sam68-Fyn interaction while UCS15A inhibited 8% of the interaction (Oneyama et al. 2003). According to our finding, 2c at the concentration of 50 μM showed 50-60% inhibition of Hck activation (Hassan et al. 2009). The significant of Hck activation by Nef in this study was demonstrated to be associated with the downregulation or maturation arrest of M-CSF receptor (or Fms) of which leading to the aberration of macrophages (Hiyoshi et al. 2008; Hassan et al. 2009). In addition, the inhibition level of Nef-activated Hck was comparable with the recovery level of the functional M-CSF receptor (Hassan et al. 2009). The mechanism of 2c inhibition was proposed by interfering with the Nef PxxP motif of which required for the binding with SH3 domain of Hck and Hck activation (Hassan et al. 2009). For the inhibitory effect of 2c on Nef-mediated MHC I downregulation, 2c partially inhibited

this function of Nef in the primary CD4^{+} T cells (Dikeakos et al. 2010). In detail, 2c was demonstrated to specifically disrupt the Nef-SFK interaction of which required for the multi-kinase complex formation and activation of the ZAP-70/Syk-PI3K cascade resulting in the internalization of cell surface MHC I, while did not affect the up-stream processes (Nef-PACS2 binding and trafficking into the TGN) (Dikeakos et al. 2010). All of these identified chemical inhibitors mainly focus on the Nef function on Hck activation. The inhibitor that can inhibit Nef-mediated viral infectivity enhancement has not been identified yet. As mentioned earlier, this function of Nef also plays a critical role to promote viral replication. Therefore, the inhibitor that can inhibit this Nef function may be beneficial to decrease the viral replication, thus reduce or delay the pathogenesis of AIDS.

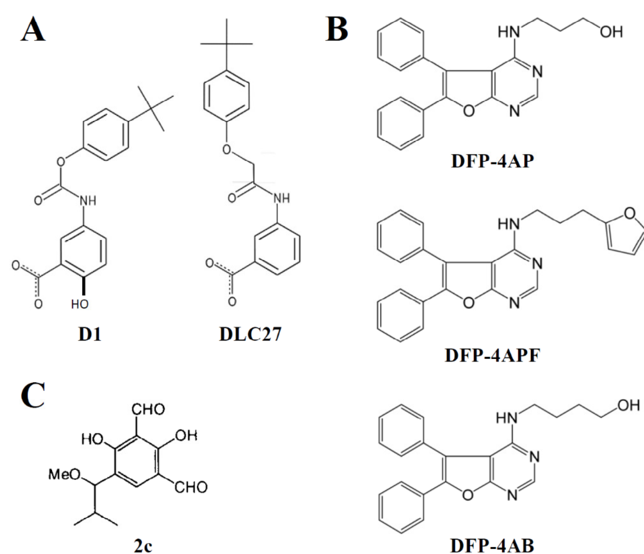


Figure 2. Chemical compounds with the abilities to inhibit Nef functions. The schematics represent the chemical structures of: *(A)* D1 and DLC27 which can inhibit the functions of Nef on Hck activation and MHC I downregulation, *(B)* DFP-4AP, DFP-4APF, and DFP-4AB which can inhibit the functions of Nef on Hck activation, *(C)* 2c which can inhibit the functions of Nef on Hck activation and MHC I downregulation (Sharma et al. 2001; Oneyama et al. 2003; Betzi et al. 2007; Emert-Sedlak et al. 2009).

According to our previous finding of the 2c inhibitor that can inhibit Hck activation and MHC I downregulation by Nef, we expand to investigate inhibitory effect of 2c on the viral infectivity enhancement by Nef. Therefore, the objective of this study is to investigate the inhibitory effect of 2c on the Nef-mediated viral infectivity enhancement. Based on the proposed inhibitory mechanisms of 2c on the Hck activation and MHC I downregulation by Nef of which expected to be occurred by interruption of the Nef PxxP motif interaction (Hassan et al. 2009; Dikeakos et al. 2010), together with

the reports showing that this PxxP motif was essential for the viral infectivity enhancement (Goldsmith et al. 1995; Saksela et al. 1995; Wiskerchen and Cheng-Mayer 1996), the possibility of 2c to inhibit Nef-enhanced viral infectivity is expected to be observed. If 2c shows the positive effect on this Nef function, together with other two inhibitory effects (on Hck activation and MHC I downregulation), will promote 2c as the potential inhibitor since can inhibit multi-function of Nef. In addition, 2c can be used to elucidate whether Nef-enhanced viral infectivity including Nef-activated Hck and Nef-downregulated MHC I, play the critical role on AIDS development in an animal model. Moreover, regarding to the controversial molecular mechanisms of Nef to enhance viral infectivity as mentioned before, 2c may be used as a chemical probe to validate or reveal the underlying mechanism of this Nef function.

7. Methods

7.1. 2c compound preparation

The 2c compound used in study was prepared by two sources; from Kyowa Hakko Kogyo (Tokyo, Japan) as described (Oneyama et al. 2003), and from Sai Advantium Pharma manufacturer (Hyderabad, India). Both lots of 2c preparations were dissolved in DMSO and confirmed for the equal inhibitory effect on HIV-1 infectivity.

7.2. Plasmid preparations

Proviral plasmids. The provial NL43 wild-type plasmid (pNL4-3) and its single-point mutation in the Nef gene including R77A, K82A, D86A, F90A, G119L, and the Nef-defective (ΔNef), were used (Jere et al. 2010). The Env-defective NL43 mutant (pNL-Kp) and VSV-G expression (pVSV-G) plasmids were used to produce the VSV-G-pseudotyped viruses (Akari et al. 1999). The proviral JRFL wild-type plasmid was provided by Dr. Y. Koyanagi (Kyoto University, Kyoto, Japan) (Koyanagi et al. 1987). The mutant proviral JRFL plasmids of which the Nef gene is disrupted (ΔNef) or replaced with AxxA at its PxxP motif was used (Hassan et al. 2009).

Hck plasmids. The wild-type Hck (p56Hck) was cloned into the pcDNA3.1 vector (Invitrogen) (Hiyoshi et al. 2008). The mutant Hck including; kinase domain deleted (HckN), SH2 domain disrupted (HckN-R151S), and SH3 domain disrupted (HckN-W93F) of which cloned into the pCAGGS vector were provided by Dr. M. Matsuda (Kyoto University, Kyoto, Japan) (Tokunaga et al. 1998).

GST fusion plasmids. The control glutathione S-transferase (GST) and GST-Nef fusion plasmids including; wild-type NL43 Nef (NL43 Nef-WT), wild-type SF2 Nef (SF2 Nef-WT), T71R substitutive NL43 Nef mutant (NL43 Nef-TR), and PxxP motif disrupted NL43 Nef mutant (NL43 Nef-AxxA) were prepared used (Hassan et al. 2009). The short peptide PxxP motif of Nef (20 amino acids) fusion with GST (GST-PxxP) was prepared by amplification of Nef gene from Nef expression plasmid using the following primers; 5'-GGATCCGTGGGTTTTCCAGT-3' and 5'-GTCGACCTATAAAGCTGCCT-3'. The amplified PCR products were then sub-cloned into the pCR2.1 vector (Invitrogen) and verified by DNA sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The constructed Nef PxxP motif was then sub-cloned into the GST expression plasmid pGEX-6P-1 (GE Healthcare) for the production of Nef PxxP motif-GST fusion (GST-PxxP) in bacterial cells.

7.3. Virus preparations

In this study we used HEK293 cells (Invitrogen) as the virus producer cells. The cells were maintained in DME medium supplemented with 10% fetal calf serum (FCS). For production of viral particles, 293 cells were seeded onto 12-well tissue culture plates at a density of 1.8×10^5 cells/well and transfected with 1.6 μg/well of the proviral HIV-1 plasmid by using 4 μl/well Lipofectamine 2000 reagent (Invitrogen). For the experiment using VSV-G-pseudotyped viruses, 0.5 μg/well of the Env-defective NL43 mutant plasmid and 1.0 μg/well of VSV-G expression plasmid were co-transfected into 293 cells. For the experimental examining the inhibitory effect of SH3 domain Hck on viral infectivity, 0.8 μg/well of pNL43 plasmid and 0.2, 0.4, or 0.8 μg/well of HckN, HckN-R151S, or HckN-W93F were co-transfected into 293 cells. The total amount of plasmid was normalized to 1.6 mg/well using the pCAGGS empty vector. After 6 hr of transfection, the culture medium was replaced with fresh medium, and the cells were cultured for an additional 48 hr in the presence or absence of 2c at the indicated concentrations in each experiment. Total volume of 2c at each concentration was equally normalized by DMSO. In the experiment examining the delay adding effect, 2c was added after 24 hr of transfection. The supernatants containing the viruses were then collected, clarified by brief centrifugation at 4°C for 5 min, and stored at -80°C until using. The amount of virus was quantified by measuring the concentration of p24 Gag protein in the supernatants using the RETROtek p24 Antigen ELISA kit (ZeptoMetrix) as described in the manufacturer protocol. In addition, the ability of viral production was also measured by analyzing of the intracellular expression of viral proteins in the virus producer cells by Western blotting. To prepare the total cell lysates for Western blotting, the cells were lysed on ice with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris, and 150 mM NaCl) containing the protease inhibitors (1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 30 min. The total cell lysates were then clarified by centrifugation at 14,000*g* for 15 min. The clear supernatant was collected, mixed with the SDS-PAGE sample buffer, boiled for 5 min, and stored at -20°C until the analysis. The Western blot was performed as standard protocol as described (Hiyoshi et al. 2008; Hassan et al. 2009; Hiyoshi et al. 2012). The primary antibodies used for Western blot analysis were shown as follows; anti-Gag (#65-004; BioAcademia, Osaka, Japan), anti-Nef (#2949; NIH AIDS Research & Reference Program), anti-Vif (#319; NIH AIDS Research & Reference Program), and anti-actin (#C-2; Santa Cruz). Their appropriated secondary antibodies labeled with HRP (GE Health- care) were used with the Western blotting

detection reagent, Immunostar LD (Wako, Osaka, Japan) and an image analyzer ImageQuant LAS 4000 (GE Healthcare) for the analysis.

7.4. Infectivity assay

TZM-bl cells (NIH AIDS Research & Reference Program) were used as the target or indicator cells for the viral infectivity assay. The cells were maintained in DME medium supplemented with 10% FCS. For viral infectivity assay, the cells were seeded onto 96-well tissue culture plates at a density of 6×10^3 cells/well and inoculated with the appropriated concentration of viruses (determined by p24 Gag protein) as showed in each experiment. In detail, the stock viruses prepared by 293 cells (as mentioned in the viral preparation step) were diluted with DME medium containing 10% FCS and 20 μg/ml DEAEdextran (MP Biomedicals, Solon, OH), added into the target cells (150 μl/well) and incubated overnight. The viral suspension was then replaced with the fresh DME medium containing 10% FCS and additionally incubated for 48 hr. In the experiment examining the effect of 2c by direct adding to the target cell, the stock virus preparing without 2c was used and instead the 2c was added to together with diluted viruses into the TZM-bl cells. The viral infectivity was examined by the activity of endogenous expression of the β-galactosidase enzyme which is conjugated with the viral LTR-promoter thus inducible by the viral Tat in the infected cells. The β-Galactosidase Enzyme Assay System (Promega) was used to examine the β-galactosidase activity. Briefly, the infected cells were washed twice with PBS, lysed with 1× Reporter Lysis Buffer (RLB) (100 μl/well) for 15 min. The supernatants containing β-galactosidase were then clarified by brief centrifugation and the 50 μl of clear supernatants were then mixed with 50 μl of 2× Assay Buffer. The enzymatic reactions were then incubated at 37 °C for 30 min and stopped by adding 150 μl of 1M Sodium Carbonate. Their absorbance was then measured at 405 nm using a Multiskan microplate reader (Thermo Electron).

7.5. Replication assay

For the viral replication assay in Jurkat cells, the cell pellet at a density 1×10^6 cells were inoculated by re-suspension in the 500 μl of the diluted NL43 viruses (prepared from 293 cells as described in the viral preparation step) for 2 hr at 37°C in the presence or absence of 2c as indicated in the result. The azidothymidine (AZT) (NIH AIDS Research & Reference Program) was used as a positive control inhibitor. An equal total volume of the adding 2c or AZT was normalized by DMSO. After inoculation, the cells were washed twice with PBS, re-suspended into 1 ml of RPMI1640-10%

FCS, and cultured for 3 days at the same conditions of 2c or AZT. Every 2 days, the infected cells were diluted 1:5 with RPMI1640-10% FCS and continually cultured at the same conditions of 2c or AZT. The remained cell suspensions containing the viruses were kept for analysis by brief centrifugation and stored at -80°C. The viral replication at day 5, 7, and 9 post-infection were analyzed by quantification of p24 Gag proteins using the RETROtek p24 Antigen ELISA kit.

The replication assay with macrophages was performed. Heparinized venous blood was collected from healthy donors, after informed consent was obtained in accordance with the Declaration of Helsinki. The approval for this study was obtained from the Kumamoto University Medical Ethical Committee. The peripheral blood mononuclear cells (PBMC) were isolated using Pancoll reagent (PAN BIOTECH, Germany). The monocytes were allowed adhering by incubation at 37°C for 1 hr. The non-adherent cells were then removed by extensive washing with PBS. The adherent monocytes were differentiated into macrophages by culturing with RPMI1640-10% FCS containing 100 ng/ml rhM-CSF (a gift from Morinaga Milk Industry, Kanagawa, Japan). After 3 days, the culture mediums were replaced with fresh complete media and continually cultured for another 2 days before using. For the viral infection step, the macrophages were inoculated with 250 µl of the diluted JRFL HIV-1 viruses (prepared from 293 cells as described in the viral preparation step) for 2 hr at 37°C in the presence or absence of 2c or AZT as indicated in the result. After inoculation, the cells were washed twice with PBS to remove the unbound viruses and cultured with RPMI1640-10% FCS containing 100 ng/ml rhM-CSF, at the same conditions of 2c or AZT. Every 3 days, haft volume of the culture medium containing viruses were collected and replaced with the same volume of fresh medium. The collected culture mediums were clarified by brief centrifugation before stored at -80°C until analysis. The viral replication at day 6, 9, and 12 post-infection were then monitored by measuring the concentration of p24 Gag proteins using the RETROtek p24 Antigen ELISA kit.

Viral replication assay in the U87MG cells expressing CD4 and CCR5 (NIH AIDS Research & Reference Program) was examined by seeding the cells (maintained in DME medium supplemented with 15% FCS) at a density 2.5×10^4 cells onto 24-well tissue culture plate. The cells were then inoculated with JRFL viruses (prepared from 293 cells as described in the viral preparation step) for overnight at 37°C in the presence or absence of 2c as indicated in the result. After inoculation, the cells were washed twice with PBS to remove the unbound viruses and continually cultured with DME medium-15% FCS at the same conditions of 2c. The cell morphologies were observed after 5 day post-infection by microscope under the magnification of 10× and 20× (Nikon Eclipe TS100).

7.6. GST pull-down assay

To produce the GST-fusion proteins, the control GST or GST-Nef fusion proteins cloned in the pGEX-6P-1 vector were transformed into *E. coli* BL21 cells (GE Healthcare), and grown in LB medium containing 50 μg/ml ampicillin overnight at 37°C. The bacterial cells were then induced with 1 μM IPTG (Sigma) and cultured for 3 hr to produce the GST-fusion proteins. The cell pellets were harvested and lysed with BugBuster Protein Extraction Reagent containing 1 unit/ml rLysozyme and 25 unit/ml Benzonase Nuclease (all from Novagen). The cell lysates were then clarified by high speed centrifugation at 16,000 ×g, 4°C for 20 min, and their supernatants were collected and purified with the GST-Bind Resin (Novagen). After extensive washing with the GST Bind/Wash Buffer (Novagen), the resin was rotating incubated overnight at 4°C with the total cell lysate of the Hck-transfected 293 cells. For the competitive pull-down assay, three conditions for the incubation were designed as followed; (1) pre-incubation of the GST-SF2 Nef resin with the Hck lysates for 3 hr before adding 2c (40, 100, or 200 μM), (2) co-incubation of the GST-SF2 Nef resin, Hck lysates, and 2c together at the starting time of incubation, (3) pre-incubation of the GST-SF2 Nef resin with 2c for 4 hr, removing the unbound 2c by washing before incubation with the Hck lysates. The Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris, and 150 mM NaCl) containing protease inhibitors (1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) was used for washing or maintaining the mixture throughout the incubation. After incubation, the resin was extensively washed with the lysis buffer (as mentioned before), boiled with the same volume of SDS-PAGE sample buffer, and analyzed for the presence of Hck by Western blotting with anti-Hck antibody (clone 18; Transduction Laboratories).

7.7. 2c-Nef docking model

The molecular docking model of 2c and Nef was generated by collaboration with Dr. H. Sato (National Institute of Infectious Diseases, Tokyo). The 2c-Nef complex formation was predicted by homology modeling and docking simulation using the Molecular Operating Environment version 2007.09. (Chemical Computing Group, Canada). In detail, the model structure of HIV-1 Nef SF2 was constructed by homology modeling (Marti-Renom et al. 2000; Baker and Sali 2001; Shirakawa et al. 2008) using Nef NMR structure (PDB code: 2NEF) (Grzesiek et al. 1997) as template. The energy calculations were performed with the AMBER ff99 force field (Wang et al. 2000) and the GB/VI implicit solvent energy function (Labute 2008) during the modeling. The ASEDock module (Goto et al. 2008) was then used to achieve the docking simulation of 2c with the homology model of Nef. The

initial structure of 2c was generated by the Molecular Builder module and the binding site of 2c was then searched with the Site-Finder module. The energy calculations were performed with the MMFF94x force field (Halgren 1999; Halgren 1999) and the GB/VI implicit solvent energy function (Labute 2008) during the simulation. During the docking simulation, movement of the main chain atoms around 4.5 angstrom (A°) of the ligand binding site in Nef was restrained with a harmonic potential of 100 kcal/mol/$A°^{2}$, while the atoms in compound 2c were not constrained. The structure with the lowest score was selected as the model for this study

8. Results

8.1. Inhibitory effect of 2c on Nef-enhanced viral infectivity

We firstly confirmed and determined the enhancement effect of Nef on viral infectivity in our assay system using the standard single-round infection assay with TZM-bl cells. The viral infectivity is measured by the activity of β-galactosidase, which is conjugated with the viral LTR promoter and inducible by the infected viral Tat protein. By producing viruses in the producer 293 cells and infecting into the target TZM-bl cells, the NL43 wild-type (NL43-WT) viruses expressing Nef showed 3 to 4-fold higher infectivity than the Nef-defective (NL43-ΔNef) viruses (Figure 3). These results confirmed previous reports about the ability of Nef to increase viral infectivity (Kimpton and Emerman 1992; Aiken and Trono 1995; Miller et al. 1995; Welker et al. 1996).

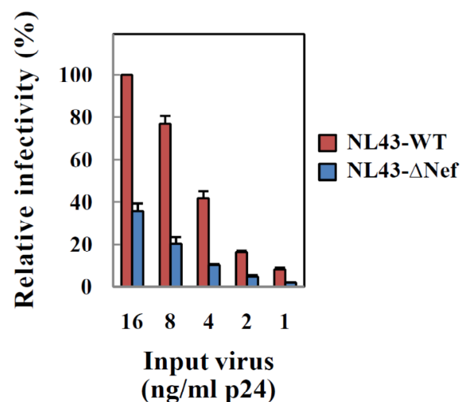


Figure 3. Nef enhances the viral infectivity. NL43 wile-type (NL43-WT) and Nef-defective (NL43-ΔNef) viruses were prepared by transfection of proviral plasmids into the producer 293 cells. The supernatants containing viruses were then quantified by p24 ELISA and inoculated into the target TZM-bl cells at the indicated p24 concentrations. After 48 hr post-infection, the viral infectivity was assayed by measuring the activity of β-galactosidase (conjugated with LTR thus inducible by the infected viral Tat protein). The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the relative viral infectivity.

We nest examined the inhibitory effect of 2c compound (Figure 2C) on viral infectivity by producing viruses in the presence of 2c and determining the effect of these viruses on the viral infectivity. From this experiment, 2c showed significantly inhibited viral infectivity of the NL43-WT viruses in the dose-dependent manner (Figure 4A). Both high (4 ng/ml) and low (2 ng/ml) viral inputs or inoculations were inhibited by 2c. Importantly, such inhibitory effect was not observed in the NL43-

ΔNef viruses suggesting that the inhibitory effect of 2c is dependent on Nef (Figure 4A). For the NL43-WT viruses, the inhibitory effect of 2c was significantly detected at the minimal concentration of 25 μM while at the concentration of 50 μM, 2c inhibited 50% of the viral infectivity (Figure 4A). This inhibitory effect was not due to inhibition of virus production, since total viruses (determined by p24 Gag protein levels) produced in the presence or absence of 2c was not different (Figure 4B). In addition, the intracellular expressions of viral proteins at least Gag and Nef (in the NL43-WT viruses) were not affected by 2c (Figure 4C). Therefore, it was clear that 2c did not affect step of viral production and release.

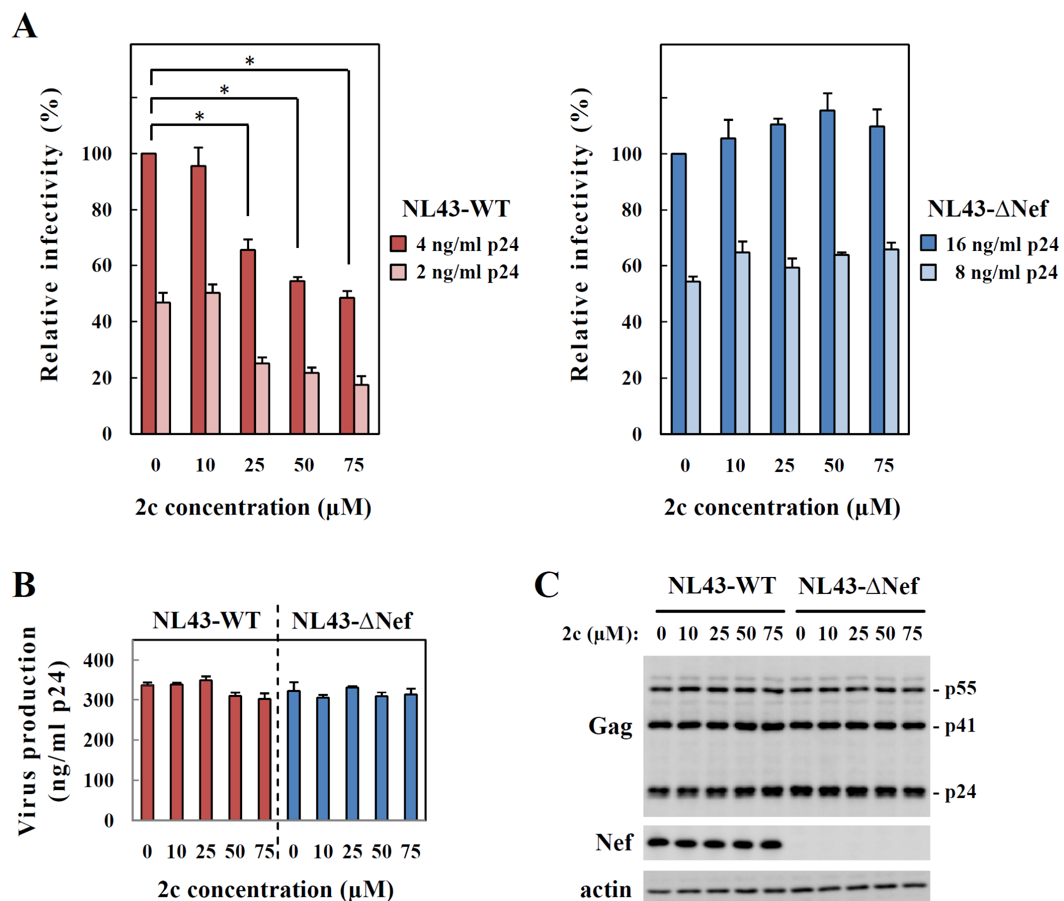


Figure 4. 2c inhibit viral infectivity of the NL43 wild-type viruses. *(A)* Inhibitory effect of 2c on the infectivity of NL43 wile-type (NL43-WT) and Nef-defective (NL43-ΔNef) viruses. The viruses were produced by transfection of the proviral plasmids into the producer 293 cells in the presence or absence of the indicated concentrations of 2c. The supernatants containing viruses were then quantified by p24 ELISA and inoculated into the target TZM-bl cells at the indicated p24 concentrations. The higher p24 concentrations of the NL43-ΔNef viruses were used to obtain the comparable infectivity level with the NL43-WT viruses. The viral infectivity was assayed after 48 hr post-infection. *(B)* Effect of 2c on the viral production. The p24 Gag protein concentrations of NL43-WT and NL43-ΔNef viruses produced in the presence or absence of indicated concentrations of 2c were quantified by p24 ELISA and compared. *(C)* The intracellular expression levels of

viral Gag (p55, p41, and p24) and Nef in the virus producer 293 cells. The total cell lysates were prepared from the virus producer cells for Western blot analysis using anti-Gag and anti-Nef antibodies by using anti-actin as the internal control. The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the relative infectivity or the absolute p24 concentration (ng/ml). The statistically differences at the *p*-value < 0.05 are indicated (*).

In the experiment in Figure 4, we used the supernatants of producer cells as a viral stock. Since the supernatants contained 2c (< 5 μM), we next tested whether 2c had a direct effect in the target TZM-bl cells. To this end, 2c was directly added to the target TZM-bl cells together with the viruses produced in the absence of 2c. As shown in Figure 5A, no inhibitory effects of 2c were observed in these conditions even at the 25 μM concentration of 2c. In contrast, even delayed addition of 2c into the virus producer 293 cells after 24 hr of the proviral plasmid transfections, the inhibitory effect of 2c on the viral infectivity in the target cells were still observed (Figure 5B). These results indicated that the inhibitory effect of 2c specifically occurred in the virus producer cells during the virus production processes.

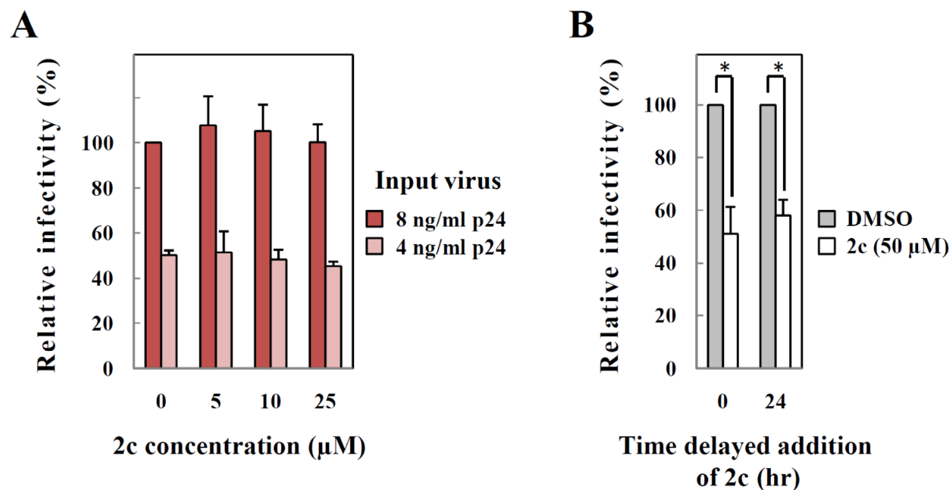


Figure 5. The inhibitory effect of 2c occurs in the virus producer cells. *(A)* Inhibitory effect of 2c when direct adding into the target TZM-bl cells instead of into the virus producer cells (293 cells). The NL43 wild-type (NL43-WT) viruses were produced in the absence of 2c and then inoculated (4 and 8 ng/ml) into the TZM-bl cells together with the addition of 2c at the indicated concentrations. The viral infectivity was assayed after 48 hr post-infection. *(B)* Inhibitory effect of 2c when delayed adding into the virus producer 293 cells. The compound 2c (50 μM) was added immediately or 24 hr after the transfection of proviral NL43-WT plasmid into 293 cells. The produced viruses were then equally inoculated (4 ng/ml) into the target TZM-bl cells and assayed for viral infectivity. The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the relative viral infectivity and the statistically differences at the *p*-value < 0.05 are indicated (*).

Nef has been shown to enhance the viral infectivity. Of interest, Nef was dispensable when VSV-G pseudotyped HIV-1 viruses were used (Aiken 1997; Chazal et al. 2001). Indeed, when added to the 293 cells producing VSV-G pseudotyped viruses, 2c failed to inhibit the infectivity of these viruses even used at the high concentration of 75 µM (Figure 6). This result also supports the specific effect of 2c on Nef.

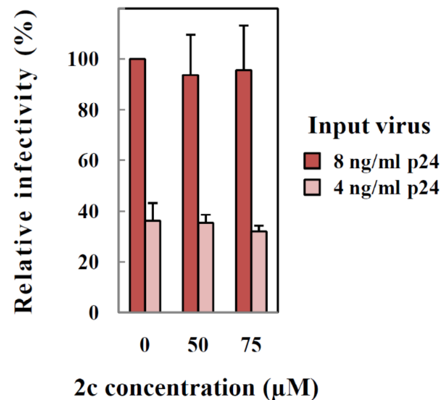


Figure 6. No inhibitory effect of 2c on the VSV-G pseudotyped viruses. The NL43 VSV-G pseudotyped (NL43-VSV-G) viruses were prepared by co-transfection of the Env-defective NL43 mutant and VSV-G expression plasmids into the producer 293 cells in the presence or absence of the indicated 2c concentrations. The produced viruses were then inoculated (4 and 8 ng/ml) into the target TZM-bl cells. The viral infectivity was assayed after 48 hr post-infection. The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the relative viral infectivity.

8.2. Inhibitory effect of 2c on Nef-promoted viral replication

According to the ability of 2c to inhibit viral infectivity of which referred to the single-round virus infection (Kimpton and Emerman 1992; Miller et al. 1995), we expected that 2c might also inhibit the multiple-rounds of virus infections or as well-known in the term of "viral replication". In addition, to confirm that the inhibitory effect of 2c was occurred by specific-targeting at the Nef protein, the host cells of which viral replication is independent of Nef protein, which is Jurkat cells (Emert-Sedlak et al. 2009) and dependent on Nef protein which are the primary macrophages and U87MG cells (Miller et al. 1994; Emert-Sedlak et al. 2009) were used. The viral replication levels of NL43-WT and NL43-ΔNef viruses were no different (Figure 7, in the absence of 2c and AZT) of which consistency with the previous reported that viral replication in this cell lines is independent on Nef (Emert-Sedlak et al. 2009). As expected, 2c did not affect viral replication in the Jurkat cells (Figure 7).

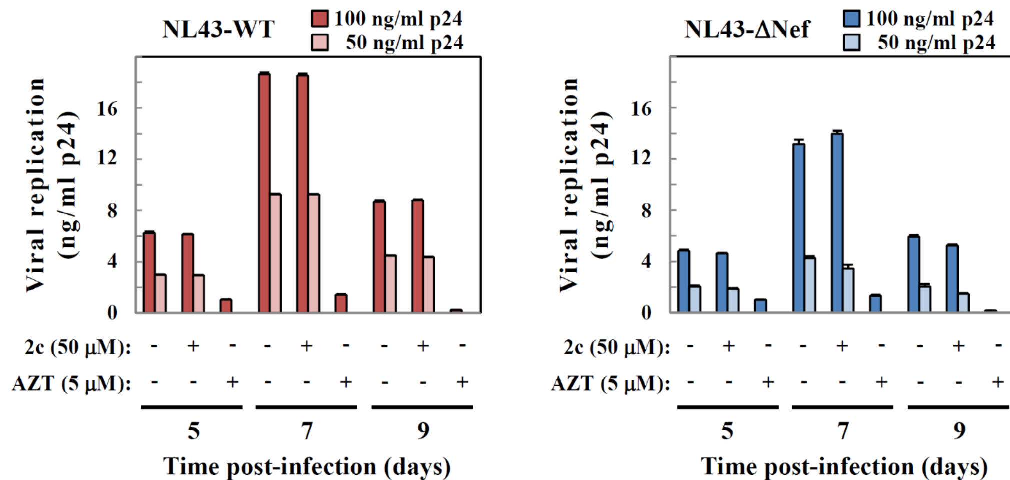
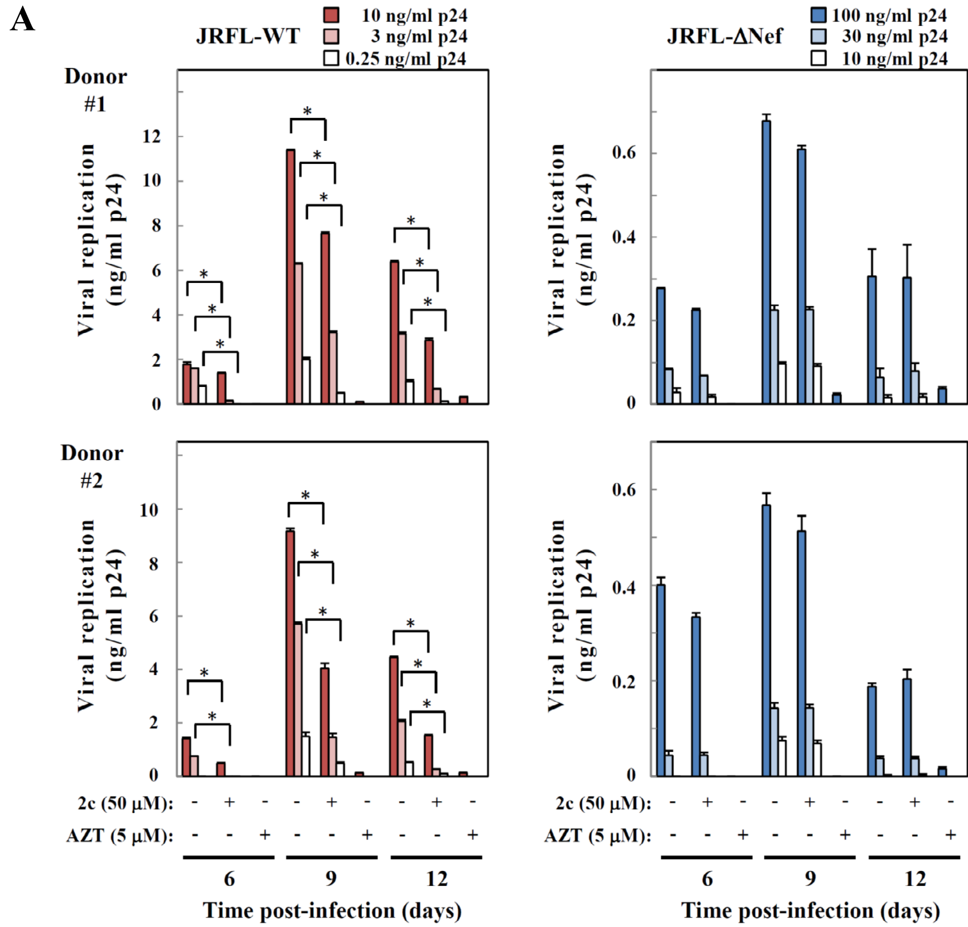


Figure 7. 2c did not inhibit viral replication in the Jurkat cells. The NL43 wild-type (NL43-WT) and Nef-defective (NL43-ΔNef) viruses were prepared in the absence of 2c from the producer 293 cells by transfections of the proviral plasmids. The supernatants containing viruses were quantified by p24 ELISA and inoculated into the Jurkat cells as the indicated p24 concentrations for 2 hr, in the presence or absence of 2c (50 µM) by using AZT (5 µM) as the positive control inhibitor. After 2 hr of inoculation, the Jurkat cells were washed up with the complete mediums to remove the unbound/uninfected viruses. The cells were then continually cultured in the presence or absence of 2c (50 µM) or AZT (5 µM) for 9 days. The culture mediums were collected (4/5 volume) (for the replication assay by p24 ELISA) and replaced with the same volume of the fresh completed mediums at the day 5, 7, and 9 post-infection. The data are shown as the mean value ± SD of the absolute p24 concentrations (ng/ml) and the statistically differences at the *p*-value < 0.05 are indicated (*).

In contrast, when using the primary macrophages, 2c showed significant inhibitory effect on viral replication of the JRFL wild-type (JRFL-WT) viruses throughout the course of replication assay (Figure 8A). Viral replication assay of the JRFL Nef-defective (JRFL-ΔNef) viruses was performed by using higher virus inoculation to increase the level of viral replication into the detectable level (by p24 Gag ELISA) and comparable with that of the wile-type viruses. In this assay, 2c did not show inhibitory effect against the replication of Nef-defective viruses (Figure 8A). The inhibitory effect of 2c on another Nef-dependent viral replication cells, U87MG (expressing CD4 and CCR5) was further determined. The JRFL-WT viruses expressing Nef efficiently replicated in this cells representing by the severe formation of the syncytia or giant cells in contrast with the JRFL-ΔNef viruses (Figure 8B). 2c showed inhibitory effect on the replication of wild-type viruses which was indicated by the reduction of syncytia formation (Figure 8B). In addition, cytotoxicity of 2c was not observed even at the highest concentration, 100 µM (Figure 8B). Altogether demonstrated that 2c inhibited viral replication in the Nef-dependent manner.


A
JRFL-WT
10 ng/ml p24
3 ng/ml p24
0.25 ng/ml p24
JRFL-ΔNef
100 ng/ml p24
30 ng/ml p24
10 ng/ml p24
Donor #1
Donor #2
Viral replication (ng/ml p24)
2c (50 μM):
AZT (5 μM):
Time post-infection (days)

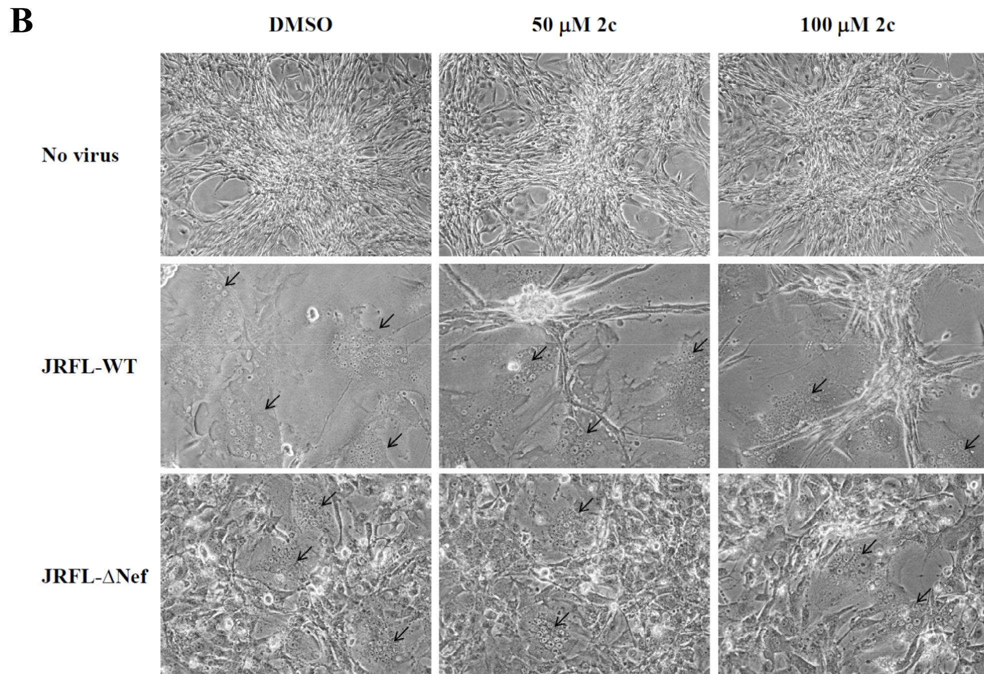

B
DMSO
50 μM 2c
100 μM 2c
No virus
JRFL-WT
JRFL-ΔNef

Figure 8. 2c inhibits viral replication in the primary macrophages and U87MG cells. *(A)* Inhibitory effect of 2c on the JRFL wild-type (JRFL-WT) and Nef-defective (JRFL-ΔNef) virus replications in the primary macrophages (monocyte-derived macrophages). The viruses were prepared in the absence of 2c from the producer cells (293 cells) by transfections of the proviral plasmids. The supernatants containing viruses were quantified by p24 ELISA and inoculated into the macrophages as the indicated p24 concentrations for 2 hr, in the presence or absence of 2c (50 µM) by using AZT (5 µM) as the positive control inhibitor. The higher p24 concentrations of the JRFL-ΔNef viruses were used to obtain the comparable infectivity level with the JRFL-WT viruses. After 2 hr of inoculation, the macrophages were washed up with the complete mediums to remove the unbound/uninfected viruses. The infected macrophages were then continually cultured in the presence or absence of 2c (50 µM) or AZT (5 µM) for 12 days. Half volumes of the culture mediums were collected (for the replication assay by p24 ELISA) and replaced with the same volume of the fresh completed mediums at the day 6, 9, and 12 post-infection. The data are shown as the mean value ± SD of the absolute p24 concentrations (ng/ml) and the statistically differences at the *p*-value < 0.05 are indicated (*). *(B)* Inhibitory effect of 2c on the JRFL-WT and JRFL-ΔNef virus replications in the U87MG cells (expressing CD4 and CCR5). The viruses were prepared in the absence of 2c and inoculated into U87MG cells overnight. The cells were then continually cultured in the presence of 2c (50 µM or 100 µM) for 5 days. The non-infected cells were also cultured to examine the cytotoxicity effect of 2c. The morphologies of non-infected and infected cells were observed under microscope at the 10× or 20× of magnification, respectively. The arrows indicated syncytia or giant cell formations resulting from the virus infection.

8.3. 2c directly targets Nef in the similar interaction as the SH3 domain of Hck

To better understanding of the inhibitory effect of 2c on Nef-mediated enhancement of viral infectivity and replication, we generated the molecular docking model of 2c and Nef. As shown in Figure 7, the predicted amino acid binding sites of 2c on the Nef surface were R77, K82, A83, D86, I87, F90, Q118, and Y120 (the amino acid positions were referenced from the NL43 strain virus). According to the model, 2c seemed to bind at the hydrophobic pocket of Nef including the PxxP motif of which was reported to be required for the infectivity enhancement (Goldsmith et al. 1995; Saksela et al. 1995; Wiskerchen and Cheng-Mayer 1996).

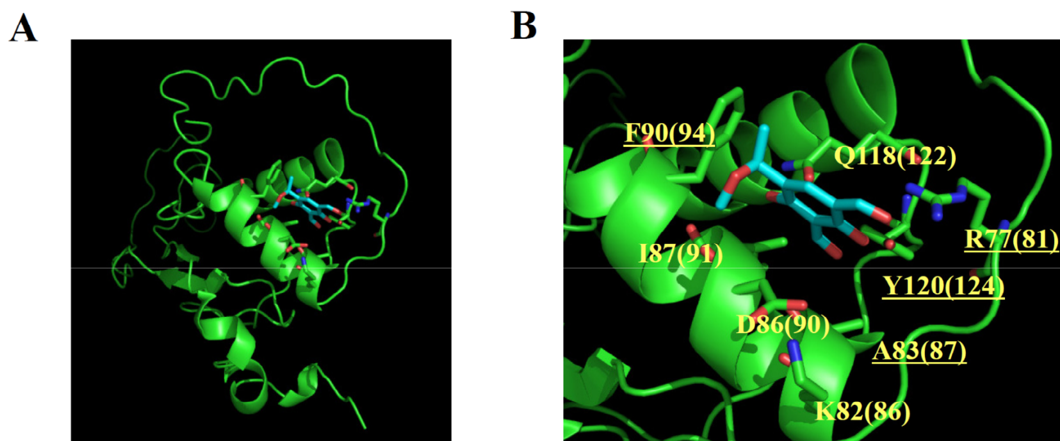


Figure 9. Molecular docking model of the 2c and Nef. *(A)* 2c (blue color) showed direct targeting around the hydrophobic groove including the PxxP motif of Nef molecule. *(B)* The predicted amino acids binding sites of 2c on Nef molecule. The amino acid positions of Nef are referenced from the NL43 strain (the comparable amino acid positions of the JRFL strain also showed in the bracket). The underline amino acid positions represent the overlapping binding positions of 2c and Hck on Nef molecule (Choi and Smithgall 2004).

To verify those binding sites of the 2c on Nef, the single-point mutant Nef viruses at the predicted amino acid positions including R77A, K82A, D86A, F90, and G119L were used. These Nef mutant viruses were produced in the presence of 2c (50 µM) and inoculated into TZM-bl for viral infectivity assay. The results showed that these Nef mutant viruses failed to abolish the inhibitory effect of 2c (Figure 10A). These data indicated that there was no critical amino acid residue for the 2c binding on Nef molecule. Interestingly, the inhibitory effect of 2c was also observed in the G119L mutant viruses (Figure 10A and 10B), of which was reported as the partial-defective in the viral infectivity (Jere et al. 2010).

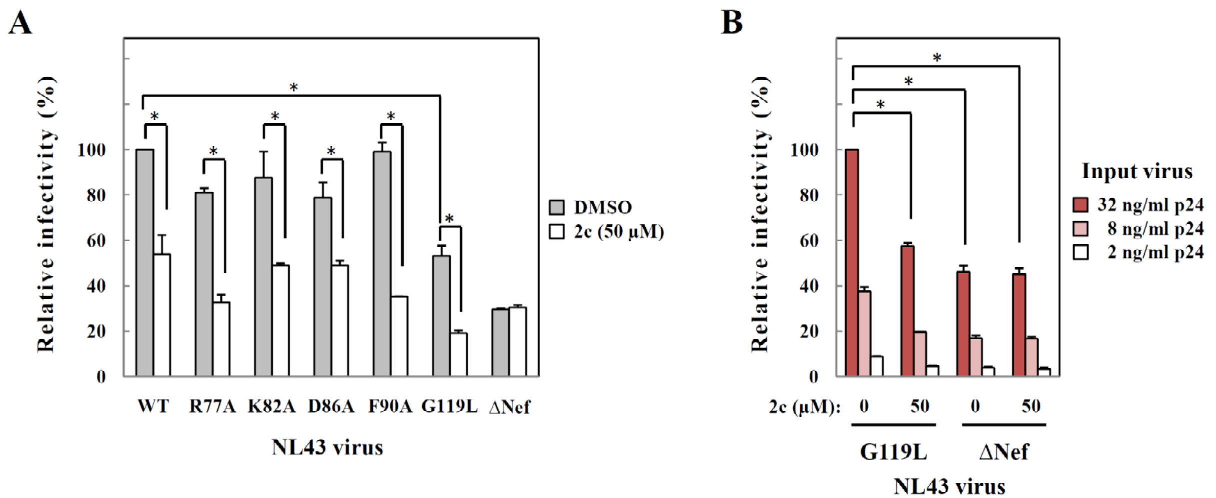


Figure 10. No inhibitory effect of 2c on the single-point Nef mutant viruses. *(A)* Inhibitory effect of 2c on the infectivity of NL43 wild-type (WT), Nef mutants (R77A, K82A, D86A, F90A, and G119L), and Nef-defective (ΔNef) viruses. The viruses were produced in the presence or absence of 2c at the concentration of 50 µM in the producer 293 cells. The supernatants containing viruses were then quantified by p24 ELISA and equally inoculated (10 ng/ml) into the target TZM-bl cells. The viral infectivity was assayed after 48 hr post-infection. *(B)* The different p24 concentrations (as indicated) of the G119L and ΔNef viruses from *(A)* were inoculated into the TZM-bl cells and assayed for the viral infectivity. The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the relative infectivity and the statistically differences at the p-value < 0.05 are indicated (*).

Since the predicted amino acid R77 (see Figure 9) is localized in the PxxP motif of Nef (P69-P78, refers from the NL43 strain virus) of which was reported to be involved with the viral infectivity enhancement (Goldsmith et al. 1995; Saksela et al. 1995; Wiskerchen and Cheng-Mayer 1996), the Nef PxxP motif-disrupted (AxxA) virus was used. In this experiment, the JRFL strain virus expressing SF2 Nef (JRFL SF2-Nef-WT) was used instead of the NL43 strain to represent the broadly inhibitory effect of 2c. Similarly to the previous results, 2c did not affect the viral production step indicated by the p24 concentration of the produced viruses in the supernatants (Figure 11A) and the intracellular expression levels of viral proteins including Gag, Nef (for the JRFL SF2-Nef-WT and the Nef PxxP motif-disrupted or JRFL SF2-Nef-AxxA viruses), and Vif in the virus producer cells (Figure 11B). In the absence of 2c, the viral infectivity of JRFL SF2-Nef-AxxA virus was ~5 fold lower than that of the JRFL SF2-Nef-WT virus while extensively lower (~100 fold) for the JRFL-ΔNef viruses (Figure 11C). As expected, the JRFL SF2-Nef-AxxA virus was able to abolish the inhibitory effect of 2c (Figure 10D). However, when compared to the inhibitory effect of 2c on the JRFL SF2-Nef-WT virus, the JRFL SF2-Nef-AxxA virus partially abolish the effect of 2c (Figure 11D). These results suggested that 2c targeted Nef at least through the interaction with the PxxP motif.

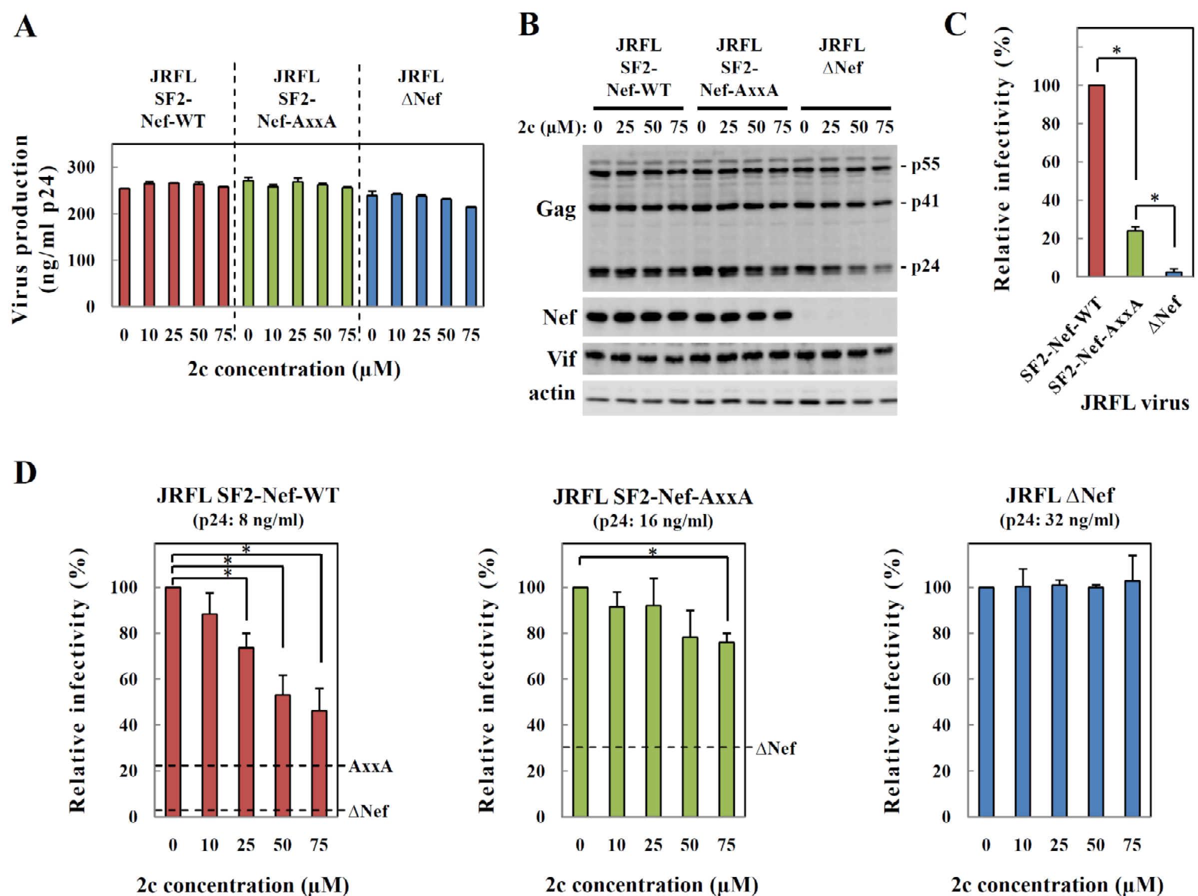


Figure 11. 2c inhibits viral infectivity of the Nef PxxP motif-disrupted viruses. *(A)* Effect of 2c on the viral productions of JRFL SF2-Nef wild-type (JRFL SF2-Nef-WT), Nef PxxP motif-disrupted (JRFL SF2-Nef-AxxA), and Nef-defective (JRFL-ΔNef) viruses. The viruses were produced by transfection of the proviral plasmids into the producer 293 cells in the presence or absence of the indicated concentrations of 2c. The supernatants containing viruses were then quantified by p24 ELISA and compared. *(B)* The intracellular expression levels of viral Gag (p55, p41, and p24), Nef, and Vif in the virus producer cells (293 cells). The total cell lysates were prepared from the virus producer cells for Western blot analysis using anti-Gag, anti-Nef, and anti-Vif antibodies by using anti-actin as the internal control. *(C)* Viral infectivity of JRFL SF2-Nef-WT, JRFL SF2-Nef-AxxA, and JRFL-ΔNef viruses. The viruses produced in the absence of 2c from *(A)* were equally inoculated (8 ng/ml) into the target TZM-bl cells. After 48 hr post-infection, the viral infectivity was. *(D)* Inhibitory effect of 2c on the viral infectivity of JRFL SF2-Nef-WT, JRFL SF2-Nef-AxxA, and JRFL-ΔNef viruses. The viruses produced from *(A)* were inoculated into TZM-bl cells as the indicated p24 concentrations and assayed for viral infectivity. The higher p24 concentrations of the JRFL SF2-Nef-AxxA and JRFL-ΔNef were used to obtain the comparable infectivity levels with the JRFL SF2-Nef-WT viruses. The infectivity levels of JRFL SF2-Nef-AxxA and JRFL-ΔNef viruses without 2c were also indicated. The assays were performed in triplicate and repeated from the two independent experiments. The data are shown as the mean value ± SD of the absolute p24 concentration (ng/ml) or the relative infectivity. The statistically differences at the p-value < 0.05 are indicated (*).

We next investigated how 2c targets Nef to inhibit its function of the viral infectivity enhancement. According to the previous finding that the viral infectivity was also inhibited by the SH3 domain of Hck protein (a Src family kinase expressed in phagocytes (Guiet et al. 2008)) (Tokunaga et al. 1998; Breuer et al. 2011; Kuo et al. 2012). Together with the evidences that Nef binds to Hck with the high affinity through the interaction of Nef PxxP motif and Hck SH3 domain (Lee et al. 1995; Saksela et al. 1995; Moarefi et al. 1997) and our result showing that the PxxP motif was involved with the binding of 2c to inhibit viral infectivity (Figure 9 and 11), we hypothesized that 2c might use the same mechanism the SH3 domain of Hck to target Nef and inhibit viral infectivity enhancement. To clarify this hypothesis, we firstly confirmed the inhibitory effect of the SH3 domain of Hck on viral infectivity by producing the viruses from co-expression of the Hck plasmid. Since the wild-type Hck is maintained in the inactive form by the intra-molecular inhibitory interactions occurred from the binding of the SH2 linker and the SH3 domain and from the binding of the phosphorylated tyrosine at the C-terminus with the SH2 domain (Figure 12A) (Moarefi et al. 1997; Lerner and Smithgall 2002; Guiet et al. 2008), the wild-type Hck might not effectively inhibit viral infectivity. Therefore, the Hck mutant lacking of the kinase domain (HckN) of which its SH2 domain is free from the intra-molecular inhibitory interactions was used. In addition, the SH3 domain mutant Hck N (HckN-W39F) and SH2 domain mutant Hck N (HckN-R151S) were also included (Figure 12A). As expected, the viral

infectivity was reduced by the HckN and HckN-R151S but not by the SH3 domain mutant HckN-W39F (Figure 12B).

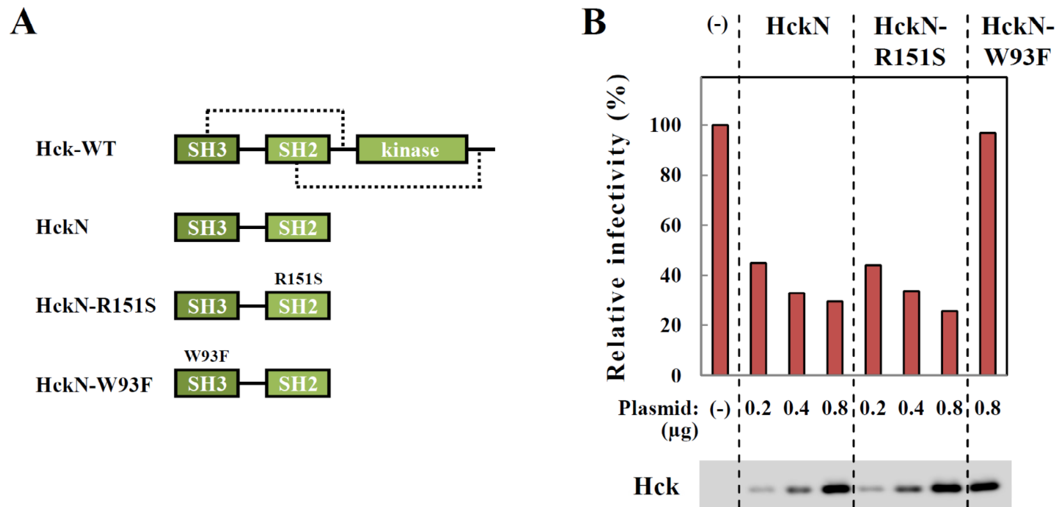


Figure 12. The SH3 domain of Hck inhibits viral infectivity. *(A)* Schematics of the Hck wild-type (Hck-WT), kinase domain deleted (HckN), SH2 domain mutant (HckN-R151S), and SH3 domain mutant (HckN-W93F). The intra-molecular inhibitory interactions of Hck-WT are indicated. *(B)* Inhibitory effect of SH3 domain of Hck on the viral infectivity of NL43 wild-type (NL43-WT) viruses. The viruses were produced by the co-transfection of proviral plasmid and HckN, or HckN-R151S, or HckN-W93F by varying amount of the Hck plasmids as indicated. The supernatants containing viruses were then quantified by p24 ELISA and equally inoculated (8 ng/ml) into the target TZM-bl cells. The viral infectivity was assayed after 48 hr post-infection. The intracellular expression levels of Hck in the virus producer 293 cells were analyzed by Western blotting of the total cell lysates with anti-Hck antibody.

To prove the direct binding of 2c with Nef of which expected to be similar to the direct binding of SH3 domain of Hck with PxxP motif of Nef, the GST pull-down assay was used. The Nef protein was conjugated with GST and bound with the GST binding resin. In addition to the NL43 strain Nef wild-type (NL43 Nef-WT), the SF2 strain Nef wild-type (SF2 Nef-WT) of which stronger binds with Hck was used (Figure 13A). The SF2 substituted NL43 mutant Nef (NL43 Nef-TR) by the point-mutation at T71 of NL43 Nef and the NL43 Nef-TR PxxP-disrupted mutant (NL43 Nef-AxxA) were also included (Figure 13A). We firstly confirmed the direct binding of Nef PxxP motif with the SH3 domain of Hck by incubating the GST-Nef resins with the total cell lysates from the transfection of Hck-WT, HckN, HckN-R151S, or HckN-W39F. After the pull-down assay and Western blotting, all of the GST-Nef resins were not able to bind with the SH3 domain mutant HckN-W39F while the GST-

Nef-AxxA failed to bind with the Hck proteins (Figure 13B). The SF2 Nef-WT showed stronger interaction with the Hck thus was used for the later assays (Figure 13B).

We further clarified the direct binding of 2c with Nef by designing the GST pull-down assay into three conditions consist of: *(1)* pre-incubation of the GST-SF2 Nef and Hck lysate (wild-type) before addition of 2c, *(2)* co-incubation of the GST-SF2 Nef, Hck lysate, and 2c at the beginning time of incubation, and *(3)* pre-incubation of the GST-SF2 Nef and 2c before allowing binding with the Hck lysate (Figure 13C). Pre-incubation of 2c with Nef effectively blocked Hck to bind with Nef (condition 3, Figure 13C, right panel), while in the co-incubation condition the competitive binding between 2c and Hck were occurred (condition 2, Figure 13C, middle panel). In contrast, 2c failed block Hck after allowing Hck-Nef binding (condition 1, Figure 13C, left panel). These results demonstrated that 2c directly bound with Nef in the similar manner as the Hck.

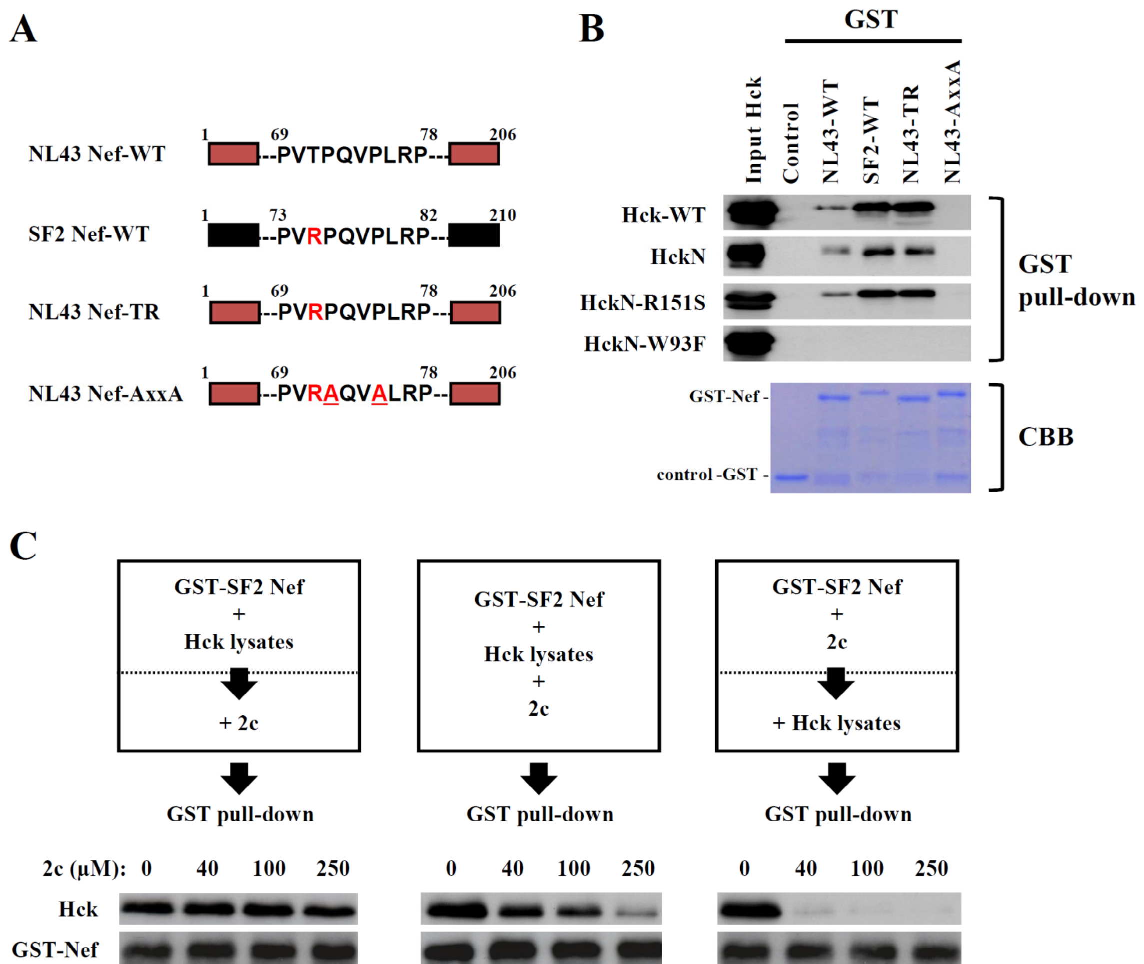


Figure 13. 2c targets Nef at the similar region as the targeting by SH3 domain of Hck. *(A)* Schematics of the GST-Nef fusions consist of: NL43-strain wild-type Nef (NL43 Nef-WT), SF2-strain wild-type Nef (SF2 Nef-WT), SF2-substituted NL43 mutant Nef (NL43 Nef-TR, by the point-mutation at T71), and NL43 Nef-TR PxxP-disrupted mutant (NL43 Nef-AxxA, by the point-mutations at P72 and P75). The GST-Nef fusion proteins were prepared by expressions in bacterial cells (*E. coli*) and purified with the GST binding resins. *(B)* GST pull-down assay of the GST-Nef resins from *(A)* with the total cell lysates from the transfections of Hck wild-type (Hck-WT), kinase domain deleted (HckN), SH2 domain mutant (HckN-R151S), or SH3 domain mutant (HckN-W93F). The GST-Nef resins were incubated with the Hck lysates for 12 hr. After that the resins were extensively washed up for the unbound Hck and subjected to the Western blot analysis using anti-Hck antibody. The amount of initial GST-Nef fusion proteins used, were quantified by the Coomassie brilliant blue (CBB) staining. *(C)* Pre-incubation GST pull-down assay. Three incubation conditions for the GST pull-down assay were designed as follows: *(left panel)* the GST-Nef resins were pre-incubated with the Hck lysates for 3 hr before adding 2c at the indicated concentrations, *(middle panel)* the GST-Nef resins, Hck lysates, and 2c were co-incubated together at the beginning time of incubation, *(right panel)* the GST-Nef resins were firstly pre-incubated with 2c at the indicated concentrations for 4 hr, the resins were then extensively washed up to remove the unbound 2c before adding of the Hck lysates. The mixtures were incubated for 12 hr and the unbound Hck were extensively washed up before detection of the pulled-down Hck by Western blotting using anti-Hck antibody and anti-GST (as the internal control).

In addition, we further specified that the interaction of 2c with Nef was occurred at the PxxP motif of Nef by using the short peptide of Nef containing only the PxxP motif (SF2-PxxP) (Figure 14A). The ability of Nef SF2-PxxP to bind with Hck was compared with the full-length Nef SF2-WT by GST pull-down assay. The Nef SF2-PxxP showed weaker interaction with the SH3 domain of Hck (Figure 14B) of which might be occurred from the lacking of another SH3 binding domain or unable to from the appropriated tertiary structure for the SH3 binding. However, the binding of Nef SF2-PxxP with Hck was detectable and was used for the co-incubation (condition 2, as explained before) and pre-incubation (condition 3, as explained before) assays (Figure 14C). Same as the previous results (Figure 13C), pre-incubation of 2c with the PxxP motif (Nef SF2-PxxP) totally blocked Hck to bind with Nef (Figure 14C, right panel), while 2c partially blocked Hck in the co-incubation condition (Figure 14C, right panel). These results confirmed that 2c bound Nef through the PxxP motif of which similar to the binding of Hck.

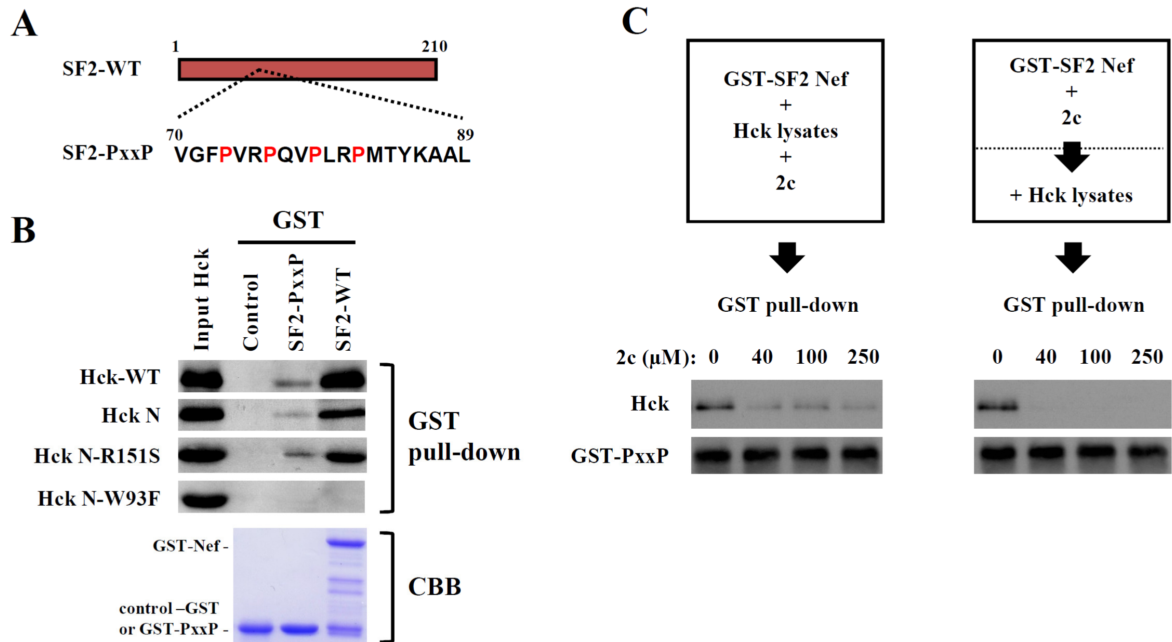


Figure 14. 2c directly targets Nef at the PxxP motif. *(A)* Schematics of the GST fusion proteins of SF2 Nef wild-type (SF2-WT) and the short peptide (20 amino acids) containing PxxP motif (SF2-PxxP). The GST-Nef fusion proteins were prepared by expressions in bacterial cells (*E. coli*) and purified with the GST binding resins. *(B)* GST pull-down assay of the GST-Nef resins from *(A)* with the total cell lysates from the transfections of Hck wild-type (Hck-WT), kinase domain deleted (HckN), SH2 domain mutant (HckN-R151S), or SH3 domain mutant (HckN-W93F). The GST-Nef resins were incubated with the Hck lysates for 12 hr. After that the resins were extensively washed up for the unbound Hck and subjected to the Western blot analysis using anti-Hck antibody. The amount of initial GST-Nef fusion proteins used, were quantified by the Coomassie brilliant blue (CBB) staining. *(C)* Pre-incubation GST pull-down assay. Two incubation conditions for the GST pull-down assay were used as follows: *(left panel)* the GST-Nef resins, Hck lysates, and 2c at the indicated concentrations were co-incubated together at the beginning time of incubation, *(right panel)* the GST-Nef resins were firstly pre-incubated with 2c at the indicated concentrations for 4 hr, the resins were then extensively washed up to remove the unbound 2c before adding of the Hck lysates. The mixtures were incubated for 12 hr and the unbound Hck were extensively washed up before detection of the pulled-down Hck by Western blotting using anti-Hck antibody and anti-GST (as the internal control).

9. Discussion

The first small chemical compound, 2c that can inhibit Nef-mediated viral infectivity enhancement was successfully identified from this study. Although previously Bentizi group (2007) and Emert-Sedlak (2009) succeeded to identify the chemical inhibitors for Nef (D1, DLC27 and DFP-4AP, DFP-4APF, DFP-4AB respectively), their inhibitory effects were base on inhibition of the Hck activation by Nef. These inhibitors interfere the binding between Nef and Hck through disruption of the PxxP motif (of Nef) and SH3 domain (of Hck) interaction (Betzi et al. 2007; Emert-Sedlak et al. 2009). The Hck protein was well-known as the high affinity binding partner of Nef (Lee et al. 1995; Saksela et al. 1995; Moarefi et al. 1997). However, the physiological function of Hck activation by Nef is not well-understood. Only resulting in the maturation arrest of M-CSF receptor was clearly demonstrated occurring by the Nef-mediated Hck activated (Hiyoshi et al. 2008; Hassan et al. 2009; Hiyoshi et al. 2012). In contrast, many reports suggested that enhancement of viral infectivity by Nef might participate in the promotion of viral replication and disease progression (Kimpton and Emerman 1992; Chowers et al. 1994; Miller et al. 1994; Spina et al. 1994; Iafrate et al. 2000; Brenner et al. 2006). Therefore, the inhibitor that can inhibit Nef-mediated viral infectivity enhancement might have higher impact than that of the inhibitor for Nef-mediated Hck activation. In addition to inhibit Mef-activated Hck, the compound D1 and DLC27 also showed partial inhibitory effect on the Nef-downregulated MHC I but did not inhibit Nef-downregulated CD4 of which similar to our 2c compound (Betzi et al. 2007; Dikeakos et al. 2010). However, the author did not show or mention about the effect of D1 and DLC27 on viral infectivity enhancement by Nef (Betzi et al. 2007). If D1 and DLC27 can inhibit this function of Nef, by the comparative study with our 2c compound may be helpful for the identification of the Nef domain that responsible for this Nef function. In addition, this data may be useful for further development of the more potent inhibitor for Nef-enhanced viral infectivity. For the DFP-4AP and its analogs, these inhibitors exactly target at the Hck molecule (on the ATP binding sites) (Emert-Sedlak et al. 2009), thus may not show the inhibitory effect on viral infectivity enhancement by Nef or other Nef functions (MHC I and CD4 downregulations).

Together with the previous reports (Hassan et al. 2009; Dikeakos et al. 2010), our 2c compound showed broad range inhibitory effects to the three functions of Nef including viral infectivity enhancement, Hck activation, and MHC I downregulation. The inhibitory effect of Nef-mediated CD4 downregulation was also tested but 2c failed to inhibit this function of Nef (Dikeakos et al. 2010). Since the PxxP motif of Nef is required for those three functions of Nef (Goldsmith et al. 1995; Saksela

et al. 1995; Wiskerchen and Cheng-Mayer 1996; Hung et al. 2007; Hassan et al. 2009; Dikeakos et al. 2010) and this study demonstrated that the PxxP motif of Nef was one of the binding sites of 2c, therefore 2c is able to inhibit these three functions of Nef. However, the efficiency of inhibition by 2c among these three functions of Nef are different by strong effect on Nef-activated Hck, modest effect on Nef-enhanced infectivity, and partial effect on Nef-downregulated MHC I (Hassan et al. 2009; Dikeakos et al. 2010). Since the PxxP motif of Nef is mainly required for the Hck interaction (Lee et al. 1995; Moarefi et al. 1997) while other domains of Nef are involved for infectivity enhancement or MHC I downregualtion (acidic domain, E62-E65) (Hung et al. 2007)), thus the inhibition efficiency of 2c are different among these three functions of Nef.

We hypothesized that the inhibitory mechanism of 2c on Nef-enhanced viral infectivity may occur from the interruption of Nef with an unidentified cellular protein of which the interaction plays role in the production of high infectious viruses (Figure 15). This hypothesis is based on the experimental evidences that 2c could compete the SH3 domain containing protein (Hck) to bind with the PxxP motif of Nef (Figure 13 and 14). In addition, from the 2c-Nef docking model showed that 2c interacted with Nef at the positions, R77, K82, A83, D86, I87, F90, Q118, and Y120 (Figure 9) while it was well-demonstrated that Hck interacts with Nef by using its SH3 domain to bind with the P72, P75, R77, A83, F90, W113, H116, and Y120 of Nef (Choi and Smithgall 2004). These two evidences indicate the overlapping binding sites of 2c and Hck on Nef molecule which are the R77, A83, F90, and Y120. Therefore, 2c is able to compete the SH3 containing protein (Hck) to bind with Nef. This inhibitory mechanism is thus expected to be occurred in the virus producer cell by interfering the interaction of Nef with an unidentified cellular protein of which important for the production of high infectious viruses (Figure 15).

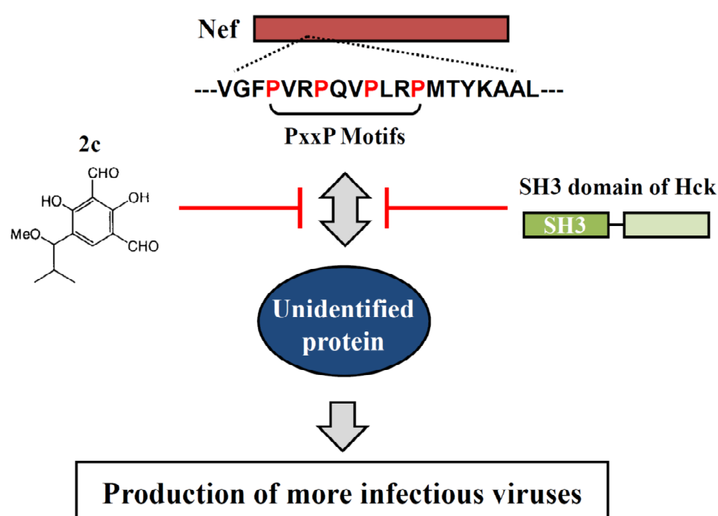


Figure 15. The proposed inhibitory mechanism of 2c on the Nef-mediated viral infectivity enhancement. The compound 2c uses the similar interaction as the SH3 domain of Hck to bind with Nef at least at the PxxP motif. These blockings may inhibit the interaction of Nef with an unidentified cellular protein of which playing role in the production of high infectious viruses. Therefore, 2c shows the inhibit effect on the viral infectivity enhancement by Nef.

The evidence from our preliminary experiments suggested that 2c did not inhibit Nef-enhanced viral infectivity through interruption of the Nef-dynamin 2 interaction of which was reported to downregulate the CD4-liked cellular molecule resulting in increasing of the viral Env function (Figure 1F) (Pizzato et al. 2007). In the immunoprecipitation experiments, 2c could not disrupt the binding between Nef and dynamin 2 (data not shown). In addition, 2c did not affect the intracellular localization of Nef (data not shown) of which was reported to associate with the viral infectivity enhancement (Welker et al. 1998). Another possible inhibitory mechanism by 2c is interfering of the lipid content of virions of which was reported as the important component for viral entry by fusion through the plasma membrane (Zheng et al. 2001; Campbell et al. 2002; Guyader et al. 2002; Zheng et al. 2003). In addition, our result also showed that no inhibitory effect of 2c on viral infectivity of the VSV-G pseudotyped viruses (Figure 6) of which was reported to enter the cell by pH-dependent endocytosis and independent of Nef on its infectivity (Aiken 1997; Chazal et al. 2001). Moreover, Nef was reported to induce multiple genes involved with the lipid biosynthesis (Figure 1B and 1C) (van 't Wout et al. 2005), especially the cholesterol, an essential component of lipid raft presented in the viral envelop (Zheng et al. 2001; Zheng et al. 2003), 2c may impair this function of Nef thus resulting in the production of low infectivity viruses. In addition, 2c may interfere the function of Nef on removal or relocalization of the cholesterol efflux protein, ABCA1 (Figure 1 D) (Cui et al. 2012), resulting in the insufficient intracellular cholesterol supplement for the production of infectious viruses. Alternatively, 2c may interfere the Nef-facilitated virion incorporation of the cellular adhesion molecule such as ICAM-1 (Figure 1 A) (Bounou et al. 2002; Beausejour and Tremblay 2004), therefore reduces the binding of virion to the target cells. By using 2c as a chemical probe, it is possible to prove whether 2c inhibit Nef-mediated viral infectivity through these proposed mechanisms or not. In addition, the positive result will be helpful to validate these proposed underlying molecular mechanisms.

Both *in vivo* studies in animal models and HIV-1infected patients have proved that Nef is a pathogenic factor for AIDS development (Kestler et al. 1991; Deacon et al. 1995; Kirchhoff et al. 1995; Hanna et al. 1998; Hanna et al. 1998; Salvi et al. 1998). However, the molecular functions of Nef on

the pathogenesis are diverse (as showed in the introduction part) and continually to be discovered. Therefore, identification of the most critical Nef function is helpful to reveal the real target for the efficient drug design and development. By using the HIV-1 infected animal model with 2c inhibitor treatment, at least we can distinguish whether Nef-enhanced viral infectivity including Nef-activated Hck and Nef-downregulated MHC I play the major role on the disease development. The potential of 2c on the infected patient viruses is evaluable by sub-cloning of the clinical isolated Nef gene into the JRFL or NL43 and examining for the inhibitory effect of 2c. The more effective inhibitor is identifiable by screening of the 2c-liked compound from the chemical compound libraries. 2c is the example of multifunctional inhibitor which can inhibit three functions of Nef including viral infectivity enhancement, Hck activation, and MHC I downregulation. This property is important for anti-HIV drug development to reduce the toxicities from multidrug treatment and their complications during the therapeutic course.

10. Conclusion

We succeeded to identify the first small chemical compound that can inhibit Nef-mediated viral infectivity enhancement. At the concentration of 50 μM, 2c inhibits 50% of the viral infectivity in single-round infection assay without any detectable effect on the viral production. The inhibitory effect of 2c was observed only in wild-type viruses but did not for the Nef-defective (ΔNef) viruses suggesting 2c targets Nef. In addition, 2c also reduced the viral replication in the Nef-promoted viral replication cells including primary macrophages and U87MG throughout the course of infection. The mechanism of 2c inhibition was then studied from the 2c-Nef molecular docking model. The model indicated R77, K82, A83, D86, I87, F90, and Q118 of Nef are interacted with 2c. However, single-point mutation of these amino acids in Nef molecular clones failed to abolish the inhibitory effect of 2c. In contrast, the Nef proline-rich (PxxP) motif mutant (AxxA) molecular clone partially rescued the inhibitory effect of 2c on virion infectivity. These results indicated that the PxxP motif, rather than a single amino acid residue, is required (at minimum) for the 2c binding. The direct binding of 2c and Nef PxxP motif was then proved by GST pull-down assay using the competitive pre-incubation conditions. 2c showed direct binding with the PxxP motif of Nef in a similar manner to the SH3 domain of Hck. Altogether raises the possibility that Nef may interact with an unidentified cellular molecule at least through the PxxP-SH3 interaction of which finally leading to the production of more infectious viruses. 2c interrupts this interaction of Nef, therefore able to inhibit the infectivity of this virus.

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