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RAPID COMMUNICATION

Single oral administration of the novel CXCR4 antagonist, KRH-3955, induces an efficient and long-lasting increase of white blood cell count in normal macaques, and prevents CD4 depletion in SHIV-infected macaques: a preliminary study

Tadashi Nakasone · Sei Kumakura · Michiko Yamamoto · Tsutomu Murakami · Naoki Yamamoto

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Abstract We evaluated the long-term effects of the single oral administration of a new CXCR4 antagonist, KRH-3955, on elevation of white blood cell (WBC), neutrophil and lymphocyte counts in normal cynomolgus monkeys. In the monkeys treated with 0, 2, 20, 200 mg/kg of the compound, WBC, neutrophil and lymphocyte counts increased dramatically at 2 days after treatment. This effect was dose-dependent, and these cell counts remained elevated 15 days after drug treatment. Since neutrophils are the most abundant WBCs in circulation and bone marrow neutrophil exhaustion impairs the response to bacterial infections, it is intriguing to exploit this pharmacological increase of neutrophils as a tool to address its influence on viral infections in vivo. The SHIV infection studies using the SHIV-KS661c/cynomolgus monkey model showed that a single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before virus exposure did not prevent infection, although it did prevent CD4 cell depletion in 3/3 monkeys. Furthermore, single oral administration of the drug 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys. This prevention of CD4 cell depletion was observed in both blood and lymphoid tissues. These results show that natural course of the SHIV infection is modulated by artificial increase of neutrophils and lymphocytes caused by KRH-3955 in the cynomolgus monkey model.

Keywords CXCR4 antagonist · WBC mobilization · SHIV · HIV

Abbreviations

CXCR4	C-X-C motif receptor 4
CCR5	C–C motif receptor 5
SDF-1	Stromal-derived factor-1
CBC	Complete blood cell count
PrEP	Pre-exposure prophylaxis
SHIV	Simian human immunodeficiency virus
PBL	Peripheral blood lymphocytes
PB	Peripheral blood
LT	Lymphoid tissue
LN	Lymph node
WBC	White blood cell
AZT	Zidovudine
IC50	Half maximal inhibitory concentration

Background

Leukocytopenia places individuals at increased risk of infection. Among various leukocytes, neutrophils play an

T. Nakasone (✉) · T. Murakami · N. Yamamoto
AIDS Research Center, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
e-mail: nakabone@nih.go.jp

T. Murakami
e-mail: tmura@nih.go.jp

S. Kumakura
Kureha Corporation, 3-3-2 Nihonbashi-Hamacho, Chuo-ku, Tokyo 103-8552, Japan
e-mail: sei53@kureha.co.jp

M. Yamamoto
Faculty of Pharmaceutical Sciences, Suzuka University of Medical Sciences, 3500-3 Minami-Tamagaki-cho, Suzuka, Mie 513-8670, Japan
e-mail: ymmt@suzuka-u.ac.jp

N. Yamamoto (✉)
Department of Microbiology, National University of Singapore, 5 Science Drive 2, #15-02 Centre for Translational Medicine, Singapore 117599, Singapore
e-mail: micny@nus.edu.sg

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important early defensive role against infection. Neutropenia—a decrease in the neutrophils count—is the most important indicator of infection risk [1]. Neutrophils occupy about 70 % of total leukocyte population. Under normal settings, only a small fraction less than 2 % of the neutrophil pool is circulating in the periphery, whereas most are found to be stored in the bone marrow [2, 3]. Upon exposure to infection, neutrophils are mobilized from the bone marrow and epithelium, and control invading pathogens in the periphery through its specialized innate immune activities such as phagocytosis, release of soluble anti-microbials including granule proteins, and generation of neutrophil extracellular traps [4]. Indeed, it is reported that interruption of the neutrophil supply is detrimental to the control of bacterial infections [5, 6]. After engagement of neutrophils in the process of bacterial killing, they tend to die, while granulocyte colony-stimulating factor is up-regulated to induce granulopoiesis and to replenish the reservoir in bone marrow [7]. The average lifespan of non-activated neutrophils in the circulation is about 5 days but once activated, they survive only for 1–2 days [8]. Through its interaction with CXCR4, SDF-1 retains the leukocyte and stem cells in the bone marrow by a process referred to as homing [9]. AMD3100, a novel antagonist of CXCR4, has been shown to specifically antagonize this interaction and recruit WBC, including neutrophils and CD34 + hematopoietic stem cell to the peripheral blood (PB) in human studies [10].

We have previously reported that unique CXCR 4 antagonists, the KRH series of compounds, exhibit potent and selective anti-HIV-1 activity [11, 12]. One of the derivatives, KRH-3955 is orally bioavailable (25.6 % bioavailability) and has high anti-HIV activity in vitro compared with AMD3100 [12, 13]. Furthermore, KRH-3955 efficiently inhibits the replication of HIV in a human peripheral blood lymphocyte-severe combined immunodeficiency mouse model after a single oral administration, even when given 2 weeks before HIV challenge [12]. Since this long elimination half-life of KRH-3955 (99.0 ± 13.1 h) after a single administration to animals suggests the long-term accumulation of the compound within tissues, it is intriguing to address whether KRH-3955 can also increase the WBC counts in normal monkeys by single oral treatment regimen and modulate the SHIV infection in the SHIV-KS661c/cynomolgus monkey (*Maccaca fascicularis*) model developed as a non-human primate (NHP) AIDS model [14–17].

In the present work, we evaluated initially the long-term efficacy of KRH-3955 on increase of WBC, neutrophils and lymphocytes in normal monkeys through single oral challenge of the drug. Having confirmed that all these WBC populations were efficiently mobilized to PB, we conducted further experiments using our cynomolgus monkey model.

Methods

Animal care

Drug toxicity study with monkeys was carried out in Medicinal Safety Research Laboratories, Sankyo Co. Ltd. (717 Horikoshi, Fukuroi, Shizuoka, 437-0065 Japan). The monkey infection experiments were conducted at the Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBIO), Japan, in accordance with requirements specifically stated in the laboratory biosafety manual of the World Health Organization. They were housed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals and were maintained in accordance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of NIBIO, Japan. Both IACUCs of NIBIO and the National Institute of Infectious Diseases (NIID), Japan, approved the study. Both guidelines are in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research". The use of SHIV was also approved by both Institutional Advisory Committees for the Biosafety of Living Modified Organisms of NIBIO and NIID with the recognition (Dai17-17) of the Japanese Minister of Education, Culture, Sports, Science and Technology, 2005. Ethical standards incorporated into these guidelines and into our routine laboratory procedures include a maximum reduction in the number of animals, a psychological enrichment program, frequent contact with other animals (visual, auditory, and olfactory), regular veterinary supervision and care.

Drugs

The CXCR4 antagonist, KRH-3955 (*N*, *N*-dipropyl-*N*-[4-({[(1*H*-imidazol-2-yl) methyl][(1-methyl-1*H*-imidazol-2-yl) methyl] amino} methyl) benzyl]-*N*-methylbutane-1, 4-diamine tri-(2*R*, 3R)-tartrate), and the CCR5 antagonist, SCH-D, were synthesized and purified by Kureha Corporation [12].

Viruses

SHIV-KS661c, molecularly cloned from SHIV-C2/1, was used in this study. The SHIV-C2/1 stock comprised plasma obtained by serum passages of p-SHIV (derived from SHIV-89.6) in cynomolgus monkeys [14–17]. SHIV-KS661c, SHIV-C2/1, and SHIV-89.6 were CXCR4-tropic viruses. SHIV-KS661c was propagated in CEMx174 cells and was confirmed to be genetically identical to the major sequences of the parent virus. SHIV-KS661c has been

shown to infect cynomolgus monkeys both intravenously and intra-rectally and to induce high-peak viremia and drastic CD4 cell depletion within 2 weeks of inoculation [18–23]. Another SHIV-89.6P used in this study was brother strain of SHIV-KS661c and CXCR4-tropic [24]. Both virus stocks were kept at −80 °C and were thawed immediately prior to use.

In vitro drug susceptibility testing

The susceptibility of SHIV-KS661c to KRH-3955 was determined as follows: Peripheral blood lymphocytes (PBL) obtained from naïve cynomolgus monkeys were stimulated with concanavalin-A (5 μg/ml, Con-A, SIGMA) for 2 days. Con-A-activated PBL (1×10^7 cells/well) were exposed to 100 $TCID_{50}$ of SHIV-KS661c or SHIV-89.6P for 4 h. After extensive washing, cells (2×10^5 cells/well) were incubated with various concentrations of the drugs. The amount of SIV p27 antigen produced in the culture supernatants was measured using an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corp., Buffalo, NY) 12 days after infection. The IC50 was calculated using XLfit analysis software (ID Business Solutions). Zidovudine (AZT, SIGMA) and Vicrovic (SCH-D, Schering-Plough) were used for comparison. All assays were carried out in duplicate in 96-well culture plates.

Drug toxicity study design

A single-dose toxicity study was conducted on male and female cynomolgus monkeys over a 15-day observation period. KRH-3955 was administered at dose levels of 0, 20, 200, and 2,000 mg/ml. Compound was formulated in water and was administered intragastrically (i.g.) via a nasal feeding tube under anesthesia, followed by additional water to wash out any compound remaining in the tube. All groups consisted of two animals/sex. The general condition of the animals including appetite, activity, and body weight was carefully observed. In the toxicity study, blood chemistry, complete blood cell counts (CBC), and WBC populations were automatically measured during the observation period. Phospholipidosis was observed as vacuolation in hematoxylin-eosin staining, and the tissues with vacuolation were characterized using Sudan Black B staining at 200 mg/kg in monkey.

Animal infection study design

Nine cynomolgus monkeys were enrolled and divided into three groups, each containing three animals. Group 1 (G1) was given a single dose of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure; Group 2 (G2) was treated with a single dose of KRH-3955 (100 mg/kg) 2 weeks before viral exposure; and Group 3 (G3, naïve control group) was not treated with any drugs. The study was divided into two research reports: this report and another [25]; group 3 was the same in both papers. KRH-3955 in water was administered i.g. with additional washing same as the above. All monkeys were then intra-rectally challenged with 10 times the AID_{50} (50 % animal infectious dose) of a highly pathogenic SHIV-KS661c. The general condition of the animals including appetite, activity, and body weight was carefully observed. Blood chemistry, CBC, absolute CD4 cell counts, and plasma virus RNA copy number were measured frequently for more than 12 weeks. Finally, the monkeys were euthanized for virological analysis and analysis of the CD4 population in lymphoid tissues.

Real-time RT-PCR quantification of SHIV RNA in plasma

Plasma viral loads were evaluated using real-time reverse transcriptase polymerase chain reaction (RT-PCR) with a TaqMan probe as previously reported [18–20, 25]. Briefly, viral RNA was extracted from the plasma and purified using the QIAamp Viral RNA Mini Kit (Qiagen). For quantitative analysis of the RNA, the TaqMan system (Applied Biosystems) was used with primers and probes targeting the SIVmac239 gag region. The viral RNA was amplified using a QuantiFast Probe RT-PCR Vial Kit (Qiagen) with primers and TaqMan probes. The fluorescence intensity of the RT-PCR product was quantitatively monitored using an Opticon 2 (former MJ Research). The plasma viral load, measured in duplicate, was calculated based on the standard curve for control RNA and on the RNA recovery rate. To obtain the RNA recovery rate, 10^5 copies of SHIV-KS661c were extracted and purified using the same kit in parallel with the sample treatment. The recovered RNA was also amplified at the same time as that of the samples. The limit of detection was approximately 500 RNA copies/ml.

CD4 cells in blood and lymphoid tissues

The absolute CD4 cell count in the PB was measured as previously described [18–20, 25]. Briefly, 50 μl of whole blood was incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource), Phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton–Dickinson), or peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton–Dickinson). After red blood cell lysis using FACS lysis solution (Becton–Dickinson), the cells were analyzed along with reference beads (Beckman Coulter) using a FACS Calibur (Becton–Dickinson) and Cell Quest software (Becton–Dickinson). The monkey lymphoid cells used for

flow cytometric analysis were prepared from thymus, spleen, and lymph node (LN) tissues obtained at necropsy. The cells were stained with the same three antibodies described above and the CD4 population analyzed.

Results

Dramatic and persistent increase of WBC count upon treatment with KRH-3955 of normal monkeys

In the hematological examination in normal monkeys, WBC, neutrophils, and lymphocytes counts increased dramatically in each treatment group (Fig. 1), whereas RBC counts were unchanged with a slight decrease at 2 days of treatment. The peak effects were observed at 2 days after dosing between 20 and 2,000 mg/kg. Strikingly, this enhancing effect of WBC was seen even 15 days after drug treatment. Additional lymphocyte subset analysis indicated that the increase in count was dependent on increases in the T, B, and NK cells (data not shown). There was no increase in immature band forms based on a differential analysis performed in all cases of leukocytosis. The hematological changes are considered to be related to the pharmacological effect of KRH-3955. All the above changes recovered or tended to recover in the 15-day recovery period.

In addition to the above changes, hemoglobin and hematocrit counts decreased in the group receiving 200 mg/kg (data not shown). In the blood chemical examination, aspartate aminotransferase slightly increased in the group receiving 200 mg/kg. In the histo-pathological examination, systemic phospholipidosis related to the pharmacological effect of KRH-3955 was observed in many organs as vacuolar change in the groups receiving >20 mg/kg, which is considered to be toxicologically insignificant. In addition, skeletal muscle injury and renal damage were observed in the 200 mg/kg group.

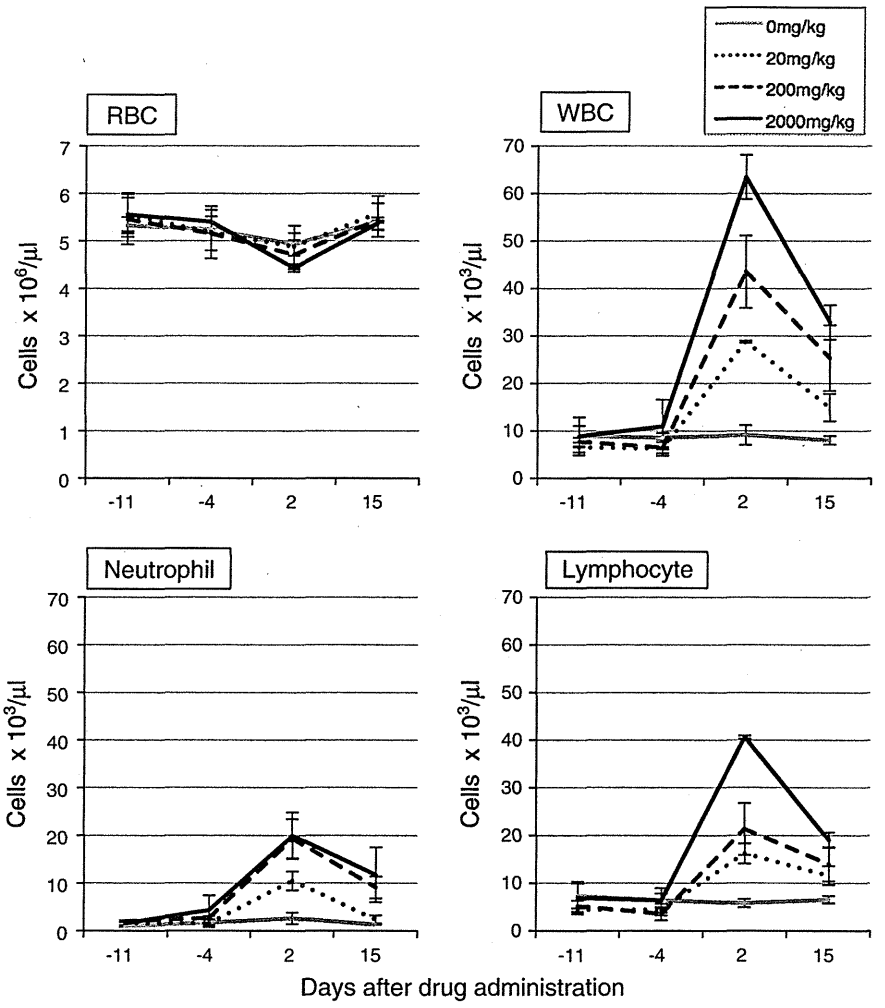

Fig. 1 Hematological examination of monkeys after treatment with KRH-3955. A single-dose study was conducted on male and female cynomolgus monkeys by a 15-day observation period. KRH-3955 dissolved in water was intragastrically administered at day 0 at dose levels of 0 (*gray*), 20 (*dot*), 200 (*dash*), and 2,000 (*black*) mg/ml. The number of red blood cells (RBC), white blood cells (WBC), neutrophils, and lymphocytes were monitored at the indicated time points. *Bars* indicate standard deviation

Anti-viral and CD4 protecting activities of KRH-3955 in SHIV-infected monkeys

In vitro effects of KRH-3955

The inhibitory activity of KRH-3955 against SHIV-Ks661c and SHIV-89.6P was examined in activated cynomolgus PBL from two different donors (Table 1). KRH-3955 inhibited the replication of SHIV-KS661c with an IC50 of 3.1–77.7 nM. Although SCH-D did not inhibit SHIV-KS661c in activated PBL isolated from monkey B (even at a concentration of up to 100 nM), it inhibited the virus in cells from monkey A with an IC50 of 37.9 nM. On the contrary, KRH-3955 inhibited the replication of SHIV-89.6P in activated PBL with an IC50 of 3.6–11.0 nM. SHIV-89.6P did replicate in the presence of SCH-D, even at concentrations up to 100 nM. SHIV-89.6P is highly pathogenic and CXCR4-tropic and has been used extensively in various experiments. AZT, used as a positive control, inhibited both viruses with an IC50 of 8.4–16.2 nM.

In vivo effect of KRH-3955 on PB

The efficacy of KRH-3955 was evaluated in the SHIV-KS661c/cynomolgus monkey model. Single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure did prevent CD4 cell depletion in 3/3 monkeys (Fig. 2, G1). But the drug did not protect monkeys from infection. In this group, two monkeys had an undetectable viral load after peak viremia. Furthermore, single administration of KRH-3955 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys (Fig. 2, G2). In this monkey, the virus did not replicate well during the course of the experiment. All six monkeys in Group 1 and Group 2 showed transient CD4 lymphocytosis (2,077–6,347 cells/μl at peak) for 4 weeks after KRH-3955 administration. We did not observe any abnormal findings in any monkeys during the course of the experiment.

Table 1 KRH-3955 inhibits SHIV replication in simian PBLs in vitro

Conc. (nM)	SHIV-KS661c (% replication)			SHIV-89.6P (% replication)		
	AZT	KRH-3955	SCH-D	AZT	KRH-3955	SCH-D
Monkey A						
100	13.1	49.0	37.1	24.3	15.8	59.5
20	30.7	68.5	51.2	42.4	28.0	74.0
4	65.2	91.8	98.4	63.2	76.7	82.3
0.8	82.7	147.9	173.5	148.6	123.7	134.6
IC50 (nM)	8.4	77.7	37.9	16.2	11.0	>100
Monkey B						
100	8.6	1.1	252.1	5.1	0.5	90.2
20	35.7	1.1	224.0	33.7	0.6	135.7
4	98.2	7.5	249.7	96.2	8.1	128.7
0.8	131.9	153.9	252.3	180.2	175.4	211.7
IC50 (nM)	15.9	3.1	>100	15.5	3.6	>100

Con-A-activated simian PBLs from two cynomolgus monkeys were exposed to SHIVs

Exposed cells were cultivated in the presence of various concentrations of the drugs for 12 days

Infection was confirmed by the presence of SIV p27 antigen and expressed as % replication

IC50 (50 % inhibitory concentration) was calculated using XLfit analysis software (ID Business Solutions)

Assays were carried out in duplicate in 96-well culture plates

The HIV RT inhibitor AZT (zidovudine) and the CCR5 antagonist, SCH-D (vicrovic), were used for comparison

In vivo effect of KRH-3955 on various lymphoid tissues

After more than 12 weeks of observation, all monkeys were killed, and the CD4 cell population in the lymphoid tissues (LTs) was analyzed. Macroscopic findings generally suggested that thymus and any LN were atrophic in those monkeys showing severe CD4 lymphocytopenia (<100 cells/μl) at necropsy. However, no atrophic tissues were found in monkeys not presenting with severe CD4 lymphocytopenia.

The CD4 cell population in the LTs of the monkeys was analyzed by flow cytometry and was expressed as ratio of CD4 cells/CD3 cells (%) (Fig. 3). In Group 1, CD4 cells were well conserved with LTs (>25 %) other than spleen (12–30 %), similar to the results seen for PB. In Group 2, CD4 cells were well conserved within the LTs of two monkeys (>20 %), except in the spleen (13 and 17 %). The CD4 cells of another monkey, which showed moderate CD4 cell depletion in PB, were not rescued in any of the LTs (2–20 %). Thus, the preventive effects of KRH-3955 on CD4 cell depletion were seen not only in PB, but also in several LTs. The total ratio of CD4/CD3, including that in the PB of animals in Group 1 and Group 2, was significantly higher ($p < 0.001$ and $p < 0.05$, respectively) than that in Group 3 (naïve control). In contrast, two monkeys in Group 3 showed severe CD4 cell depletion in all LTs (0–8 %), although the CD4 cells of another monkey were well conserved in all LTs (15–48 %).

Discussion

The increase of WBC, neutrophils, and lymphocytes counts noted in all treatment groups in monkeys receiving parenteral KRH-3955 was dramatic. At 2 days after treatment, there were 3.2–10-, 4–7.5-, and 2.8–7-fold increases for WBC, neutrophils, and lymphocytes, respectively, compared to untreated controls. Though the activity of

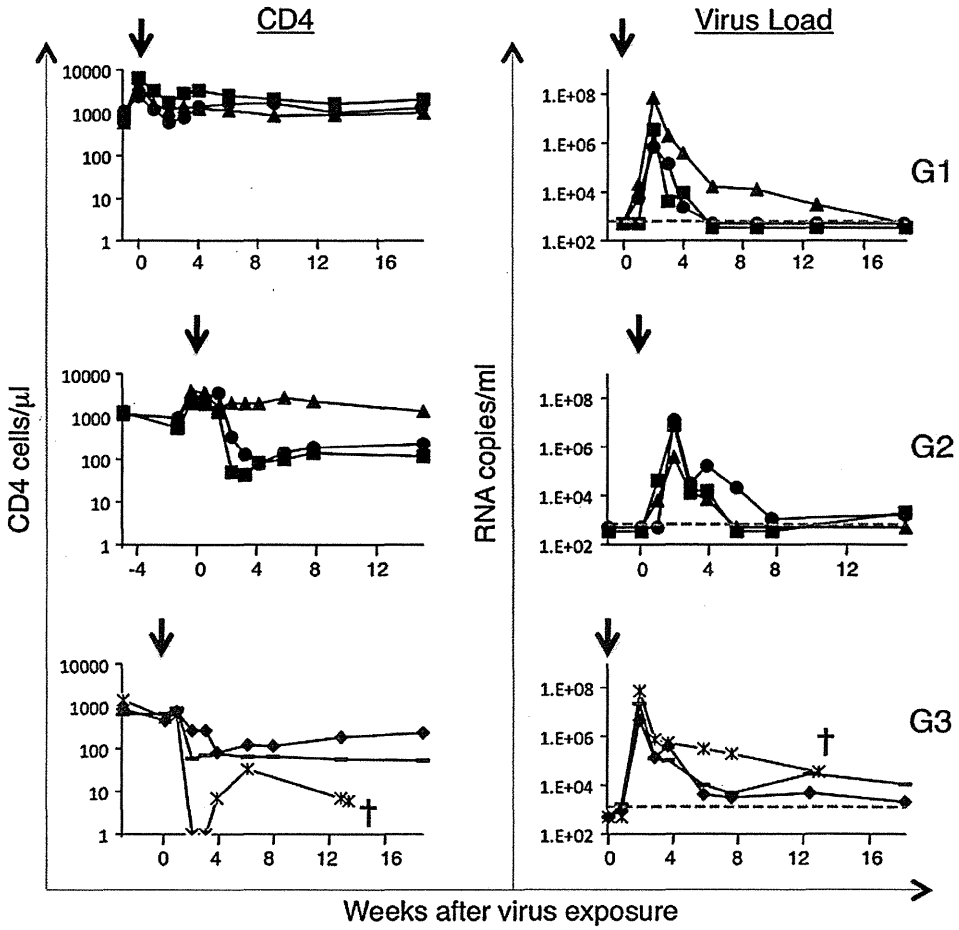

Fig. 2 Effect of PrEP on CD4 cells and virus in peripheral blood. Absolute CD4 cell counts (cells/μl) and virus RNA copy numbers (copies/ml) are shown for monkey peripheral blood and plasma, respectively, during the experiments. Each *symbol* indicates each individual monkey. *Arrows* indicate intrarectal viral challenges. *Dash* indicates threshold (<500 copies/ml) in this system. One naïve control monkey (cross in G3) showing severe CD4 cell depletion and high setpoint viremia was euthanized due to AIDS. *G1* Group 1, *G2* Group 2, *G3* Group 3

KRH-3955 was reversible, its long-lasting activity was also striking because even 15 days after treatment with this compound, significant higher counts were seen (1.8–4.1-, 1.9–9-, and 1.8–2.9-fold increases for WBC, neutrophils, and lymphocytes, respectively, compared to untreated controls). The effect was clearly dose-responsive. It has been already shown that AMD3100, a novel antagonist of CXCR4, specifically antagonizes CXCR4-SDF-1 interaction and mobilizes WBC including neutrophils and CD34 + hematopoietic stem cell counts in the PB in phase I clinical trials [10]. When administered to healthy volunteers, AMD3100 produced an increase in WBC count of 1.5–3.1 times the baseline, peaking at 6 h following the intravenous infusion and largely returning to the baseline at 24 h. We believe that an increase in WBCs induced by KRH-3955 is also CXCR4-mediated since KRH-3955 inhibits both SDF-1 binding to CXCR4 and Ca2++ signaling through the receptor in consistency with the observation with AMD3100. These data suggest that binding of KRH-3955 to CXCR4 may inhibit the chemotactic effects of SDF-1, causing mobilization of WBCs from bone marrow [1, 26].

AMD3100 is now approved as a hematopoietic stem cell mobilizer by FDA (renamed plerixafor or Mozobil™). Since KRH-3955 and AMD3100 show significant difference in terms of anti-CXCR4 efficiency and their long half-life, KRH-3955 could also be considered to use in some disease conditions, especially those where chronic treatment of the drug is needed such as warts, hypogammaglobulinemia, infections, and myelokathexis syndrome [27]. On the other hand, long-term effect of KRH-3955 may complicate the therapy, and in general, a prolonged effect of drugs in elevating WBC count is viewed as a disadvantage in selecting a new drug for the development. However, the effect inherent to this compound provides us with a unique opportunity to study a significance of neutrophils in viral and bacterial infections. Turning this to our own advantage, we attempted to address two issues using the same species of cynomolgus monkey: (1) the possible modulation of natural course of SHIV infection through artificially increasing neutrophils and lymphocytes counts and (2) possibility for this compound as a drug for PrEP using our SHIV-KS661c model. Before starting our in vivo experiments, we confirmed the inhibitory effect of

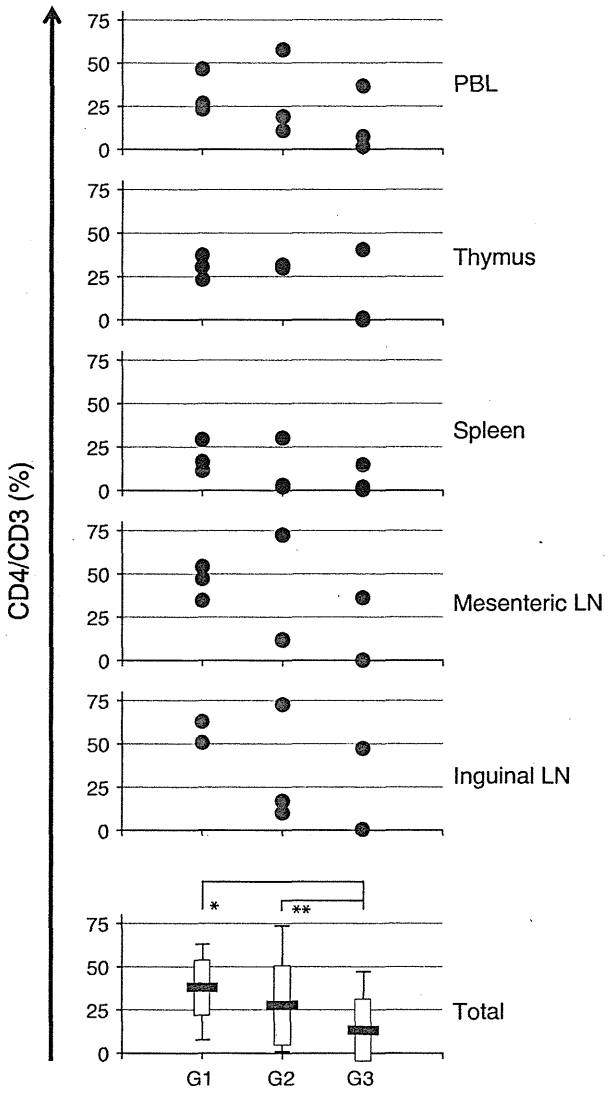

Fig. 3 Effect of pre-exposure prophylaxis against SHIV on various lymphoid tissues of monkeys. The population of CD4 cells in the various lymphoid tissues at necropsy is shown as a ratio of CD4 cells/CD3 cells (%). The ratios of CD4/CD3 in peripheral blood at necropsy are also shown. The total CD4/CD3 ratios assembled from three compartments for analysis using a one-sided Student's *t* test are shown at the *bottom*. *Narrow bars* indicate ranges from minimum to maximum. *Boxes* indicate the standard deviation. *Bold bars* indicate mean values. $*p < 0.001$, $**p < 0.05$. *PBL* peripheral blood lymphocytes, *LN* lymph node, *G1* Group 1, *G2* Group 2, *G3* Group 3

KRH-3955 against in vitro infection by predominantly CXCR4-tropic SHIV-KS661c. The results revealed that single oral administration of KRH-3955 (100 mg/kg) approximately 24 h before viral exposure effectively prevented CD4 cell depletion in 3/3 monkeys. Furthermore, single administration of the drug 2 weeks before viral exposure rescued CD4 cells in 1/3 monkeys. Prevention of CD4 cell depletion was seen not only in PB, but also in several LTs. The characteristically long half-life of KRH-3955 in LTs may contribute to its long-term preventive effects. Thus, artificial increase of WBC induced by KRH-3955 could indeed modulate natural infection process of SHIV significantly. This long half-life of KRH-3955 may contribute to the studies to address the active role of innate immunity through neutrophils in various viral infections, especially acute infection models such as influenza and dengue.

However, KRH-3955 did not protect monkeys from SHIV infection under the same experimental condition. Why did single oral administration of KRH-3955 (100 mg/kg) fail to protect from infection? One possible explanation is that SHIV-KS66c may not be "absolutely CXCR-4-tropic" but is simply "predominantly CXCR4-tropic". This could not be predicted at the stage of our in vitro studies but might be possible to determine in animal experiments where multiple types of cells are present. It is highly likely that some types of cells other than activated cynomolgus PBL which we used in vitro should be susceptible to SHIV infection in monkeys. Another possibility could be due to the experimental system we choose, that is, the single high-dose intra-rectal viral challenge. Under this condition, multiple low-dose intra-vaginal (IVAG) viral challenges would be more likely to result in HIV infection, and KRH-3955 may "miss" an opportunity to block viruses administered via single high-dose intra-rectal challenge. Future studies of multiple high-dose IVAG viral challenges might reveal whether a CXCR4 antagonist is a suitable drug for PrEP or KRH-3955 is useful for PrEP.

In conclusion, single oral administration of KRH-3955 induced long-lasting increase of neutrophils and lymphocytes very efficiently in normal monkeys and prevented CD4 depletion in SHIV-infected monkeys. This efficient and long-lasting effect of KRH-3955 may give us unique opportunity to study an active role of innate immunity, especially that of neutrophils, in various viral infection models. In terms of the usefulness of KRH-3955 as a possible drug for the future long-term intermittent PrEP, further extensive studies are needed.

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Conflict of interest The authors have no conflicts of interest to declare.

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ANNALS OF CLINICAL MICROBIOLOGY AND ANTIMICROBIALS

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Adherence to antiretroviral therapy (ART) during the early months of treatment in rural Zambia: influence of demographic characteristics and social surroundings of patients

Yuri Sasaki[1*], Kazuhiro Kakimoto[2], Christopher Dube[3], Izukanji Sikazwe[4], Crispin Moyo[4], Gardner Syakantu[4], Kenichi Komada[4,5,6], Shinsuke Miyano[4,5,6], Naoko Ishikawa[6], Kiyoshi Kita[7] and Ichiro Kai[7]

Abstract

Background: Around 70% of those living with HIV in need of treatment accessed antiretroviral therapy (ART) in Zambia by 2009. However, sustaining high levels of adherence to ART is a challenge. This study aimed to identify the predictive factors associated with ART adherence during the early months of treatment in rural Zambia.

Methods: This is a field based observational longitudinal study in Mumbwa district, which is located 150 km west of Lusaka, the capital of Zambia. Treatment naive patients aged over 15 years, who initiated treatment during September-November 2010, were enrolled. Patients were interviewed at the initiation and six weeks later. The treatment adherence was measured according to self-reporting by the patients. Multiple logistic regression analysis was performed to identify the predictive factors associated with the adherence.

Results: Of 157 patients, 59.9% were fully adherent to the treatment six weeks after starting ART. According to the multivariable analysis, full adherence was associated with being female [Adjusted Odds Ratio (AOR), 3.3; 95% Confidence interval (CI), 1.2-8.9], having a spouse who were also on ART (AOR, 4.4; 95% CI, 1.5-13.1), and experience of food insufficiency in the previous 30 days (AOR, 5.0; 95% CI, 1.8-13.8). Some of the most common reasons for missed doses were long distance to health facilities (n = 21, 53.8%), food insufficiency (n = 20, 51.3%), and being busy with other activities such as work (n = 15, 38.5%).

Conclusions: The treatment adherence continues to be a significant challenge in rural Zambia. Social supports from spouses and people on ART could facilitate their treatment adherence. This is likely to require attention by ART services in the future, focusing on different social influences on male and female in rural Zambia. In addition, poverty reduction strategies may help to reinforce adherence to ART and could mitigate the influence of HIV infection for poor patients and those who fall into poverty after starting ART.

Background

Sub-Saharan Africa contains nearly 70% of the world's HIV infections. In 2009, the average number of people living with HIV (PLWH) reached 22.5 million [1-3]. Zambia is one of the most severely affected countries in the region. Since Zambia's first reported AIDS diagnosis in 1984, the proportion of PLWH has rapidly increased, peaking in the mid-1990s at about 16% and reaching as high as 25% in some urban areas [4,5]. The Zambia HIV epidemic has moderately stabilized over the last 15 years with a very modest decline after the initial peak in prevalence [6]. Although the rate of new HIV infections has decreased, the total number of PLWH continues to rise. An estimated average of 980,000 (890,000–1,100,000) people in a population of 12.9 million have been infected with HIV in Zambia [6,7]. Adults aged 15–49 had an HIV prevalence of 13.5% in 2009, which was the 6th highest in Sub-Saharan countries [2].

* Correspondence: yurippe1006@hotmail.com
[1]Department of Infection Control and Prevention, Graduate School of Nursing, Nagoya City University, Kawasumi 1, Mizuho-ku, Nagoya-shi, Aichi 467-8601, Japan
Full list of author information is available at the end of the article

The National HIV/AIDS/STD/TB Council (NAC) in Zambia became operational in 2002. One of its key priorities is the provision of care, treatment and support to PLWH [3]. In 2004, the Ministry of Health (MoH) in Zambia offered antiretroviral therapy (ART) at four clinics in Lusaka, the capital of Zambia. The government declared that the entire ART service package would be provided free of charge in the public sector, with a goal of universal access to HIV care and treatment [8].

As more than half of the population lives in rural areas where there is poor access to health services [9], the MoH aimed to develop approaches to expand services by strengthening the existing public health care system and expressed its intention to expand HIV testing and treatment facilities to all districts and as close to households as possible [10]. With this effort, 283,863 (68%) people out of an estimated total population of 416,533 who were in need of ART received it at 447 health facility sites throughout the country by 2009, and the number of sites is being expanded further [6]. While achievements have been remarkable, the universal coverage and retaining the patients on ART remain as challenges. Studies showed that 59.5% of patients in Zambia's southern province throughout a period of 15–723 days (a median follow-up of 275 days) and 62.9% of patients in Lusaka over the first 12 months (a median follow-up of 15.7 months from 12 months onwards) were adherent to ART[11,12].

Some reports also have revealed the factors associated with adherence in both urban and rural settings [8,12-18]. The common reasons raised by patients on ART for poor adherence in several qualitative studies included demographic and physical health factors (e.g. insufficient food and side effects) as well as interpersonal factors (e.g. lack of support) [15,16,18]. Other barriers were patients' mental health factors (e.g. fear of stigma/disclosure and presence of depression/ hopelessness) [16,18].

Such qualitative studies in Zambia generate information from the respondent's perspective that may facilitate culturally appropriate and effective interventions. However, few quantitative studies have investigated associations between the treatment adherence and factors identified in these qualitative studies. In addition, there is a paucity of studies which have investigated factors related to the treatment adherence during the early months of treatment. Since most complications including deaths occur within early period of treatment [8], optimizing adherence during this stage is important for ensuring long-term immunological and virological treatment success [19,20].

Thus, the objective of this study was to identify predictive factors associated with ART adherence during the early months of treatment in rural Zambia so as to propose possible interventions for future treatment strategies in this region.

Methods

Study design and study site

A field based observational longitudinal study was conducted in Mumbwa district, which is located 150 km west of Lusaka. There were one district hospital and 27 rural health centers in Mumbwa during the study period. Among the health facilities, ART services were available at the district hospital, and at eight rural health centers.

Study population

During the study period, all patients who met the criteria of treatment initiation according to the Zambia HIV National Guidelines and visited the district hospital or one of the eight rural health centers were invited to participate in the study. The eligible population for this study comprised patients who 1) were aged 16 and over; 2) were ART-naïve and newly registered for ART services from September to November 2010; and 3) agreed to give informed consent. The exclusion criteria included patients who were too ill to be interviewed. Patients were interviewed at the initiation of treatment as a baseline and six weeks later as a follow-up (Figure 1).

Study tools

Structured questionnaires for both baseline and follow-up interviews were developed, following the generic tools developed by World Health Organization (WHO) [21] and the AIDS clinical trial group (ACTG) adherence follow-up questionnaires [22]. The questionnaires were first developed in English. After being translated into Nyanja (dominant regional language), these were back-translated to English to ensure its clarity and consistency. The questionnaires covered respondents' sociodemographic characteristics, ART adherence, disclosure status,

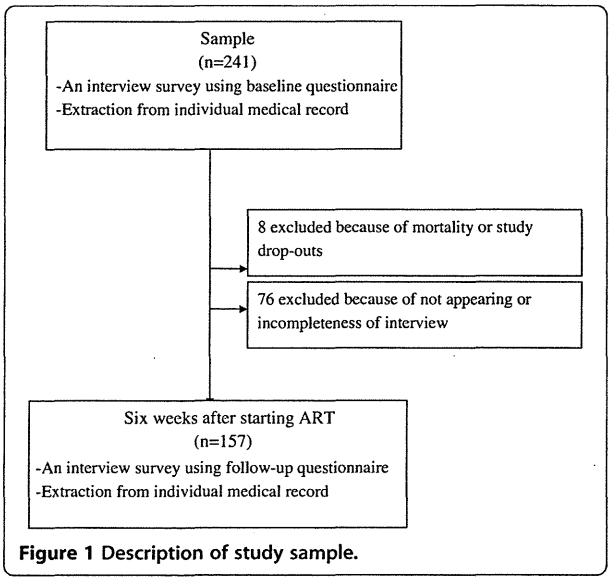

Figure 1 Description of study sample.

physical and mental health related characteristics. WHO HIV/AIDS stage [23], weight, CD4 cell count, and Tuberculosis (TB) status were extracted from individual medical records.

In this study, full adherence to ART was defined as when a patient had never skipped prescribed drugs and had followed time restrictions during the previous four days before the interview. Scores for internalized AIDS-related stigma and cognitive/affective depression were measured using scales developed and validated by Kalichman [24]. To simplify the administration, the items were responded to dichotomously, 1=agree and 0=disagree; and scale scores represent the sum total of endorsed stigma items, range 0–6. The cognitive/affective depression subscale is an 11-item measure that assesses symptoms of depression over the previous seven days, 0=no days, 1=1-2 days, 2=3-4 days, and 3= 5–7 days. The median of each scale score was used as the cutoff point between patients who had self-stigma or depressive symptoms and who did not.

Food insufficiency was measured by using one closed-ended question following the Sociodemographic Module of the Client Instrument developed by WHO as a generic tool for operational research on HIV testing, treatment and prevention [21]. Patients were asked to recall the frequency with which there was not enough food in the month prior to interview: never, sometimes, often, or almost always. A similar single question assessment of food insufficiency has been validated in previous studies [25,26].

Data collection

Before the commencement of the study, a three-day training course on research protocols, administration of questionnaires, and ethics was conducted for eight interviewers. Then field surveys were carried out from September 2010 to March 2011.

Data analysis

Data obtained from the questionnaire surveys were analyzed with SPSS version 19 statistical software. Baseline characteristics of participants were compared between patients who were adherent to ART six weeks after starting the treatment and those who were not, using Pearson's chi-square test and Fisher's exact test. Multiple logistic regression analysis was performed to identify the predictive factors associated with ART adherence. The variables of which the associated p value level was less than 0.1 were entered into the multiple logistic regression model. If the variable was highly correlated with the other variable, one of them was removed from the model. An adjusted odds ratio (AOR) was calculated for the levels of the other factors included in the model.

Ethical considerations

This study was approved by the Research Ethics Committee of the University of Tokyo, the Biomedical Research Ethics Committee of the University of Zambia, and the Institutional Ethics Committee of the National Center for Global Health and Medicine (NCGM). Written informed consent was obtained from respondents at the beginning of the interview, after the study was explained to them. They were informed that participation in the study was voluntary.

Results

Description of study sample

During the field research, 241 patients who were newly registered for ART services were enrolled and interviewed enrolled and interviewed (Figure 1). After the baseline survey, 84 could not be interviewed six weeks later because six patients had passed away, two patients referred to the other health facilities, and 76 patients did not answer questions completely at the baseline or did not appear on the appointment date and we could not reach their contact address. The sociodemographic, physical and mental health of these patients did not differ from the patients included in the analysis except for the required time to reach the health facilities (Table 1). Therefore, data from the remaining 157 were used for statistical analysis.

Demographic characteristics of patients on ART

Seventy-four (47.1%) of patients visited the district hospital for ART services (Table 2). The median age was 35 years old (range: 18–68), 94 (59.9%) were female, and 105 (66.9%) were married. Around half of patients (n = 77, 49.4%) had not completed the standard primary education of seven years and 107 (68.2%) were farmers. Sixty-one (38.9%) reported experiences of food insufficiency in the previous 30 days.

With regard to the transportation, 127 (80.9%) did not need to pay the transportation fee to access the district hospital or rural health centers. Over half of patients (n = 80, 51%) travelled to the health facility on foot and 85 (54.8%) required more than one hour for this journey. The general physical and mental health of patients was also surveyed. One hundred thirty-eight (87.9%) had a functional status of being able to work, and 11 (7.1%) were smear-positive for TB. Over half of patients had self-stigma (n = 87, 56.5%) and were depressed (n = 92, 58.6%). In addition, 89 (85.6%) disclosed their HIV status to their spouse, and 36 (29.3%) spouses were also receiving ART (Table 3).

Status of ART adherence

Among 157 patients on ART who were included in this study, 94 (59.9%) were fully adherent and 63 (40.1%)

Table 1 Characteristics of patients who were excluded compared with those included in the study

	Excluded (n=84) n(%)	Included (n=157) n(%)	p-value
Site			
Mumbwa District Hospital	42 (50.0)	74 (47.1)	0.671
Rural Health Centers	42 (50.0)	83 (52.9)	
Age			
<35 years old	40 (47.6)	70 (46.1)	0.817
≥35 years old	44 (52.4)	82 (53.9)	
Sex			
Female	43 (51.2)	94 (59.9)	0.195
Male	41 (48.8)	63 (40.1)	
Education			
No or primary incomplete	39 (46.4)	77 (49.4)	0.665
Primary complete or more	45 (53.6)	79 (50.6)	
Marital status			
Married	46 (55.4)	105 (66.9)	0.080
Not married	37 (44.6)	52 (33.1)	
Occupation			
Agriculture	49 (58.3)	107 (68.2)	0.128
Others	35 (41.7)	50 (31.8)	
Transportation			
On foot	50 (59.5)	80 (51.0)	0.204
Others	34 (40.5)	77 (49.0)	
Time required for transportation to health facilities by above method			
Within one hour	51 (61.4)	70 (45.2)	0.017
More than one hour	32 (38.6)	85 (54.8)	
Transportation cost			
Zero	72 (85.7)	127 (80.9)	0.347
More than zero	12 (14.3)	30 (19.1)	
Lack of food during the past 30 days			
Yes	27 (32.5)	61 (38.9)	0.334
No	56 (67.5)	96 (61.1)	
Functional status			
Working	69 (85.2)	138 (87.9)	0.556
Ambulant or bed lid	12 (14.8)	19 (12.1)	
Tuberculosis			
Positive	9 (11.1)	11 (7.1)	0.286
Negative or not sure	72 (88.9)	145 (92.9)	
Self-stigma at baseline			
Yes (Score: 2-6)	52 (62.7)	87 (56.5)	0.359
No (Score: 0-1)	31 (37.3)	67 (43.5)	
Depressive symptoms at baseline			
Yes (Score: 13-33)	50 (62.5)	92 (58.6)	0.562
No (Score: 0-12)	30 (37.5)	65 (41.4)	

Pearson's chi-square test.

Table 2 Sociodemographic, physical mental health characteristics of patients who were adherent and non-adherent to ART

	Total (n=157) n(%)	Not full adherence (n=63) n(%)	Full adherence (n=94) n(%)	p-value
Site				
Mumbwa District Hospital	74 (47.1)	32 (50.8)	42 (44.7)	0.452
Rural Health Centers	83 (52.9)	31 (49.2)	52 (55.3)	
Age				
<35 years old	70 (46.1)	25 (41.7)	45 (48.9)	0.381
≥35 years old	82 (53.9)	35 (58.3)	47 (51.1)	
Sex				
Female	94 (59.9)	30 (47.6)	64 (68.1)	0.010*
Male	63 (40.1)	33 (52.4)	30 (31.9)	
Education				
No or primary incomplete	77 (49.4)	30 (48.4)	47 (50.0)	0.844
Primary complete or more	79 (50.6)	32 (51.6)	47 (50.0)	
Marital status				
Married	105 (66.9)	45 (71.4)	60 (63.8)	0.321
Not married	52 (3.1)	18 (28.6)	34 (36.2)	
Occupation				
Agriculture	107 (68.2)	26 (41.3)	24 (25.5)	0.038*
Others	50 (31.8)	37 (58.7)	70 (74.5)	
Transportation				
On foot	80 (51.0)	36 (57.1)	44 (48.8)	0.204
Others	77 (49.0)	27 (42.9)	50 (53.2)	
Time required for transportation to health facilities by above method				
Within one hour	70 (45.2)	29 (46.8)	41 (44.1)	0.742
More than one hour	85 (54.8)	33 (53.2)	52 (55.9)	
Transportation cost				
Zero	127 (80.9)	50 (79.4)	77 (81.9)	0.690
More than zero	30 (19.1)	13 (20.6)	17 (18.1)	
Lack of food during the past 30 days				
Yes	61 (38.9)	14 (22.2)	47 (50.0)	<0.001***
No	96 (61.1)	49 (77.8)	47 (50.0)	
Knowing HIV status				
Within 30 days	65 (41.7)	37 (39.8)	28 (44.4)	0.562
More than 30 days	91 (58.3)	56 (60.2)	35 (55.6)	
Functional status				
Working	138 (87.9)	58 (92.1)	80 (85.1)	0.190
Ambulant or bed lid	19 (12.1)	5 (7.9)	14 (14.9)	
Tuberculosis				
Positive	11 (7.1)	5 (7.9)	6 (6.5)	0.722
Negative or not sure	145 (92.9)	58 (92.1)	87 (93.5)	

Pearson's chi-square test; *p<0.05;***p<0.001.

Table 3 Interpersonal characteristics of patients who were adherent and non-adherent to ART

	Total (n=157) n(%)	Not full adherence (n=63) n(%)	Full adherence (n=94) n(%)	p-value
Disclose to First wife/husband if married				
Yes	89 (85.6)	35 (77.8)	54 (91.5)	0.048 [a*]
No	15 (14.4)	10 (22.2)	5 (8.5)	
First additional sexual partner				
Yes	13 (59.1)	7 (63.6)	6 (54.5)	1.000 [b]
No	9 (40.9)	4 (36.4)	5 (45.5)	
People in community know my HIV status even though I did not tell them				
Yes	48 (38.6)	21 (37.5)	27 (31.0)	0.424 [a]
No or unsure	95 (66.4)	35 (62.5)	60 (69.0)	
First wife/husband's HIV status if married (including divorced, separated, widow/widower)				
Positive	58 (44.6)	20 (37.7)	38 (49.4)	0.190 [a]
Negative or unsure	72 (55.4)	33 (62.3)	39 (50.6)	
First wife/husband is (was) on ART (including divorced, separated, widow/widower)				
Yes	36 (29.3)	10 (20.0)	26 (35.6)	0.062 [a]
No or unsure	87 (70.7)	40 (80.0)	47 (64.4)	
First additional sexual partner's HIV status				
Positive	0 0.0	0 0.0	0 0.0	
Negative or unsure	21 (100.0)	10 (100.0)	11 (100.0)	
First additional sexual partner is on ART				
Yes	0 0.0	0 0.0	0 0.0	
No or unsure	18 (100.0)	9 (100.0)	9 (100.0)	

a: Pearson's chi-square test; b: Fisher's exact test; *p<0.05.

were non-adherent to their treatment six weeks after starting ART.

Factors associated with full ART adherence

Bivariate analysis indicated that being female (p = 0.010), disclosure to spouses (p = 0.048), and experiences of food insufficiency in the previous 30 days (p < 0.001) were positively associated with adherence to ART, but being farmers (p = 0.038) were negatively associated (Tables 2 & 3). Additionally, the proportion of patients who were adherent to ART was likely to be higher among patients whose spouse was also receiving ART than patients whose spouse was not, although this was not statistically significant (p = 0.062) (Table 3).

To identify the factors associated with full ART adherence, multiple logistic regression analysis was performed. Full adherence was associated with being female [AOR, 3.3; 95% Confidence interval (CI), 1.2 - 8.9], having a spouse who were also receiving ART (AOR, 4.4; 95% CI, 1.5 - 13.1) and experience of food insufficiency in the previous 30 days (AOR, 5.0; 95% CI, 1.8 - 13.8) (Table 4).

Table 4 Multiple logistic regression analysis of factors affecting adherence to ART (n=96)

Variable	B	SE	β	p	AOR	95% CI
Gender (Females)	0.21	0.09	0.21	0.021*	3.26	1.20-8.90
Experience of food insufficiency in the previous 30 days	0.30	0.09	0.31	0.002**	5.00	1.81-13.76
Disclose HIV status to spouse	0.20	0.12	0.15	0.130	2.85	0.73-11.06
Spouse on ART	0.26	0.09	0.25	0.007**	4.44	1.50-13.12
R^2	0.28					

B: Unstandardized coefficient; SE: Standard error; β: Standardized coefficient; p value at <0.05* & <0.01**; AOR: Adjusted odds ratio; CI: Confidence interval; R^2: Coefficient of determination. The variables of which the associated p value level was less than 0.1 by an univariate analysis were entered into a multiple logistic regression model.

Reasons for missed doses

Among patients who were not adherent to ART (n=63), 39 gave reasons for missed doses. Some of the most common reasons were long distance to health facilities (n = 21, 53.8%), food insufficiency (n = 20, 51.3%), being busy with other activities such as work (n = 15, 38.5%), being depressed (n = 5, 12.8%), and forgetfulness (n = 4, 10.3%) (Table 5).

Discussions

Status of ART adherence in the Mumbwa district

Based on a self-report, 59.9% were considered to be fully adherent to ART six weeks after starting the treatment. The adherence was lower than that found in studies conducted in other countries in Sub-Saharan Africa, which have shown a full medication adherence of 76% [27]. Although a simple comparison is not accurate because of large variation between the surveys, the difference could be explained as follows.

First, it could be related to the different approach for measuring ART adherence. As long-term viral suppression requires consistent and high level dose adherence accompanied by optimal inter-dose intervals [28], adherence to dose as well as adherence to schedule was considered in this study. Those who never skipped prescribed drugs and followed time restrictions during the previous four days were considered to be fully adherent. In contrast, most other studies that have measured ART adherence using patient self-reporting have only looked at adherence to dose instructions [27].

Second, possible difference in access to treatment among patients who were in need for ART in Zambia and other countries in Sub-Saharan Africa could also have influenced the result. Nearly 70% of those in need for treatment have accessed it in Zambia in both urban and rural areas. On the other hand, studies in other African countries were conducted mostly in urban areas [27] where patients have relatively easy access to health facilities. It could be expected that relatively high levels of adherence in other studies may decline as treatment access expands to rural area where people have poor access to the health facility.

Table 5 Reasons for missed doses (multiple answers) (n=39)

	n(%)
Long distance to health facilities	21 (53.8)
Not enough food	20 (51.3)
Were busy with other things like work etc.	15 (38.5)
Felt depressed, overwhelmed, hopelessness	5 (12.8)
Forgot	4 (10.3)
Felt sick or ill	3 (7.7)
Ran out of pills	2 (5.1)
Traditional prohibition	2 (5.1)
Felt asleep or slept through dose tine	1 (2.6)
Had a change in daily routine	1 (2.6)
Had problem taking pills at specific times with meals	1 (2.6)
Sold out the pills	1 (2.6)

Factors related to full adherence to ART

In a multivariate analysis, gender remained a significant factor after adjusting for potential confounding variables. In a study in rural Uganda, female patients had a significantly higher CD4 cell count at the initiation of ART and lower mortality six months later than male patients, as female patients had more opportunities to access care and start treatment at less advanced stages of HIV, potentially through their participation in prevention of mother-to-child transmission (PMTCT) programs [29]. In Zambia, PMTCT initiative was launched in 1999 and expanded such that an estimated 69% of pregnant women living with HIV had received antiretroviral (ARV) drugs for PMTCT by the end of 2009 [30]. As the case of Uganda, national PMTCT services in Zambia may have contributed to earlier access to ART and supported better ART adherence for a larger group of HIV-positive female, since early ART results in less AIDS progression and death with no increase in adverse events or loss of virologic response compared to deferred ART [31].

Although not determined in this study, sex differences in treatment response and side effects could also have contributed to the outcomes observed in this study. Another study showed that female patients had better responses to treatment compared with that of male patients, and side effects related to ARV drugs were more frequently observed in male patients than in female patients [32].

Additionally, female patients may have had greater motivation to adhere to ART as suggested by other study that female patients caring for children emphasize their role as primary care to children and their children are known to be a facilitating factor in adherence among them [33].

However, other studies have also shown that HIV-positive females often experience gender-related barriers to accessing health services, thus affecting ART adherence [34,35]. For example, many females have to obtain permission from a male spouse or a relative to seek HIV care, which is difficult when females have to ask for money and take time away from household chores. In addition, where costs for treatment are involved, families may prioritize paying for male's treatment [34]. Gender-based violence has also affected female's access and ART adherence [36]. Although these barriers were not found in this study, the issue demands further exploration,

particularly given the different social influences on male and female [37].

Regarding HIV disclosures, over 80% disclosed their status to their spouse. This was positively associated with treatment adherence in bivariate analysis, although it was not seen in the final analysis. The spousal disclosure rate was relatively higher in this study than in a study conducted in another part of rural Zambia in 2005–2006 [12]. The higher rate of disclosure in this study may be attributed to the establishment of peer counselors or treatment supporters during the past few years in Zambia, which encourages disclosure and treatment adherence in the district hospital and rural health centers. However, disclosure could still have both positive and negative consequences. Disclosure has the potential to yield much-needed social support. Alternatively, it may also lead to stigmatization, discrimination, abandonment or gender-based violence mentioned above after disclosing their HIV status to their spouse or partners. Strategies are needed to take account of HIV positive patients who want to disclose HIV status safely to their surroundings.

Having a treatment partner such as a spouse, family member, friends or peer counselor is known to be positively associated with ART adherence [38]. In this study, having a spouse who is also on ART was found to be positively associated with ART adherence. Spouses on ART might play a role as a treatment partner more readily than spouses not on ART because they have a better understanding of treatment adherence for their partners and themselves. While it could not be determined from the results whether spouses were supportive, ART programs should consider the potential benefit of treatment support provided by persons close to patients, especially from those on ART.

A number of qualitative studies have reported that food insufficiency is an important barrier to ART adherence [15,16,39,40]. In urban Peru, Franke et al. [26] found that individuals who reported food insufficiency in the month prior to interview were more likely to experience suboptimal adherence than those who did not. In three rural ART clinics located in another part of rural Zambia, patients who had skipped a meal because of a lack of food in the past week were more likely to have poor adherence [41].

However, on the contrary, this study found that the experience of food insufficiency in the previous 30 days from the baseline interview date was positively associated with treatment adherence. This may be explained by enhanced social support targeting people living in such extreme poverty that they cannot afford to buy food described as below. A pilot study in Zambia found that individuals with food insufficiency who received nutritional support demonstrated significantly better ART adherence compared with a group who did not receive this support [13]. The World Food Program (WFP) has been implementing a program in Zambia since 1967 and is committed to providing food assistance to approximately 2.3 million people in Zambia in 2011 [42]. Patients who were extremely poor and suffered from insufficient food at the initiation of ART might more easily receive such assistance compared with patients who were not so poor that they ever experienced food insufficiency at the time, although it is difficult to rely only on such explanation to account for everything since some donors have suspended their assistance in Mumbwa.

On the other hand, one of the most cited reasons for missing doses by this study population was also 'food insufficiency', and the experience of food insufficiency in the previous 30 days from the follow-up interview date was also associated with poor treatment adherence by bivariate analysis (data not shown). This paradoxical finding could be understood by considering the timing of the interviews. The question about reasons for missing doses was asked six weeks after starting ART, while the positive association was found between treatment adherence and the experience of food insufficiency in the previous 30 days from the baseline interview date. Therefore, patients who experienced food insufficiency in the 30 days previous to ART initiation might have received food supplementation or counseling afterwards, which might have supported their adherence positively as mentioned above. However, patients who were not so poor thus they could not receive food assistance at the initiation of ART might have needed financial assistance after starting the treatment because of transportation fee and loosing wages due to long waiting times for a clinic visit. They might eventually have fallen into poverty and food insecurity after starting ART, and could not adhere to treatment, even if the ART services are provided free of charge in Zambia.

Moreover, some patients may have been taught and believed that ARV drugs always need to be taken with food and some might have missed the medication as they missed their meal due to the food insufficiency. Thus, individuals who missed ARV drugs might consider food insufficiency the reason. However, more studies are needed to better understand the association between ART adherence and food issues, because a single question was used to assess food insufficiency in this study, which is only one aspect of the issues.

In addition to 'food insufficiency', the frequently cited reasons for missing doses in this study were 'long distance to health facilities' and 'being busy with other things like work'. This observation is consistent with findings in other studies [16,18,27,39,43-54].

Although time required for transportation to the health facilities and transportation fees were not significantly

associated with treatment adherence, accessing to treatment facilities can be a problem for many patients living in Mumbwa. This is supported by the result that it took over one hour to reach health facilities in half of patients on ART in this study. Because of this long distance to access to health facilities in Mumbwa, patients who missed doses might report that the distance from home to the district hospital or rural health centers caused the disruption in ART adherence.

In addition, patients whose occupation was agriculture were more likely to have poor adherence. It is possible that patients who worked in agriculture had difficulty coming for health facilities because of their work's seasonal nature. This is probably supported by our finding that 'being busy with other things like work' was the major reason for missing doses.

To enhance understanding of self-stigma and depressive symptoms among patients on ART in Zambia, the associations between these psychological factors and treatment adherence were assessed. Although these factors have been identified as factors associated with poor adherence in multiple studies, no associations were found in this study. The limited numbers of subjects with poor adherence may have prevented the identification of potential associations. More work is needed to investigate patients' self-stigma and depressive symptoms with having to adhere to lifelong regimens.

Limitations

This study has several limitations. First, assessment of treatment adherence based on a self-report may be subject to recall and social desirability bias that may result in under-reporting of missed pill intakes. Thus, an overestimation of adherence is possible. However, there is evidence that a simple self-report adherence questionnaire provides a sensitive measure of non-adherence that predicts viral rebound and is almost always reliable [55-57]. It is also an inexpensive and quick method to use in a field research and resource poor setting.

Second, we could only include those individuals who initiated ART at the target health facilities, and returned six weeks later. Hence, we may also have slightly overestimated the actual adherence levels of this population, and our sample may not be enough to detect significant associations between patients who were adherent to ART six weeks after starting the treatment and who were not. However, this influence is likely to be limited because there were no significant differences in basic sociodemographic or health characteristics between patients who were included or excluded in this study except for the required time to access health facilities (Table 1).

Third, in relation to recent changes in Zambia, this study was conducted in a rural area where ART services have been initiated. Therefore, it is difficult to generalize the study findings to the population in areas where ART services are not yet available, although there were no differences in patients' characteristics between those who visited the district hospital and rural health centers.

Future challenges

This study investigated patients' adherence to ART over a short period of time, because the initial response to ART has long-term prognostic significance, and optimizing adherence in the early months is important for ensuring long-term immunological and virological success [19,20]. However, long-term analyses are clearly needed to fully assess factors related to treatment adherence and to allow some generalizability of the results.

In addition, one of the most cited reasons for missing doses was long distance to health facilities, and only difference in basic sociodemographic or health characteristics between patients who were included and excluded in this study was required time to access health facilities. It is therefore necessary to examine the association between travel-related variables and adherence to ART in detail in future research, although they did not predict the adherence in another study in rural Zambia [14].

Finally, recent changes such as the adoption of free access to ART in Zambia may have some implications for the study results. While it is expected that this policy will reduce financial constraints, the high level of other health expenditures still experienced by patients suggests that the detrimental influence of out-of-pocket payments will certainly not be fully eliminated. Further studies are needed to assess and examine this policy for treatment adherence and its interruptions over longer time scales.

Conclusions and recommendations

Social supports from spouses and people on ART could facilitate their adherence to ART. This is likely to require attention by ART services in the future, focusing on different social influences on male and female in rural Zambia. In addition, poverty reduction strategies could help to reinforce adherence to ART and mitigate the influence of HIV infection for poor patients and those who fall into poverty after starting ART.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YS, KKa, SM, NI, KKi and IK carried out data analysis and drafted this manuscript. YS, CD, IS, CM, GS, KKo, SM and NI helped to collect data and participated in coordinating the study design to involve trained interviewers. KKa, KKi and IK helped with the design of this study. All authors read and approved the final manuscript.

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Author details

[1]Department of Infection Control and Prevention, Graduate School of Nursing, Nagoya City University, Kawasumi 1, Mizuho-ku, Nagoya-shi, Aichi 467-8601, Japan. [2]School of Nursing, Osaka Prefecture University, 3-7-30, Habikino-shi, Osaka 583-8555, Japan. [3]Mumbwa District Health Office, P.O.Box 830018, c/o Mumbwa, Zambia. [4]Ministry of Health, Ndeke House, P.O.Box 30205, Lusaka, Zambia. [5]Japan International Cooperation Agency (JICA), Nibancho Center Building 5-25, Niban-cho, Chiyoda-ku, Tokyo 102-8012, Japan. [6]Department of International Medical Cooperation, National Center for Global Health and Medicine (NCGM), 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan. [7]Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

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ORIGINAL ARTICLE

Activation of the AKT/cyclin D1/Cdk4 survival signaling pathway in radioresistant cancer stem cells

T Shimura[1], N Noma[1], T Oikawa[1], Y Ochiai[1], S Kakuda[1], Y Kuwahara[1], Y Takai[2], A Takahashi[3] and M Fukumoto[1]

Radioresistance, which is a major cause of failure of radiotherapy (RT), is proposed as one of the intrinsic characteristics of cancer stem cells (CSCs) whose unique DNA damage response (DDR), efficient DNA repair and resistance to apoptosis are thought to confer the phenotype. We have isolated surviving CSCs by exposure to long-term fractionated radiation for 82 days from HepG2 and A172 cells (82FR-31NR cells). 82FR-31NR cells exhibited CSC properties, such as high expression of CSC marker CD133 and the ABC transporters (MDR1 and BCRP1), and high tumorigenic potential after transplantation into nude mice. The advantage of our isolated CSCs is that they can proliferate in as the same growth medium as that of parental cells without loss of CSC properties. Therefore, we can analyze DDR of non-stem cells and CSCs without any influences caused by different culture conditions. 82FR-31NR cells showed efficient DNA repair of radiation-induced DNA damage and radioresistance with activation of the AKT/cyclin D1 survival signaling pathway. In contrast, DNA damage persisted for a long time after irradiation in parental cells compared with isolated CSCs. Persisted DNA damage induced apoptosis in parental cells without activation of the AKT/cyclin D1 pathway. Therefore, inhibition of the AKT/cyclin D1 pathway by an AKT inhibitor, API-2, or cyclin D1 siRNA resulted in a loss of efficient DNA repair and radiosensitization of 82FR-31NR cells. Furthermore, knockdown of Cdk4 by its siRNA or a Cdk4 inhibitor was sufficient to suppress radioresistance of CSCs. In this study, we present a newly discovered DDR regarding the AKT/cyclin D1/Cdk4 pathway in response to radiation in CSCs. Combination of fractionated RT and reagents targeting the AKT/cyclin D1/Cdk4 pathway to eradicate CSCs would be effective therapeutic modality.

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INTRODUCTION

Radiotherapy (RT) is one of the major modalities of cancer treatment with excellent tumor control, preservation of normal tissues and less systemic influences. General protocol of fractionated RT consists of daily exposures to a fraction dose of around 2 Gy for 5–7 weeks. Although tumors receive a large total dose by multiple fractionated radiation (FR), they sometimes recur with radioresistance. Repopulation of surviving tumor cells during fractionated RT limits the efficacy of RT and is the major cause of failure of RT.[1] Tumors consist of a heterogeneous population of cells that contain a subpopulation of cancer stem cells (CSCs) that are defined by the capacity of self-renewal and the generation of heterogeneous lineages of cancer cells.[2] Tumor radioresistance is thought to be caused by CSCs, which harbor preferential activation of the DNA damage response (DDR), efficient DNA repair machinery and resistance to apoptosis.[3–6] Therefore, targeting CSCs is likely to be the key to cure cancer for the development of more effective fractionated RT.[7] Despite recent progress in CSC study, our knowledge of the molecular target for suppressing radioresistance of CSCs remains to be answered.

Stem cell medium containing epidermal growth factor and basic fibroblast growth factor without serum is required for *in vitro* culture of CSCs. The PI3K/AKT pathway is activated in stem cell medium, which is important in the maintenance and survival of CSCs *in vitro*.[8–10] Accumulating evidence suggests that the PI3K/AKT signaling pathway is a major contributor to tumor radioresistance. Active AKT, a common mediator of cell survival signals induced by radiation through multiple intracellular signaling pathways,[11,12] suppresses apoptosis. AKT positively regulates cyclin D1 expression through inactivation of glycogen synthase kinase 3β (GSK3β). The AKT-mediated phosphorylation of glycogen synthase kinase 3β on serine9 decreases its kinase activity for Thr286 of cyclin D1, which inhibits the nuclear export and the cytoplasmic proteasomal degradation of cyclin D1.[13,14] Thus, the activation of the AKT pathway leads to nuclear accumulation of cyclin D1 resulting in cell proliferation. Overexpression of cyclin D1 is strongly correlated with the poor prognosis of oral, and head and neck squamous cell carcinoma after RT or chemo-RT. Cyclin D1 is considered as a therapeutic target for these cancers.[15] We have previously reported that the AKT/glycogen synthase kinase 3β/cyclin D1 pathway is implicated in acquired radioresistance of tumor cells triggered by long-term FR.[16] Targeting the pathway completely suppressed tumor regrowth after FR *in vivo*.[17] However, the precise role of AKT/cyclin D1 pathway in radioresistance of CSCs remains to be elucidated.

In this study, we have isolated CD133-positive CSCs from HepG2 and A172 by exposure to FR for 82 days. These cells showed

[1]Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan; [2]Department of Radiology and Radiation Oncology, Hirosaki University School of Medicine, Hirosaki, Japan and [3]Advanced Scientific Research Leaders Development Unit, Gunma University, Maebashi, Japan. Correspondence: Dr M Fukumoto, Department of Pathology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Sendai 980-8575, Japan.
E-mail: fukumoto@idac.tohoku.ac.jp
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