NOVEL LOW MOLECULAR WEIGHT SPIRODIKETOPIPERAZINE DERIVATIVES POTENTLY INHIBIT R5 HIV-1 INFECTION THROUGH THEIR ANTAGONISTIC EFFECTS ON CCR5

Kenji Maeda¹, Kazuhisa Yoshimura¹, Shiro Shibayama², Hiromu Habashita³,
Hideaki Tada², Kenji Sagawa², Toshikazu Miyakawa¹, Manabu Aoki¹,
Daikichi Fukushima², and Hiroaki Mitsuya¹,⁴

¹Department of Internal Medicine II, Kumamoto University School of Medicine, Kumamoto 860-0811; ²Exploratory Research Laboratories and ³Department of Medicinal Chemistry, Minase Research Institute, Ono Pharmaceutical Co. LTD, Osaka 618-8585;
⁴Experimental Retrovirology Section, Medicine Branch,
National Cancer Institute, Bethesda, MD 20892.

*Running Title; R5-HIV Inhibition by Spirodiketopiperazine Derivatives

*Correspondence should be addressed to: H.M; e-mail: hmitsuya@helix.nih.gov

Postal address: Dr. Hiroaki Mitsuya
Department of Internal Medicine II
Kumamoto University School of Medicine
1-1-1 Honjo, Kumamoto 860-0811, Japan

Phone: 81-96-373-5156
Facsimile: 81-96-363-5265
E-mail: hmitsuya@helix.nih.gov

Copyright 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Summary

Novel low molecular weight spirodiketopiperazine derivatives which potently inhibit R5 human immunodeficiency virus type 1 (HIV-1) infection through their antagonistic effects on CCR5 were identified. One such compound E913 (Mr. 484) specifically blocked the binding of MIP-1α to CCR5 (IC$_{50}$: 0.002 µM) and MIP-1α-elicited cellular Ca$^{2+}$ mobilization (IC$_{50}$: ~0.02 µM). E913 potently inhibited the replication of laboratory and primary R5 HIV-1 strains as well as various multi-drug resistant monocyte/macrophage-tropic (R5) HIV-1 at IC$_{50}$ values of 0.03 to 0.06 µM. E913 was inactive against T cell-tropic (X4) HIV-1; however, when combined with a CXCR4 antagonist AMD-3100, E913 potently and synergistically inhibited the replication of dualtropic HIV-1 and a 50:50 mixture of R5 and X4 HIV-1. Antagonism in anti-HIV-1 activity was not seen when E913 was combined with the reverse transcriptase inhibitor zidovudine or protease inhibitors. E913 proved to compete with the binding of antibodies to CCR5 which recognize the C-terminal half of the second extracellular loop (ECL2B) of CCR5. E913 and its analogs are acid-resistant and orally bioavailable in rodents. These data warrant that spirodiketopiperazine derivatives be further developed as potential therapeutics for HIV-1 infection.


**Introduction**

Highly active antiretroviral therapy (HAART) has brought about a major impact on the AIDS epidemics in the industrially advanced nations (1, 2), however, eradication of HIV-1 appears to be currently impossible, in part, due to the viral reservoirs remaining in blood and infected tissues (3, 4). The limitation of antiviral therapy of AIDS is exacerbated by complicated regimens, the development of drug resistant HIV-1 variants (5), and a number of inherent adverse effects. Successful antiviral drugs, in theory, exert their virus-specific effects by interacting with viral receptors, virally encoded enzymes, viral structural components, viral genes or their transcripts without disturbing cellular metabolisms or functions (2). However, at present, no antiretroviral drug or agent is likely to be completely specific for HIV-1 and to be devoid of toxicity or side effects in the therapy of AIDS, which has been a critical issue since patients with AIDS and its related diseases will have to administer antiretroviral therapy for a long period of time, perhaps for the rest of their lives (4). Thus, the identification of new antiretroviral drugs which have unique mechanisms of action and produce no or least minimal side effects remains an important therapeutic objective (2). In this respect, certain chemokine receptor antagonists might produce no or minimal toxicity.

Approximately 1% of Caucasians have a gene encoding a mutant form of CCR5 called delta-32, which is known to contribute to their resistance against HIV-1 infection (6, 7). Such CCR5-deficient individuals apparently do not have significant health problems and CCR5 knockout mice do not show grave pathological defects (6, 8). Such apparently limited effects of the lack of CCR5 render this receptor an attractive target for possible intervention of HIV-1 infection. It should be noted, however, that CCR5-lacking mice have some aberrant immunological defects and compromised defence to some pathogens (8, 9). Most recently, CCR5-lacking HIV-1- and hepatitis C virus-coinfected individuals were shown to have significantly higher levels of hepatitis C virus than their counterparts who have the normal form of CCR5 (10). Hence, the
sustained, long-term suppression of the effects of chemokines and/or chemokine receptors may produce adverse effects and caution should be used in the development of chemokine receptor antagonists as potential therapeutics.

In this study, we designed, synthesized, and identified a novel small non-peptide CCR5 antagonist, E913, and its related spirodiketopiperazine derivatives, which show potent HIV-1-specific antiviral activity, and examined the effects of E913 combined with other classes of anti-HIV agents. We demonstrate that E913 and its related compounds block the infectivity and replication of laboratory, clinical strains of HIV-1, highly drug-resistant HIV-1 variants, dualtropic HIV-1, and mixed populations of X4 and R5 variants when properly combined with other classes of anti-HIV-1 agents.
Experimental Procedures

Reagents - Spirodiketopiperazine derivatives including E913 were newly designed and synthesized as will be described elsewhere. The structures of E913 and three selected such compounds examined in this study are illustrated in Figure 1: E910, 1-butyl-2,5-dioxo-3-(2-methylpropyl)-9-(6-phenylhexyl)-1,4,9-triazaspiro[5,5]undecane; E913, 1-butyl-2,5-dioxo-3-cyclohexylmethyl-9-(1,4-benzodioxan-6-ylmethyl)-1,4,9-triazaspiro[5.5]undecane; E916, 1-butyl-2,5-dioxo-3-cyclohexylmethyl-9-(2-phenylimidazol-5-ylmethyl)-1,4,9-triazaspiro[5.5]undecane; and E917, 1-butyl-2,5-dioxo-3-(2-methypropyl)-9-[(4-phenoxyphenyl)methyl]-1,4,9-triazaspiro[5.5]undecane. A CCR5 antagonist TAK779 and a CXCR4 antagonist AMD-3100 were synthesized as previously described (11, 12). Zidovudine (3'-azido-3'-deoxythymidine or AZT) was purchased from Sigma (St. Louis, MO.). Unlabeled chemokines [macrophage inflammatory protein-1α (MIP-1α), macrophage chemoattractant protein-1 (MCP-1), macrophage-derived chemokine (MDC), and stromal cell-derived factor-1α (SDF-1α)] and [125I]-labeled chemokines (MIP-1α and MCP-1) were purchased from Peprotech (Rocky Hill, NJ) and NEN life Science (Boston, MA), respectively.

Cells and Viruses - The Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (CHO-dhfr(-);ATCC#CRL-9096, Manassas, VA) and were maintained in Ham’s F-12 medium (GIBCO BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS: Sigma) and 50 U/ml penicillin and 50 µg/ml streptomycin. Peripheral blood mononuclear cells (PBM) were isolated from buffy coats with Ficoll-Hipaque density gradient centrifugation and cultured at a concentration of 10^6 cells/ml in RPMI 1640-based culture medium supplemented with 10% fetal calf serum (FCS: HyClone, Logan, UT) and antibiotics with 10 µg/ml phytohemagglutinin (PHA) for 3 days prior to use. Hela-CD4-LTR-β-gal indicator cells expressing human CCR5 (CCR5-MAGI)(13) were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (Bethesda, MD). CCR5-MAGI cells
were maintained in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FCS, 200 µg/ml G418, 100 µg/ml hygromycin B, and 100 µg/ml zeomycin.

A panel of HIV-1 was employed for drug susceptibility assays: HIV-1_{LAI} (14), HIV-{1}_{NL4-3} (15), HIV-1_{BaL} (16), HIV-1_{89.6} (17), and HIV-1_{ERS104pre} [a clinical HIV-1 strain isolated from a drug-naive AIDS patient (18)]. Four clinical HIV-1 strains: HIV-1_{JSL}, HIV-1_{MM}, HIV-1_{TM}, and HIV-1_{MOKW}, were also employed. HIV-1_{JSL}, HIV-1_{MM} and HIV-1_{TM} were isolated from patients with AIDS who had received 9-11 anti-HIV-1 drugs in the past 32-83 months and were highly resistant to a number of antiviral agents as tested in vitro (19). HIV-1_{MOKW} was isolated from a drug-naive Japanese patient with AIDS and was confirmed to lack any known drug resistance-associated amino acid mutations. All clinical HIV-1 strains were passaged once or twice in PHA-PBM. Nucleotide sequences of the polymerase- and protease-encoding regions were determined for the clinical HIV-1 strains as previously described (19), and the culture supernatants were stored at –70°C until use.

Generation of a CCR5 Expressing Cell Line - Human CCR5 cDNA was amplified with polymerase chain reaction from a human placenta cDNA library, purified, and subcloned into a mammalian expression vector pEF6/V5-His (Invitrogen, Carlsbad, CA), in which the V5-His tag epitope was deleted. CHO cells were transfected with thus obtained CCR5 plasmids by using DMRIE-C (Gibco BRL), selected in the presence of 5 µg/ml blasticidin S hydrochloride (Kaken Pharmaceutical, Tokyo), and CHO cells stably expressing CCR5 (CCR5-CHO cells) were obtained.

Chemokine Binding Studies - CCR5-CHO cells (1.2 x 10^5 cells/well) were plated onto 48-well, flat-bottomed culture plates, incubated for 18-24 hours, rinsed once with Ham’s F-12 medium containing 20 mM Hepes and 0.5% BSA. These adherent CCR5-CHO cells were exposed to 0.1 nM [^{125}I]-labeled MIP-1α in the presence of varying concentrations of a test compound at room temperature for 40 min, washed thoroughly with cold
phosphate-buffered saline (PBS), and lysed with 0.5 ml of 1 N NaOH. The radioactivity in 0.5 ml of the cell lysates was determined with COBRA $\gamma$-counter (#5010; Packard, Tokyo). The non-specific binding of the labeled chemokine to the cells was determined based on the radioactivity from the wells added with 100 nM non-radiolabeled chemokine. The inhibition by test compounds of the binding of 0.1 nM $[^{125}]$I-labeled MCP-1 to CCR2-expressing CHO (CCR2-CHO) cells was similarly determined.

Assays for Inhibition of Cytosolic Ca$^{2+}$ Mobilization - CCR5-CHO cells ($3 \times 10^4$ cells/well) were plated onto 96-well, flat-bottomed microtiter culture plates, incubated for 18-24 hours, loaded with 5 mM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) for 60 min at $37^\circ$C in Ham’s F-12 containing 20 mM Hepes and 2.5 mM probenecid, and washed once with Hanks solution containing 20 mM Hepes and 2.5 mM probenecid. A test compound was added at varying concentrations and the cells were exposed to MIP-1$\alpha$ at a concentration of 30 nM, and a relative increase of the cytosolic Ca$^{2+}$ level at 3 min after the MIP-1$\alpha$ exposure was determined with Spectrofluorometer FDSS-2000 and 4000 (Hamamatsu Photonics, Shizuoka, Japan). The inhibition by a test compound of the cytosolic Ca$^{2+}$ mobilization elicited by MCP-1 (30 nM), MDC (10 nM) or SDF-1$\alpha$ (30 nM) was determined using the same conditions.

FACS Analysis - CCR5-CHO cells were employed for the chemokine binding inhibition assay. The confluent CCR5-CHO cells were lifted off with 1 mM EDTA/PBS, washed once with F-12 medium, suspended in 0.75% FCS-containing F-12 (FACS staining buffer), and incubated in the presence of varying concentrations of a test compound for 15 min on ice. Fluorescein-conjugated monoclonal antibodies 45523 and 45531 (20)(R&D Systems, Minneapolis, MN) were then added to a final concentration of 13.3 $\mu$g/ml, and incubated for 30 min on ice. FACS analysis was performed with a Becton Dickinson FACSort flow cytometer using the CellQuest software (Becton Dickinson, Franklin Lakes, NJ).
Antiviral Assays and Determination of Cytotoxicity - The MAGI assay using CCR5-MAGI cells was conducted as previously described (21) with minor modifications. Briefly, CCR5-MAGI cells were plated (10^4 cells/well) and cultured in 96-well, flat-bottomed microculture plates. After 24-hour incubation, the cells were exposed to various concentrations of a test compound and HIV-1 virus in DMEM containing 15% FCS, and were stained at 48 hours of culture with chlorophenolred β-D-galactopyranoside (CPRG) as previously described (22, 23). Supernatants were removed and the cells were lyzed with 100 µl PBS containing 1% Triton X-100. A solution (100 µl) containing 10 mM CPRG, 2 mM MgCl₂, and 0.1 M KH₂PO₄ was added to each well, the mixture was incubated at room temperature in the dark for 30 min, and the optical density (wave length: 570 nm) was measured in a microplate reader (Vmax, Molecular Devices, Sunnyvale, CA). Drug concentrations that brought about 50% inhibition (IC₅₀) of the β-galactosidase activity were determined. All assays were performed in triplicate.

PHA-PBM (1x10⁶/ml) were exposed to 50 TCID₅₀ of each HIV-1 strain and cultured in the presence or absence of various concentrations of test compounds in 10-fold serial dilutions in 96-well microculture plates. The amounts of p24 antigen produced by the cells were determined on day 7 of culture by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo)(24), which has an extensive dynamic range of 10-100,000 pg/ml p24. Drug concentrations that suppressed the production of p24 Gag protein by 50% (IC₅₀) were determined by comparison with the p24 production level in drug-free control cell cultures (25, 26). All assays were performed in triplicate.

In order to assess the cytotoxicity of a test compound, PHA-PBM (1x10⁶/ml) were cultured in the presence or absence of various concentrations of the compound in 5-fold serial dilutions in 96-well microculture plates. In 7 days of culture, 100 µl of the medium was removed from each well, and 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (7.5 mg/ml) was added to each well in the plate, followed by incubation at 37°C for 3 hours. After incubation, to dissolve the
formazan crystals, 100 µl of acidified isopropanol containing 4% Triton X-100 was added and the optical density was measured in a microplate reader. All assays were performed in triplicate.
Results

Spirodiketopiperazine Derivatives as CCR5 Antagonists - We designed, synthesized, and tested approximately 130 spirodiketopiperazine derivatives and examined whether such spirodiketopiperazine derivatives blocked the binding of $^{125}$I-labeled MIP-1α to CCR5-CHO cells and MIP-1α-elicited cellular Ca$^{2+}$ mobilization. We identified 4 compounds, E910, E913, E916, and E917, which potently inhibited the binding and Ca$^{2+}$ mobilization at IC$_{50}$ values of 0.002 to 0.1 μM (Table 1).

As shown in Figure 2, both E910 and E913 blocked the binding of MIP-1α to CCR5-CHO cells, while they failed to block the binding of MCP-1, whose primary receptor is CCR2, to CCR2-CHO cells (Figure 2). We also asked whether E913 blocked the intracellular Ca$^{2+}$ mobilization induced by MDC and SDF-1α, whose primary receptors are CCR4 and CXCR4, respectively, as well as MCP-1. E913 completely blocked MIP-1α-induced Ca$^{2+}$ mobilization at 0.3 μM and beyond, however, it failed to block the Ca$^{2+}$ mobilization induced with MDC, SDF-1α and MCP-1 (Figure 3). When we asked if these compounds had agonistic effects to induce chemotaxis and Ca$^{2+}$ mobilization in CCR5-CHO cells, none induced chemotaxis or Ca$^{2+}$ mobilization (data not shown).

Potent Activity of Spirodiketopiperazine Derivatives Against R5 HIV-1 - We tested the four compounds against HIV-1$_{Bal}$ (R5 HIV-1) and HIV-1$_{Lai}$ (X4 HIV-1) using CCR5-MAGI cells and PHA-PBM as target cells, respectively. As assessed in the MAGI assay, three spirodiketopiperazine derivatives, E913, E916 and E917 were highly potent against HIV-1$_{Bal}$ with IC$_{50}$ values of 0.03, 0.07, and 0.06 μM, respectively (Table 2). In the antiviral assay using PHA-PBM as target cells, E913 was the most potent with an IC$_{50}$ value of 0.04 μM against HIV-1$_{Bal}$ although other three compounds were moderately active with IC$_{50}$ values of 0.1 to 0.5 μM (Table 2). It should be noted that E913 was least cytotoxic against PHA-PBM as assessed with the MTT assay with a selectivity index of 1,295. However, all four spirodiketopiperazine derivatives failed to block the replication of X4 HIV-1$_{Lai}$ and HIV-1$_{NL4-3}$ in both assay systems, although a CXCR4 antagonist AMD-3100 was active.
against X4 HIV-1LaI and HIV-1NL4-3. It has been reported that certain CC-chemokines enhance the replication of X4 HIV-1 while SDF-1 enhances the replication of R5 HIV-1 by more than 3-fold in vitro (27-29). However, no enhancement of X4 HIV-1 replication was seen with E913 or other derivatives as tested at concentrations up to 1 µM (data not shown).

Potent Activity of E913 against Multi-drug-resistant HIV-1 - The most potent and least cytotoxic CCR5 antagonist E913 was chosen for further testing against a variety of clinical HIV-1 isolates. Three (two R5 and one X4) clinical HIV-1 strains were isolated from patients with AIDS who had received 9-11 anti-HIV-1 drugs in the past 32-83 months and had lost response to all available antiviral regimens (19, 25)(Table 3). All these HIV-1 isolates were highly resistant to a number of antiviral agents as tested in vitro (19, 25). For example, zidovudine was highly active against the wild type HIV-1MOKW and its IC50 value was as low as 0.001 µM. However, IC50 values of zidovudine against three multi-drug resistant (MDR) HIV-1 variants (HIV-1JSL, HIV-1MM, and HIV-1TM) were 28 to 98-fold greater than that against the wild type HIV-1MOKW (Table 3). All three drug-resistant HIV-1 also showed resistance to didanosine, stavudine, saquinavir, and nelfinavir. However, E913 was invariably potent against the wild type HIV-1MOKW and two drug-resistant R5 HIV-1JSL and HIV-1MM, while it failed to block the replication of X4 HIV-1TM. By contrast, AMD-3100 was potent against X4 HIV-1TM, but failed to block all three R5 HIV-1 strains (Table 3).

Suppression of HIV-1 Replication by E913 Combined with AMD-3100 - It was thought that any CCR5 antagonist alone or CXCR4 antagonist alone would not sufficiently block the replication of HIV-1 in patients where HIV-1 exists as a quasispecies. Therefore, we asked whether a combined use of both E913 and AMD-3100 blocked more effectively the infectivity and replication of HIV-1 in PHA-PBM. Against R5 HIV-1 (HIV-1Bal and HIV-1MM) and X4 HIV-1 (HIV-1NL4-3 and HIV-1TM), no apparent potentiation of activity was seen
with the combination (Figure 4-A, B). When E913 and AMD-3100 were combined at various concentrations and tested against R5 or X4 HIV-1, no significant combination effects were observed (data not shown). However, as shown in Figure 4-C, against two dual tropic HIV-1, HIV-1$_{89.6}$ (17) and HIV-1$_{ERS104pre}$ (18) combination effects were identified. E913 at 5 $\mu$M only partially blocked the replication of HIV-1$_{89.6}$ and HIV-1$_{ERS104pre}$ (by 29% and 55%), respectively (Figure 4-C). AMD-3100 at 0.1 $\mu$M quite effectively suppressed the replication of these dual tropic HIV-1 preparations, but not completely (by 95 and 94%, respectively). However, when E913 and AMD-3100 were combined, the replication of both HIV-1$_{89.6}$ and HIV-1$_{ERS104pre}$ was completely blocked (Figure 4-C and Figure 5-A).

The observation that AMD-3100 suppressed the replication of HIV-1$_{89.6}$ and HIV-1$_{ERS104pre}$ quite effectively suggested that these two dual tropic HIV-1 preparations were predominantly of the X4 HIV-1 nature. We, therefore, re-examined the effects of the combination on the replication of HIV-1$_{89.6}$ (Figure 5-A, left panel) using various concentrations of E913 and AMD-3100 and confirmed that the combination effect was synergistic (Figure 5-A, right panel). Furthermore, we prepared a 50:50 mixture preparation of R5 HIV-1$_{BaL}$ and X4 HIV-1$_{NL4-3}$, and examined whether the combination of E913 and AMD-3100 blocked the replication of both strains. As illustrated in Figure 5-B (left panel), with a combination of 1 $\mu$M E913 and 1 $\mu$M AMD-3100, a complete inhibition of HIV-1 replication was seen. We then examined the effects of the combination of E913 and AMD-3100 on the replication of the 50:50 mixture of R5 and X4 HIV-1 using the published method by Prichard et al. (30, 35), and found that the antiviral activity seen with the combination was synergistic (Figure 5-B, right panel). When we analyzed drug interactions of E913 and a nucleoside reverse transcriptase inhibitor zidovudine or a protease inhibitor nelfinavir, an additivism was seen but no synergism or antagonism was seen (data not shown).
E913 Binds to ECL2B of CCR5 and Blocks R5 HIV-1 Replication - Finally, we asked the mechanism E913 blocks the replication of R5 HIV-1. In order to examine where E913 binds and possibly blocks the binding of R5 HIV-1 to its receptor CCR5, we employed several monoclonal antibodies known to bind to different domains of CCR5. FACS analyses revealed that E913 competitively blocked the binding of two different monoclonal antibodies, 45523 directed against multidomain epitopes of CCR5 and 45531 specific for the C-terminal half of domain B of the second extracellular loop (ECL2B) of CCR5 (20), as examined using CCR5-CHO cells (Figure 6). However, there was no E913 inhibition of the binding of a monoclonal antibody 2D7, which is known to bind to the N-terminal half or domain A of the second extracellular loop of CCR5 (20). These data strongly suggest that E913 binds to ECL2B of CCR5, presumably causing steric hindrance for the binding of HIV-1 gp120 to CCR5, thus ultimately blocking the infection by R5 HIV-1.
Discussion

It has been shown that HIV-1 undergoes phenotypic shift in the course of development of AIDS: non syncytia-inducing (NSI) R5 HIV-1 predominates in early infection, while syncytia-inducing (SI) X4 HIV-1 and those which use both CCR5 and CXCR4 for cell entry (R5X4 HIV-1 or dualtropic HIV-1) emerge as the disease progresses (31, 32). However, it is known that NSI R5 HIV-1 still exist in the late stages within the HIV-1 population of the quasispecies property (33). Hence, from a therapeutic strategy point of view, if a coreceptor antagonist(s) is used, antiviral regimens should be able to suppress mixed viral populations, in particular R5 HIV-1 and X4 HIV-1 simultaneously. In the present study, a CCR5 antagonist E913, when combined with a CXCR4 antagonist AMD-3100, potently inhibited the replication of the dual tropic HIV-1\textsubscript{89.6} and the 50:50 mixture of R5 HIV-1 and X4 HIV-1. C-C chemokines appear to affect the replication of X4 HIV-1 and influence the phenotypic HIV-1 shift seen in HIV-1-infected individuals. Margolis et al. have reported that when human lymphoid tissues are infected with X4 HIV-1 ex vivo, C-C chemokines including MIP-1\textsubscript{α}, MIP-1\textsubscript{β}, and RANTES are upregulated, presumably contributing to the phenotypic HIV-1 shift from R5 HIV-1 to X4 HIV-1 (36). It has also been reported that C-C chemokines suppress the replication of R5 HIV-1 in vitro but enhances the replication of X4 HIV-1 in CD4\textsuperscript{+} T cells (27, 28), contributing to the viral phenotypic shift. In our study, however, there was no enhancement seen in the replication of X4 HIV-1 in PHA-PBM, when the dual tropic HIV-1\textsubscript{89.6} and HIV-1\textsubscript{ERS104pre} were exposed to E913. In this regard, Kinter et al. have reported that C-C chemokines elicit signal transduction through inhibitory guanine nucleotide-binding regulatory (Gi) proteins and increase cell surface co-localization of CD4 and CXCR4, inducing enhanced X4 HIV-1 replication (37). E913 and its analogues block the binding of C-C chemokines to CCR5 and C-C chemokine-elicited cellular Ca\textsuperscript{2+} mobilization, but per se do not induce cellular Ca\textsuperscript{2+} mobilization, suggesting that E913 and its analogues do not cause CD4-CXCR4 co-localization or signal transduction in the cells so that enhanced replication of X4 HIV-1 does not occur.
Recently Mosier et al. reported that RANTES analogues such as aminooxypentane (AOP)-RANTES[2-68] and N-nonanoyl (NNY)-RANTES[2-68] rapidly selected for X4 viruses in the human PBL-SCID mouse model when those analogues were maintained at sub-inhibitory concentrations (38). Conversely, in the presence of a CXCR4 antagonist AMD-3100, R5 HIV-1 outgrew when mixtures of R5 and X4 HIV-1 strains were cultivated (39). These results reinforce the notion that regimens using chemokine antagonists should block all major HIV-1 coreceptors for effective therapy and that the clinical use of blocking agents for CCR5 or CXCR4 alone should be approached with caution. Although the combination of E913 and AMD-3100 blocked the replication of R5 HIV-1, X4 HIV-1, and R5X4 HIV-1 quite efficiently (Figure 5), studies to address whether the viral shift occurs in the presence of both antagonists are to be conducted.

In this study, we asked whether the antiviral activity of E913 combined with AMD-3100 was synergistic, additive, or antagonistic, employing the method based on the Bliss independence (Figure 5)(30) and the method of Chou and Talalay (data not shown)(34). The antiviral activity of the combination proved to be synergistic for many concentrations in both methods, in agreement with a recent report that SDF-1α and AOP-RANTES were synergistic when they were used in combination against clinical HIV-1 strains containing R5 and X4 HIV-1 (40). When we analyzed drug interactions between E913 and a nucleoside reverse transcriptase inhibitor (zidovudine) or a protease inhibitors (nelfinavir or saquinavir), additivism was seen but no synergism or antagonism was identified (data not shown). It is intriguing that no synergism was seen when E913 was combined with AZT or nelfinavir although E913 showed synergism when combined with AMD-3100. In this regard, Singer et al. recently reported that CCR5, CXCR4, and CD4 are clustered and closely apposed on microvilli of human macrophages and T cells (41). They demonstrated that CD4 molecules and the chemokine receptors are separated only by distances less than the diameter of an HIV-1 particle, thereby HIV-1 adsorption and penetration of cells are facilitated. If so, it is assumed that a CCR5 antagonist combined with a CXCR4 antagonist
can block those CD4-chemokine receptor microclusters more effectively, bringing about antiviral synergism, but no synergy occurs in combination of E913 and AZT or nelfinavir since the latter does not affect the viral adsorption onto the target cells.

Three spirodiketopiperazine derivatives examined in this study, E910, E916, and E917, were moderately toxic in vitro, but the mechanism of the toxicity is presently not known, although the cytotoxicity of these derivatives does not appear to be associated with their binding to CCR5 receptors or blocking of Ca\(^{2+}\) mobilization (data not shown). However, the cytotoxicity of E913 was insignificant with a CC\(_{50}\) value of 51.8 µM and the selectivity index of 1,295. Nevertheless, it should be noted that long-term administration of chemokine receptor antagonists may cause adverse effects in vivo. Salazar-Mather et al. have reported that when mice genetically lacking MIP-1α functions were intraperitoneally infected with murine cytomegalovirus, focal natural killer cell traffic and accumulation in infected liver were totally absent (9). Moreover, CCR5-deficient mice showed a defect in macrophage function, an enhanced delayed-type hypersensitivity reaction, and increased humoral responses to T cell-dependent antigenic challenge (8). Murai and her colleagues recently reported that administration of anti-CCR5 antibody in mice, which otherwise underwent graft-versus-host disease-associated liver injury after cell transfer, had a dramatically reduced infiltration of CCR5\(^{+}\), CD8\(^{+}\) T cells into the liver (42). These animal data suggest that the inhibition of the CC chemokine-CCR5 system compromises the host’s defense system. In this respect, our knowledge of toxicity profiles in humans upon the administration of chemokine receptor antagonists is limited. In a phase I open-label dose escalation study, AMD-3100 has been administered to healthy volunteers (43). All subjects tolerated the doses tested without any grade 2 toxicity or dose adjustment. There were seen mild, transient gastrointestinal symptoms and a transient dose-related elevation of white blood cell (WBC) counts reaching 1.5 to 3.1 times the baseline upon administration, suggesting that AMD-3100 binding to CXCR4 caused the release of WBCs from the endothelium and/or stem cells from bone marrow (43, 44). The clinical
significance of the WBC count elevation remains to be determined. Nevertheless, the sustained, long-term suppression of the effects of chemokines may result in unexpected adverse effects and such clinical trials should be conducted with caution. In fact, HIV-1-infected individuals who homozygously carry a gene encoding a mutant form of CCR5 called delta-32 which is associated with resistance to HIV-1 have been shown to have higher levels of hepatitis C virus than those who had the normal form of CCR5 (10).

In order to examine where E913 binds and blocks the binding of MIP1-α to its receptor CCR5, we employed several anti-CCR5 monoclonal antibodies. FACS analyses revealed that E913 competitively blocked the binding of two different monoclonal antibodies, 45523 directed against multidomain epitopes of CCR5 and 45531 specific against ECL2B of CCR5 (20). However, there was no E913 inhibition of the binding of a monoclonal antibody clone 2D7 which binds to ECL2A of CCR5. It is surmised that E913 causes changes in CCR5 distribution on the cell surface and blocks HIV-1 infection. However, when PHA-PBM were incubated for 2, 6, 72, and 120 hours with 1 µM of E913, no significant reduction of CCR5 on their surface was detected (data not shown). Hence, E913 does not cause CCR5 down-regulation, but binds to CCR5 and competes with those monoclonal antibodies, resulting in steric hindrance for the binding of HIV-1 gp120 to CCR5, thus ultimately blocking the infection of R5 HIV-1. It is noteworthy that E913, unlike TAK779, does not inhibit the binding of MCP-1 to CCR2 or the MCP-1-induced Ca²⁺ mobilization (Figures 2 and 3), suggesting that the CCR5 binding specificity of E913 differs from that of TAK779. It is worth noting that E913 and its analogs are acid-resistant and have an acceptable oral bioavailability in rodents (3 - 30%)(Shibayama et al., unpublished data). These data warrant that E913 and its analogs be developed as potential therapeutics for HIV-1.
Acknowledgments

We are grateful to Masayoshi Matsuo, Takao Yoshida, Toshio Yoshizawa, Hisanori Haga, and Hiromi Ogata for excellent technical assistance and to Yosuke Maeda and Tetsuya Kimura for helpful discussion. This work was supported in part by a grant from a Research for the Future Program (JSPS-RFTF 97L00705) of the Japan Society for the Promotion of Science, a Grant-in-aid for Scientific Research (Priority Areas) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Monbukagakusho), and a Grant for Promotion of AIDS Research from the Ministry of Health Welfare and Labor of Japan (Kosei-Rohdosho).
References

1. Fauci, A. S. (1999) N Engl J Med 341, 1046-50.

2. Mitsuya, H. & Erickson, J. (1999) Textbook of AIDS Medicine, Second Edition (Merigan, T.C., Bartlet, J.G. & Bolognesi, D., eds) pp751-780, Williams & Wilkins, Baltimore.

3. Finzi, D., Hermankova, M., Pierson, T., Carruth, L. M., Buck, C., Chaisson, R. E., Quinn, T. C., Chadwick, K., Margolick, J., Brookmeyer, R., Gallant, J., Markowitz, M., Ho, D. D., Richman, D. D. & Siliciano, R. F. (1997) Science 278, 1295-300.

4. Finzi, D., Blankson, J., Siliciano, J. D., Margolick, J. B., Chadwick, K., Pierson, T., Smith, K., Lisziewicz, J., Lori, F., Flexner, C., Quinn, T. C., Chaisson, R. E., Rosenberg, E., Walker, B., Gange, S., Gallant, J. & Siliciano, R. F. (1999) Nat Med 5, 512-7.

5. Kavlick, M.F., & Mitsuya, H. (2001) The Art of Antiretroviral Therapy (De Clercq, E. ed) pp.279-312, American Society for Microbiology, Washington, D.C.

6. Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A. & Landau, N. R. (1996) Cell 86, 367-77.

7. Samson, M., Libert, F., Doranz, B. J., Rucker, J., Liesnard, C., Farber, C. M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., Muyldermans, G., Verhofstede, C., Burtonboy, G., Georges, M., Imai, T., Rana, S., Yi, Y., Smyth, R. J., Collman, R. G., Doms, R. W., Vassart, G. & Parmentier, M. (1996) Nature 382, 722-5.

8. Zhou, Y., Kurihara, T., Ryseck, R. P., Yang, Y., Ryan, C., Loy, J., Warr, G. & Bravo, R. (1998) J Immunol 160, 4018-25.

9. Salazar-Mather, T. P., Orange, J. S. & Biron, C. A. (1998) J Exp Med 187, 1-14.

10. Woitas, R. P., Rockstroh, J. K., Bockmuehl, R., Schepers, K., Stoschus, B., Matz, B., Sauerbruch, T. & Spengler, U. (2001) The 8th Conference on Retroviruses and Opportunistic Infections., Chicago, IL., February 4-8, 2001 (Abstr. 79)
11. Baba, M., Nishimura, O., Kanzaki, N., Okamoto, M., Sawada, H., Iizawa, Y., Shiraishi, M., Aramaki, Y., Okonogi, K., Ogawa, Y., Meguro, K. & Fujino, M. (1999) Proc Natl Acad Sci USA 96, 5698-703.

12. De Clercq, E., Yamamoto, N., Pauwels, R., Balzarini, J., Witvrouw, M., De Vreese, K., Debyser, Z., Rosenwirth, B., Peichl, P., Datema, R. & et al. (1994) Antimicrob Agents Chemother 38, 668-74.

13. Chackerian, B., Long, E. M., Luciw, P. A. & Overbaugh, J. (1997) J Virol 71, 3932-9.

14. Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., Laurent, A. G., Dauguet, C., Katlama, C., Rouzioux, C. & et al. (1986) Science 233, 343-6.

15. Westervelt, P., Gendelman, H. E. & Ratner, L. (1991) Proc Natl Acad Sci USA 88, 3097-101.

16. Gartner, S., Markovits, P., Markovitz, D. M., Kaplan, M. H., Gallo, R. C. & Popovic, M. (1986) Science 233, 215-9.

17. Collman, R., Balliet, J. W., Gregory, S. A., Friedman, H., Kolson, D. L., Nathanson, N. & Srinivasan, A. (1992) J Virol 66, 7517-21.

18. Shirasaka, T., Yarchoan, R., O'Brien, M. C., Husson, R. N., Anderson, B. D., Kojima, E., Shimada, T., Broder, S. & Mitsuya, H. (1993) Proc Natl Acad Sci USA 90, 562-6.

19. Yoshimura, K., Kato, R., Yusa, K., Kavlick, M. F., Maroun, V., Nguyen, A., Mimoto, T., Ueno, T., Shintani, M., Falloon, J., Masur, H., Hayashi, H., Erickson, J. & Mitsuya, H. (1999) Proc Natl Acad Sci USA 96, 8675-80.

20. Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M. & Doms, R. W. (1999) J Biol Chem 274, 9617-26.

21. Uchida, H., Maeda, Y. & Mitsuya, H. (1997) Antiviral Res 36, 107-13.

22. Felgner, J. H., Kumar, R., Sridhar, C. N., Wheeler, C. J., Tsai, Y. J., Border, R., Ramsey, P., Martin, M. & Felgner, P. L. (1994) J Biol Chem 269, 2550-61.
23. Floch, V., Audrezet, M. P., Guillaume, C., Gobin, E., Le Bolch, G., Clement, J. C., Yaouanc, J. J., Des Abbayes, H., Mercier, B., Leroy, J. P., Abgrall, J. F. & Ferec, C. (1998) Biochim Biophys Acta 1371, 53-70.

24. Sakai, A., Hirabayashi, Y., Aizawa, S., Tanaka, M., Ida, S. & Oka, S. (1999) Kansenshogaku Zasshi 73, 205-12.

25. Shirasaka, T., Kavlick, M. F., Ueno, T., Gao, W. Y., Kojima, E., Alcaide, M. L., Chokekijchaisi, S., Roy, B. M., Arnold, E., Yarchoan, R. & et al. (1995) Proc Natl Acad Sci USA 92, 2398-402.

26. Tanaka, M., Srinivas, R. V., Ueno, T., Kavlick, M. F., Hui, F. K., Fridland, A., Driscoll, J. S. & Mitsuya, H. (1997) Antimicrob Agents Chemother 41, 1313-8.

27. Schols, D., Proost, P., Van Damme, J. & De Clercq, E. (1997) J Virol 71, 7300-4.

28. Moriuchi, H., Moriuchi, M. & Fauci, A. S. (1998) J Exp Med 187, 1689-97.

29. Marechal, V., Arenzana-Seisdedos, F., Heard, J. M. & Schwartz, O. (1999) J Virol 73, 3608-15.

30. Prichard, M. N., Prichard, L. E. & Shipman, C., Jr. (1993) Antimicrob Agents Chemother 37, 540-5.

31. Tersmette, M., Gruters, R. A., de Wolf, F., de Goede, R. E., Lange, J. M., Schellekens, P. T., Goudsmit, J., Huisman, H. G. & Miedema, F. (1989) J Virol 63, 2118-25.

32. Schuitemaker, H., Kootstra, N. A., de Goede, R. E., de Wolf, F., Miedema, F. & Tersmette, M. (1991) J Virol 65, 356-63.

33. Singh, A. & Collman, R. G. (2000) J Virol 74, 10229-35.

34. Chou, T. C. & Talalay, P. (1984) Adv Enzyme Regul 22, 27-55.

35. Greco, W. R., Bravo, G. & Parsons, J. C. (1995) Pharmacol Rev 47, 331-85.

36. Margolis, L. B., Glushakova, S., Grivel, J. C. & Murphy, P. M. (1998) J Clin Invest 101, 1876-80.

37. Kinter, A., Catanzaro, A., Monaco, J., Ruiz, M., Justement, J., Moir, S., Arthos, J., Oliva, A., Ehler, L., Mizell, S., Jackson, R., Ostrowski, M., Hoxie, J., Offord, R. & Fauci, A. S. (1998) Proc Natl Acad Sci USA 95, 11880-5.
38. Mosier, D. E., Picchio, G. R., Gulizia, R. J., Sabbe, R., Poignard, P., Picard, L., Offord, R. E., Thompson, D. A. & Wilken, J. (1999) J Virol 73, 3544-50.
39. Este, J. A., Cabrera, C., Blanco, J., Gutierrez, A., Bridger, G., Henson, G., Clotet, B., Schols, D. & De Clercq, E. (1999) J Virol 73, 5577-85.
40. Rusconi, S., La Setta Catamancio, S., Citterio, P., Bulgheroni, E., Croce, F., Herrmann, S. H., Offord, R. E., Galli, M. & Hirsch, M. S. (2000) J Virol 74, 9328-32.
41. Singer, II, Scott, S., Kawka, D. W., Chin, J., Daugherty, B. L., DeMartino, J. A., DiSalvo, J., Gould, S. L., Lineberger, J. E., Malkowitz, L., Miller, M. D., Mitnaul, L., Siciliano, S. J., Staruch, M. J., Williams, H. R., Zweerink, H. J. & Springer, M. S. (2001) J Virol 75, 3779-90.
42. Murai, M., Yoneyama, H., Harada, A., Yi, Z., Vestergaard, C., Guo, B., Suzuki, K., Asakura, H. & Matsushima, K. (1999) J Clin Invest 104, 49-57.
43. Hendrix, C. W., Flexner, C., MacFarland, R. T., Giandomenico, C., Fuchs, E. J., Redpath, E., Bridger, G. & Henson, G. W. (2000) Antimicrob Agents Chemother 44, 1667-73.
44. Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben Hur, H., Many, A., Shultz, L., Lider, O., Alon, R., Zipori, D. & Lapidot, T. (1999) Science 283, 845-8.
**Figure Legends**

**Figure 1.** Structures of CCR5 antagonist spirodiketopiperazine derivatives.

**Figure 2.** Inhibition of MIP-1\(\alpha\) binding to CCR5 and MCP-1 binding to CCR2 by E910 and E913.

CCR5-CHO cells and CCR2-CHO cells were exposed to 0.1 nM \[^{125}\text{I}\]-labeled MIP-1\(\alpha\) (Panel A) and 0.1 nM \[^{125}\text{I}\]-labeled MCP-1 (Panel B), respectively, and incubated for 40 min in the presence of increasing concentrations of E910 or E913. The results shown are the mean values (± S.D.) from three independent assays.

**Figure 3.** E913 blocks MIP-1\(\alpha\)-induced intracellular Ca\(^{2+}\) mobilization but fails to block MCP-1, MDC and SDF-1-induced intracellular Ca\(^{2+}\) mobilization.

E913 blocked MIP-1\(\alpha\)-induced intracellular Ca\(^{2+}\) mobilization in CCR5-CHO cells, but failed to block Ca\(^{2+}\) mobilization induced by MCP-1, MDC, or SDF-1 in CCR2-CHO, CCR4-CHO and CXCR4-CHO cells.

**Figure 4.** Effects of E913 combined with AMD-3100 on the replication of R5, X4, and dualtropic HIV-1.

Panel A: E913 (5 \(\mu\)M) completely blocked R5 HIV-1 replication but AMD-3100 (1 \(\mu\)M) totally failed, and no obvious antagonistic effect was seen. Panel B: AMD-3100 (1 \(\mu\)M) completely blocked X4 HIV-1 replication while E913 (1 \(\mu\)M) totally failed, and no obvious antagonistic effect was seen. Panel C: E913 (5 \(\mu\)M) and AMD-3100 (0.1 \(\mu\)M) partially blocked the replication of dualtropic HIV-1, while the combination of E913 and AMD-3100 completely suppressed its replication.

**Figure 5.** Effects of E913 combined with AMD-3100 on the replication of dualtropic HIV-1 and mixed HIV-1 populations.
Panel A: E913, combined with AMD-3100, effectively blocked the replication of dualtropic HIV-1_{89.6} (50 TCID\textsubscript{50})(left). The antiviral activity of the combined drugs was analyzed using the method by Prichard et al. (right)\cite{30} and found to be synergistic.

Panel B: E913, combined with AMD-3100, completely blocked the replication of the 50:50 mixture of R5 HIV-1\textsubscript{BaL} (25 TCID\textsubscript{50}) and X4 HIV-1\textsubscript{NL4-3} (25 TCID\textsubscript{50})(left). The antiviral activity of the combination was also synergistic (right).

**Figure 6.** E913 binds to the domain B of the second extracellular loop of CCR5 (ECL2B).

E913 competitively blocked the binding of two monoclonal antibodies, 45523 reactive against multidomain epitopes of CCR5 and 45531 specific for ECL2B of CCR5. Note that there was no E913 inhibition of the binding of a monoclonal antibody 2D7 which binds to the domain A of the second extracellular loop (ECL2A) of CCR5.
Table 1. Inhibitory activity of novel CCR5 antagonists in the Ca\(^{2+}\) assay and the chemokine-chemokine receptor binding assay

| Compound | MIP-1\(\alpha\) | MCP-1 | MIP-1\(\alpha\)/CCR5 | MCP-1/CCR2 | MDC/CCR4 | SDF-1\(\alpha\)/CXCR4 |
|----------|----------------|-------|---------------------|------------|----------|---------------------|
| E910     | 0.03 ± 0.005   | >30   | 0.1 ± 0.03          | 17 ± 0.5   | 17 ± 3   | 24 ± 2              |
| E913     | 0.002 ± 0.0005 | >30   | 0.02 ± 0.003        | >30        | >30      | >30                 |
| E916     | 0.007 ± 0.001  | >30   | 0.07 ± 0.03         | >30        | >30      | >30                 |
| E917     | 0.009 ± 0.0007 | >30   | 0.08 ± 0.02         | >30        | >30      | >30                 |
| Compound | HIV-1 <sub>BaL</sub> (R5) | HIV-1 <sub>LAI</sub> (X4) | HIV-1 <sub>BaL</sub> (R5) | HIV-1 <sub>NL4-3</sub> (X4) | CC<sub>50</sub> (µM) | SI<sup>b</sup> |
|----------|------------------|-------------------|------------------|------------------|----------------|----------|
| E910     | >1               | >1                | 0.54 ± 0.03      | >1               | 11.7 ± 2.7    | 22       |
| E913     | 0.03 ± 0.008     | >1                | 0.04 ± 0.04      | >1               | 51.8 ± 5.6    | 1295     |
| E916     | 0.07 ± 0.03      | >1                | 0.24 ± 0.09      | >1               | 12.0 ± 0.8    | 50       |
| E917     | 0.06 ± 0.03      | >1                | 0.16 ± 0.04      | >1               | 10.8 ± 0.9    | 68       |
| AMD-3100 | >1               | 0.001 ± 0.0001    | >1               | 0.007 ± 0.0003   | >100          | >14286   |
| AZT      | 0.06 ± 0.02      | 0.076 ± 0.023     | 0.004 ± 0.001    | 0.013 ± 0.003    | >100          | >25000   |

<sup>a</sup> The IC<sub>50</sub> values were determined with the MAGI assay and p24 assay. Cytotoxicity of the test compounds to PBM was determined with the MTT assay.

<sup>b</sup> SI: selectivity index, CC<sub>50</sub> / IC<sub>50</sub>.
|                  | wild-type R5         | MDR R5        | MDR X4        |
|------------------|----------------------|---------------|--------------|
|                  | HIV-1\textsubscript{MOKW} | HIV-1\textsubscript{JSL} | HIV-1\textsubscript{MM} | HIV-1\textsubscript{TM} |
| AZT              | 0.001 ± 0.002 (1x)  | 0.03 ± 0.19 (28x)\textsuperscript{b} | 0.04 ± 0.007 (41x) | 0.1 ± 0.06 (98x) |
| ddl              | 0.48 ± 0.12 (1x)    | 2.50 ± 0.35 (5x)    | 3.9 ± 1.1 (8x)   | 2.50 ± 0.25 (5x)   |
| d4T              | 0.015 ± 0.004 (1x)  | 0.10 ± 0.07 (9x)    | 0.09 ± 0.06 (6x) | 0.05 ± 0.02 (3x)   |
| SQV              | 0.004 ± 0.004 (1x)  | 0.08 ± 0.04 (19x)   | 0.37 ± 0.065 (93x) | 0.07 ± 0.04 (18x) |
| NFV              | 0.014 ± 0.004 (1x)  | >1 (>71x)           | >1 (>71x)         | >1 (>71x)           |
| E913             | 0.04 ± 0.03 (1x)    | 0.06 ± 0.01 (1x)    | 0.05 ± 0.06 (1x) | >1 (>25x)           |
| AMD-3100         | >1                   | >1                   | >1               | 0.008 ± 0.0006     |

\textsuperscript{a} The IC\textsubscript{50} values were determined with the p24 assay.

\textsuperscript{b} Numbers in parentheses represent fold-changes of IC\textsubscript{50} values against each isolate compared to IC\textsubscript{50} values against the wild-type HIV-1\textsubscript{MOKW}. 

---

Table 3. Anti-HIV-1 activity of E913 and AMD-3100 against HIV-1 clinical isolates in PBM.
Figure 1.
Figure 2.
Figure 3.

- MCP-1/CCR2
- MDC/CCR4
- MIP-1α/CCR5
- SDF-1α/CXCR4
Figure 4.
Figure 5.

A  HIV-1_{89.6}

B  HIV-1_{Bal} : HIV-1_{NL4-3} = 50 : 50
Figure 6.
Novel low molecular weight spirodiketopiperazine derivatives potently inhibit R5 HIV-1 infection through their antagonistic effects on CCR5

Kenji Maeda, Kazuhisa Yoshimura, Shiro Shibayama, Hiromu Habashita, Hideaki Tada, Kenji Sagawa, Toshikazu Miyakawa, Manabu Aoki, Daikichi Fukushima and Hiroaki Mitsuya

J. Biol. Chem. published online July 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105670200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts