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A simple, rapid, and sensitive system for the evaluation of anti-viral drugs in rats

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ABSTRACT

The lack of small animal models for the evaluation of anti-human immunodeficiency virus type 1 (HIV-1) agents hampers drug development. Here, we describe the establishment of a simple and rapid evaluation system in a rat model without animal infection facilities. After intraperitoneal administration of test drugs to rats, antiviral activity in the sera was examined by the MAGI assay. Recently developed inhibitors for HIV-1 entry, two CXCR4 antagonists, TF14016 and FC131, and four fusion inhibitors, T-20, T-20EK, SC29EK, and TRI-1144, were evaluated using HIV-1_{IIIB} and HIV-1_{BaL} as representative CXCR4- and CCR5-tropic HIV-1 strains, respectively. CXCR4 antagonists were shown to only possess anti-HIV-1_{IIIB} activity, whereas fusion inhibitors showed both anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities in rat sera. These results indicate that test drugs were successfully processed into the rat sera and could be detected by the MAGI assay. In this system, TRI-1144 showed the most potent and sustained antiviral activity. Sera from animals not administered drugs showed substantial anti-HIV-1 activity, indicating that relatively high dose or activity of the test drugs might be needed. In conclusion, the novel rat system established here, "phenotypic drug evaluation", may be applicable for the evaluation of various antiviral drugs *in vivo*.

1. Introduction

Numerous antiviral agents have been developed to suppress infection with viruses such as human immunodeficiency virus type 1 (HIV-1) [1], and have successfully provided excellent outcomes *in vivo*. However, the emergence of drug-resistant HIV-1 variants is a major concern in HIV therapy. Therefore, the development of novel drugs with sustained activity to resistant variants is desirable. Drugs, especially those targeting HIV-1 entry, have been recently developed and approved, such as a CCR5 antagonist, maraviroc [2], and a fusion inhibitor, enfuvirtide (T-20) [3], where both drugs effectively suppress HIV-1 in the patient even resistant to previous drugs [4,5].

In addition to CCR5, which is a main co-receptor for clinical HIV-1 strains, CXCR4 can also act as a co-receptor for HIV-1 (X4-tropic HIV-1), such as that seen for the vast majority of laboratory-adapted HIV-1 strains [6]. Thus, CXCR4 is also considered an important therapeutic target. We previously identified a β-sheet-like 14-residue peptide, T140 [7,8], and its down-sized analog, a cyclic pentapeptide FC131 (Fig. 1) [9], as potent and specific CXCR4 antagonists. Both T140 and FC131 were proved to inhibit X4-tropic HIV-1 infection *in vitro*. T140 has been further modified to TF14016 (4F-benzoyl-TN14003; BKT140) that shows more potent inhibitory effect [10].

The first fusion inhibitor, T-20, efficiently inhibits replication of HIV-1 resistant even to inhibitors for reverse transcriptase and protease [11,12]. However, the genetic barrier to overcome suppression by T-20 seems to not be high since a 1–2 amino acid(s) substitution in gp41 appears to be sufficient for resistance [13–15]. Therefore, we developed T-20EK [16] and SC29EK [17] as novel and potent fusion inhibitors that sustain their inhibitory effects on T-20 resistant HIV-1 stains. A series of systematic replacements with hydrophilic glutamic acid (E) or lysine (K) was introduced (EK motif) at the solvent-accessible site to enhance the α-helicity of the peptides by possible intrahelical electrostatic interactions [18]. T-20EK/S138A [16] was synthesized

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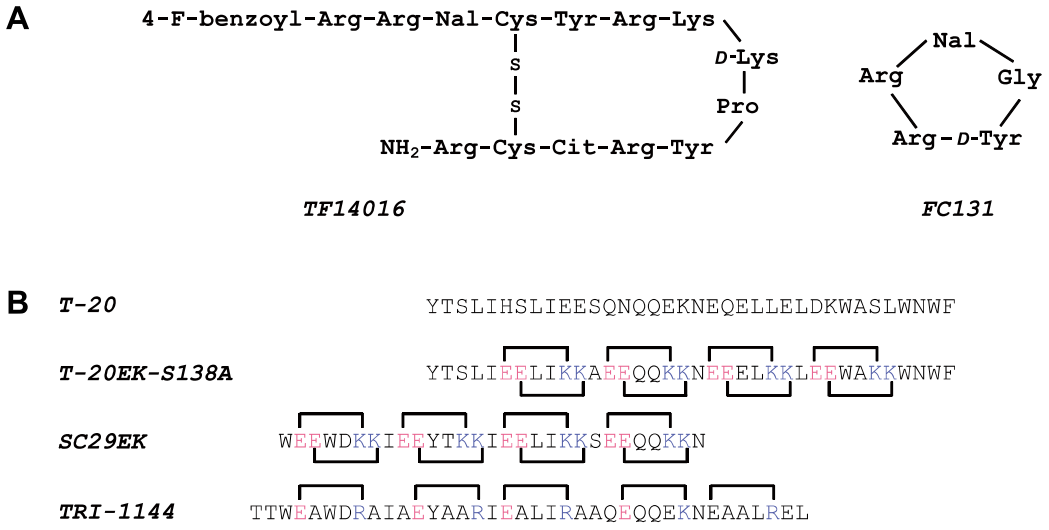

Fig. 1. Amino acid sequences of peptide-based drugs. (A) CXCR4 antagonists used in this study are shown. Nal: L-3-(2-naphthyl)alanine; Cit: L-citrulline. (B) Fusion inhibitors used are shown. T-20 is original sequenced of gp41 C-HR region. Electrostatic interactions are indicated by the linker. SC29EK and T-20EK/S138A, contain EExxKKx motif, while TRI-1144 does ExxxRxx motif. x indicates original and/or modified amino acids. Each motif creates 2 and 1 interaction(s) in each helical turn. T-20 resistance associated mutation, S138A, is introduced into T-20EK sequence (T-20EK/S138A). All peptides are N-terminally acetylated and C-terminally amidated.

with a combined rational design by the introduction of the EK motif for enhancement of α-helicity and increased affinity to mutated gp41 by S138A, a T-20 resistant associated mutation [19]. Dwyer et al. developed another fusion inhibitor, TRI-1144 (T-2635) that also exerted potent activity against T-20 resistant variants [20,21]. The amino acid sequence of TRI-1144 is also modified by substitutions with E and arginine (R), similar to the EK motif introduced into T-20EK and SC29EK.

Analyses of the efficacy and adverse effects of new drugs in animal models are important prior to their clinical application. Indeed, generally, the toxic effects, kinetics, and efficacy of new drugs are expected to be obtained by animal experiments. In the case of anti-HIV-1 drugs, the toxic effects of drug candidates can be determined by animal experiments. Furthermore, the kinetics of some drugs may be examined by some analytical methods such as liquid chromatography–mass spectrometry (LC–MS) [22] or bioimaging with labeled compounds. Unfortunately, these results may not be well-correlated with *in vivo* efficacy due to degradation and/or modification of drugs, and the detection of false positives of similar component(s) *in vivo* [23]. The efficacy of anti-HIV-1 drugs is, so far, hard to examine *in vivo* due to the lack of convenient animal infection models with low cost. One of the main obstacles to establish appropriate animal models is restricted infection of small animals with HIV-1, such as for mice, rats, and ferrets. An HIV-1 receptor-transgenic rat model has been developed for the analysis of HIV-1 infection *in vivo*; however, the levels of plasma viremia in infected rats were modest and not sustained [24,25]. Monkeys infected with simian immunodeficiency virus-HIV chimeric virus (SHIV) is the only model for the evaluation of HIV-1 replication [26], but comes at a high cost, especially for animal infection facilities. Taken together, novel rapid, simple, and sensitive HIV-1 infection models with low cost, such as those in small animals, are urgently needed to be established.

Here, we established a new system to evaluate the anti-HIV-1 activity of drugs and its kinetics in rats in addition to their toxic effects. The bioavailability of anti-HIV-1 drugs in sera was determined for the assessment of antiviral activity *in vitro*. The *in vivo* efficacy of various peptide-based entry inhibitors, such as TF14016, FC131, T-20EK/S138A, SC29EK, and TRI-1144, were assessed using this model and may be useful for the *in vivo* assessment of novel entry inhibitors.

2. Materials and methods

2.1. Drugs and cells

CXCR4 antagonists, TF14016 and FC131, and fusion inhibitors, T-20, T-20EK/S138A, SC29EK and TRI1144, were synthesized as previously described [7,9,16–18,20]. For *in vitro* drug susceptibility assays and *in vivo* administration, the test drugs were dissolved in 50% dimethyl sulfoxide (DMSO; 2 mM) and sterile water (3 or 10 mg/1.5 mL), respectively. MAGI CCR5 cells (HeLa CD4/CCR5/LTR-β-galactosidase cells) were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID: from Dr. Julie Overbaugh [27–29] and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum [30].

2.2. Administration of drugs

Animal experiments were performed in the Biotechnical Center of the Japan SLC, in accordance with the institutional ethical guidelines. To examine the pharmacological kinetics in sera, rats were used for collection of sera. Drugs were used at 3 mg/1.5 mL/kg of T-20, 3 mg/1.5 mL/kg of TF14016, 10 mg/1.5 mL/kg of FC131, 10 mg/1.5 mL/kg of SC29EK, 10 mg/1.5 mL/kg of T-20EK/S138A, and 3 mg/1.5 mL/kg of TRI1144, and were intraperitoneally administrated to six groups of six male SD rats (7 weeks). Sera were then harvested 0.5, 1, 2, 4, 8, and 12 h from the administrated rat, and stored at −80 °C.

2.3. MAGI assay

The anti-HIV-1 activity of drugs in rat sera after drug administration was detected by the MAGI assay, as previously described [31]. Briefly, MAGI CCR5 cells were transferred to 96-well plates at 1×10^4 cells per well. On the following day, serially-diluted drugs or rat sera were added to cells in triplicate with HIV-1 preparations (HIV-1_{IIIB} or HIV-1_{BaL}). After 48 h, cultured cells were fixed with 1% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde in phosphate-buffered saline (PBS), and were stained with 0.4 mg/mL 5-bromo-4-chloro-3-indolyl-2-D-galactopyranoside (X-gal). Blue

cells were counted by observation under a light microscope. The 50% effective concentration was defined as the serum dilution fold or drug concentration that inhibited virus infection in 50% of the wells.

3. Results

3.1. Anti-HIV-1 activity of drugs in vitro

Prior to animal experiments, the anti-HIV-1 activity of test drugs *in vitro* was determined by the MAGI assay. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4- and R5-tropic HIV-1 strains, respectively. TF14016 exerted most potent anti-HIV-1 activity *in vitro* compared to other inhibitors as shown in Table 1. As expected, the two CXCR4 antagonists, TF14016 and FC131, inhibited replication of only HIV-1_{IIIB}, but not HIV-1_{BaL}, which uses CCR5 for its entry. All four fusion inhibitors, T-20EK/S138A, SC29EK, TRI-1144 and T-20, comparably inhibited replication of both HIV-1_{IIIB} and HIV-1_{BaL}. Among newly developed fusion inhibitors, T-20EK/S138A showed the strongest inhibitory effect both on HIV-1_{IIIB} and HIV-1_{BaL}. Our antiviral data are similar to previous observations for TF14016 and FC131 [7–10,32], T-20EK/S138A [16], SC29EK [17], and TRI-1144 [20,21].

3.2. Anti-HIV-1 activity of CXCR4 antagonists in rat

First, we examined background anti-HIV-1 activity in four PBS-injected rat sera as negative controls. In the control rat sera, anti-HIV-1_{IIIB} and HIV-1_{BaL} activities were detected (Fig. 2). Rat sera showed antiviral activity up to the 90- and 160-fold dilution for HIV-1_{IIIB} and HIV-1_{BaL} (Fig. 2; shown as a baseline activity).

The two CXCR4 antagonists, TF14016 and FC131, were intraperitoneally injected into six rats and sera were withdrawn at the indicated time as shown in Fig. 2. Drug activities were detected up to 4 h, with peak time point at 1 h after the administration. Surprisingly, sera from two rats injected with TF14016 and four rats with FC131 also weakly showed anti-HIV-1_{BaL} activity (data not shown). However, both CXCR4 antagonists were generally effective only against HIV-1_{IIIB}.

3.3. Anti-HIV-1 activity of fusion inhibitors in rat

Anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities were detected in four rat sera and all six rat sera, respectively, that were administered T-20. Anti-HIV-1 activity of T-20 in rats was detected up to 8 h with a peak time point 1–2 h after administration. Anti-HIV-1_{IIIB} activities were detected in sera of six rats injected with SC29EK, T-20EK/S138A, and TRI-1144, which were detected up to 3, 8, and 8 h, respectively, with serum peak levels at 1–2 h after administration. Anti-HIV-1_{BaL} activities were detected in sera with SC29EK, T-20EK/S138A, and TRI-1144 with similar extent with these for HIV-1_{IIIB}. These results indicate that in rats, intraperitoneally injected drug activities were present in sera and may exert anti-HIV-1 activity *in vivo*. Among these, TRI-1144 showed stable and relatively sustained activity.

3.4. Effect of heat inactivation

To identify component(s) for baseline anti-HIV-1 activity in rat sera, we examined heat inactivation. As expected, non-specific anti-HIV-1 activity in sera decreased in a time-dependent manner. At 1000-fold dilution of sera, non-specific activity was completely abolished (Fig. 3); unfortunately the drugs tested in the study were not heat stable and irreversible even at 56 °C (data not shown). However, when administered a physiological dose, anti-HIV-1 activity was detectable even without heat inactivation (Fig. 2). Therefore, the rat model system proved to be adequate to evaluate the efficacy of drugs.

3.5. Toxic effect of drugs in rats

All peptides tested showed no apparent lethal effect at the administered dosages, except for FC131, where one rat succumbed from unknown causes at a dose of 30 mg/kg.

4. Discussion

To develop effective and safe antiviral agents, *in vitro* screening systems are established for some viruses, while *in vivo* evaluation systems using small animals are hampered by limited infection efficiency and the need for specialized facilities. In the case of animal models for HIV-1, animal models are largely restricted [33]. In the present study, we describe the establishment of a novel evaluation system of anti-HIV-1 drugs through *in vitro* detection of anti-HIV-1 activity in the sera of rats administrated drugs using the MAGI assay. The *in vivo* efficacies of five potential entry inhibitors were evaluated. In this system, only TRI-1144 consistently showed potent and sustained activity compared with T-20. The glutamic acid–arginine (ER) modification, but not the glutamic acid–lysine (EK) modification and/or alanine substitutions to the peptide (Fig. 1), may have beneficial effects on stability and efficacy, resulting in sustained anti-HIV-1 activities. The simple and convenient *in vivo* efficacy evaluation system established in this study not only reveals whether drugs exert anti-HIV-1 activity *in vivo*, but also provides *in vivo* kinetics without the need for infectious animal facilities. Moreover, this system can be used for the evaluation of not only anti-HIV-1 drugs *in vivo*, but also of drugs against other viruses *in vivo*. Nonetheless, the sera produced by the rats can be also applied to resistant virus variants and clinical isolates resulting in a reduction of the number animal experiments required.

Other methods, such as a high performance liquid chromatography (HPLC), may provide accurate measurement of the drug concentration in sera and was performed in this study. Even after administration of FC131 at 30 mg/kg, we could only detect FC131 at the peak concentration (data not shown). In a case of small amount of agents with extremely high activity, it is possible to fail to detect by HPLC. For more sensitive detection by HPLC, further labeling, such as with radioisotopes, may be needed. In addition, HPLC analysis can detect drugs that have been modified and/or degraded by *in vivo* metabolism when they are spectrometrically indistinguishable. However, our system detects only the active

Table 1
Anti-HIV-1 activity of drugs *in vitro*.

Virus	EC_{50}[a] (nM)					
	TF14016	FC131	T-20	T-20EK/S138A	SC29EK	TRI-1144
HIV-1_{IIIB}	0.3 ± 0.0	17.4 ± 5.7	42.3 ± 7.6	2.0 ± 0.5	8.3 ± 1.3	4.6 ± 0.6
HIV-1_{BaL}	>10,000	>10,000	16.2 ± 4.9	0.4 ± 0.2	1.4 ± 0.2	0.4 ± 0.2

[a] Antiviral activity, shown as EC_{50}, was determined using the MAGI assay. Each EC_{50} represents the mean ± SD obtained from at least three independent experiments. HIV-1_{IIIB} and HIV-1_{BaL} were used as representative X4 and R5 HIV-1 strains, respectively.

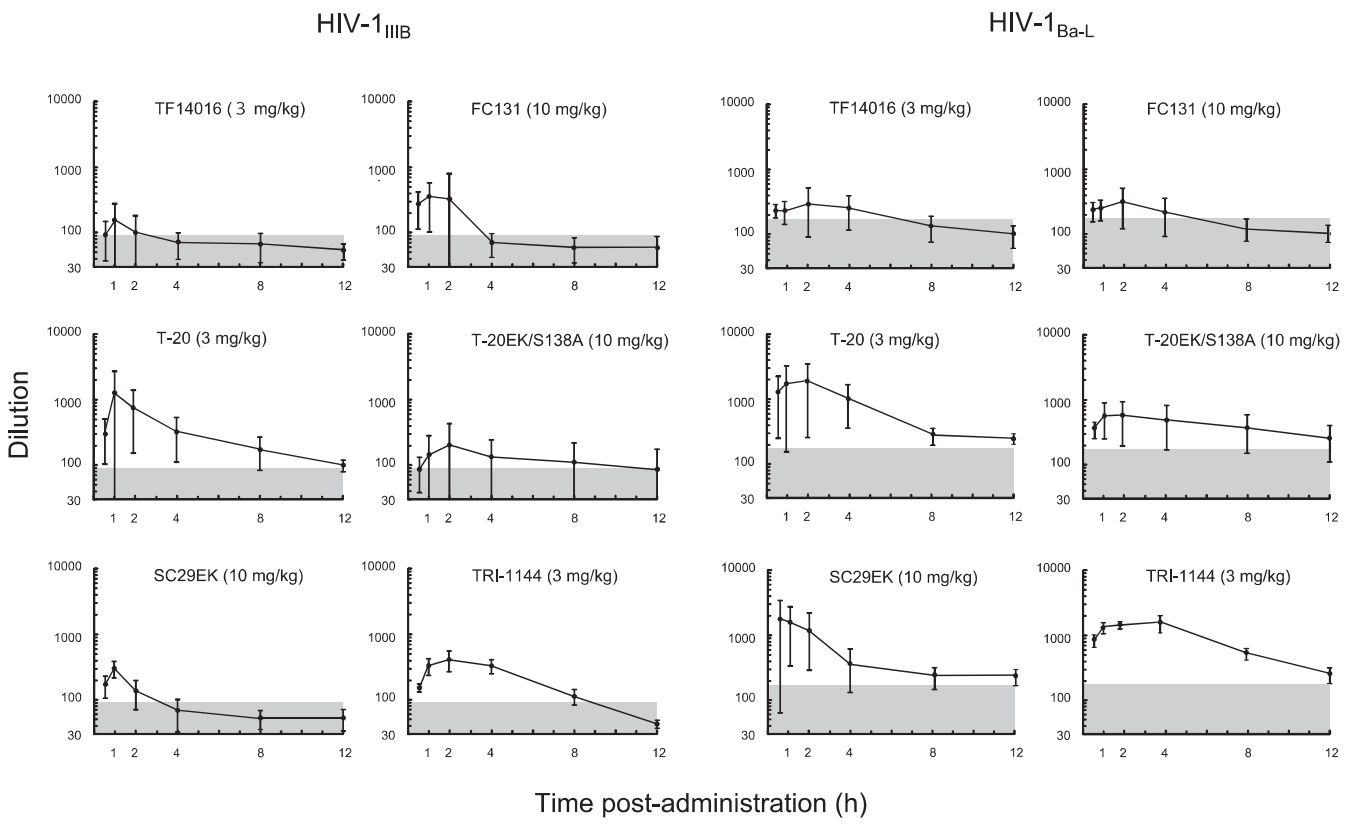

Fig. 2. Anti-HIV-1 activity of drugs *in vivo*. Six groups of six rats were administered each drug by intra-peritoneal injection and rat sera were harvested at different time points post-administration. All serum samples were analyzed by MAGI assay for 50% inhibition of infections of HIV-1_{IIIB} and HIV-1_{BaL}. This experiment was performed in triplicate for each rat. Data represent mean ± SD of from six rats. Gray shade indicates average results of age-matched rat sera as negative controls.

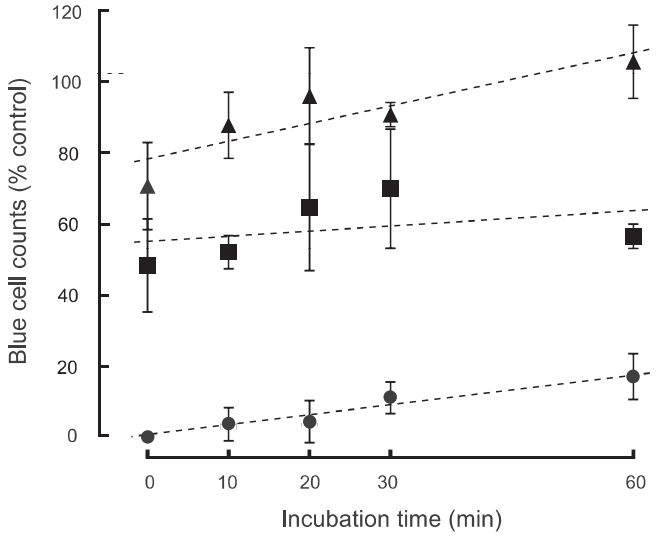

Fig. 3. Heat inactivation of sera. Rat sera without heat activation were examined using the MAGI assay. Heat inactivation was performed at 56 °C. Ten-fold dilutions of sera were resistant to heat inactivation even after 1 h inactivation (~20%). At the 1000-fold dilution, most of the inherent inhibitory effect was removed.

form of the agents, and in addition provides direct comparison of the tested drugs *in vitro* and *in vivo*, since the assay utilizes identical evaluation by the MAGI assay. In comparison, the rat *in vitro* system revealed that TRI-1144 showed strong and sustained activity compared with T-20EK/S138A and SC29EK. In this study, we only performed intraperitoneal injection that may have an effect on drug metabolism(s). Further experiments, such as subcutaneous injection, for which TF14016 shows greater efficacy [34,35], should be performed and compared with other administration roots.

The two CXCR4 antagonists analyzed in this study, TF14016 and FC131, showed moderate anti-HIV-1_{BaL} activity *in vivo*, and sera from two rats administered T-20 inhibited HIV-1 infection less efficiently (data not shown). These unexpected data might result from the relatively high background caused by non-specific inhibitory component(s) in sera. As shown in Fig. 2, sera from rats not administered drugs also showed moderate anti-HIV-1_{IIIB} and anti-HIV-1_{BaL} activities. Therefore, the development of a reagent or method for removal of background activity in rat sera may improve the accuracy and sensitivity of this *in vivo* drug efficacy evaluation method. For instance, serum albumin [36], lactoferrin [37,38], and transferrin [39] may influence HIV replication. Unfortunately, the drugs used in this study were all peptide-derived agents, therefore, heat-inactivation may reduce antiviral activity. Therefore, administration of relatively high doses of drug may be required to overcome this inhibition.

In conclusion, we established a novel, simple and rapid system for the phenotypic evaluation of anti-HIV-1 drugs in a rat model. This system may also be applicable for the analysis of other antiviral drugs for viruses that do not have an appropriate infection model in rodents, and/or useful for the initial screening, such for dosing, administration root decision and other factors, prior to actual animal infection experiments. In this system for HIV infection, TRI-1144 displayed the most potent anti-HIV-1 activity *in vivo* of the six drugs analyzed.

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