SUPPLEMENTARY INFORMATION

Novel CDK9 Inhibitor Prevents Replication of Broad DNA Viruses

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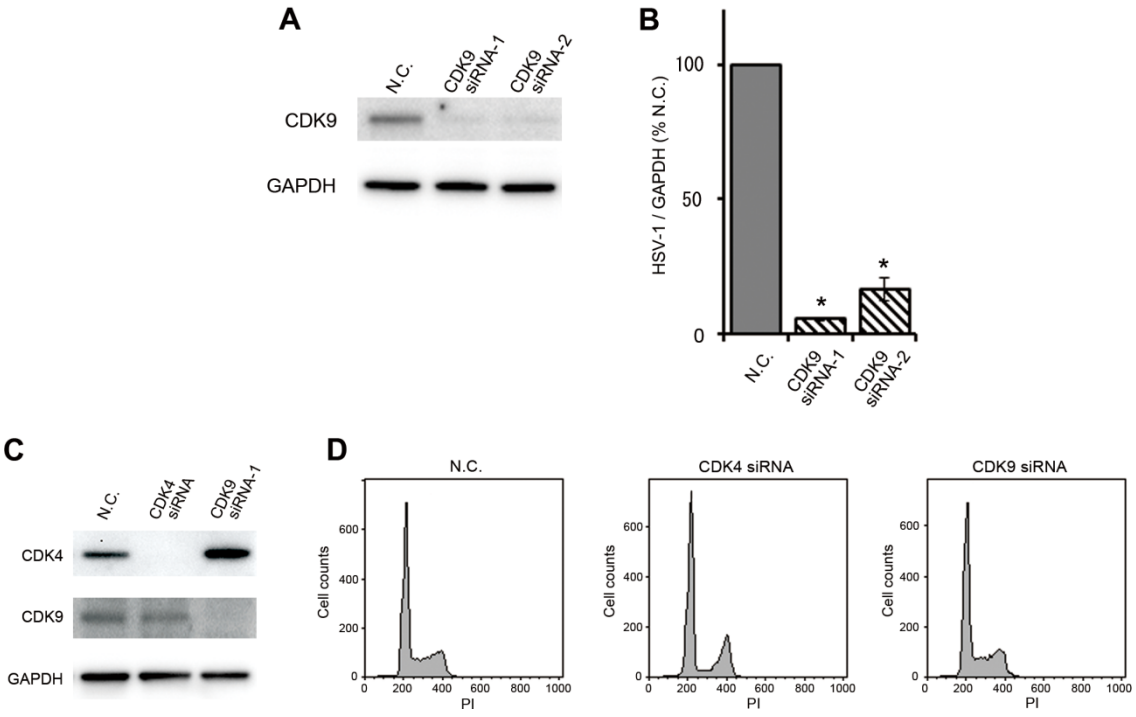
Supplementary Results

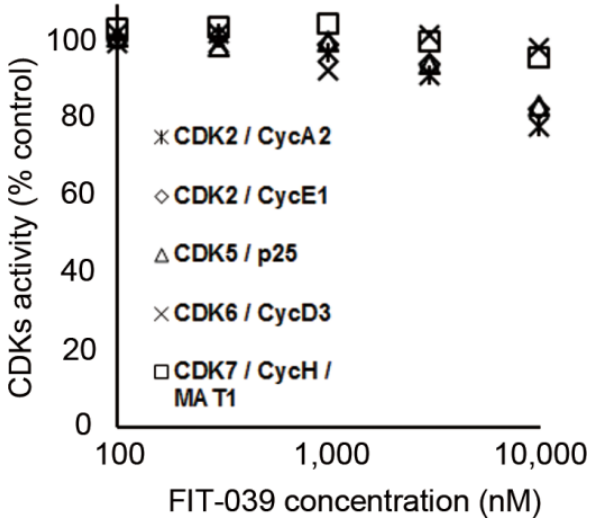
Supplementary Figure 1



Knockdown of CDK9 suppressed the replication of HSV-1.

(A) Knockdown of CDK9 by siRNA-1 and -2 (see Methods) in HeLa cells. Total cell lysates were subjected to Western blotting with antibodies against CDK9 and GAPDH. (B) Replication of HSV-1 was suppressed in CDK9-knockdown HeLa cells. The genomic DNA of HSV-1 replication was analyzed by real-time PCR. Scramble oligo was used as a negative control (N.C.). Each point represents the average ± standard deviation of the results from three experiments preformed in duplicate. Asterisks indicate significant differences (* $P < 0.0001$) versus N.C. as determined by the Student's t test (B). (C) Knockdown of CDK4 or CDK9 in HeLa cells by siRNAs. Total cell lysates were subjected to Western blotting with antibodies against CDK4, CDK9, and GAPDH. (D) Knockdown of CDK9 did not affect the cell cycle, compared to that of CDK4. Cells were stained with propidium iodide and analyzed by flow cytometry.

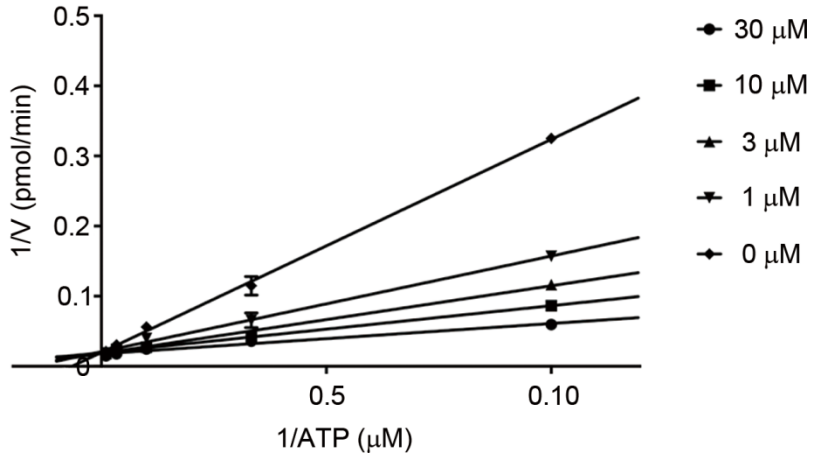
Supplementary Figure 2



***In vitro* kinase assay of other CDKs**

An increased amount of FIT-039 did not inhibit CDK2/cyclinA2, CDK2/cyclinE1, CDK5/p25, CDK6/cyclinD3, and CDK7/cyclin/MAT1.

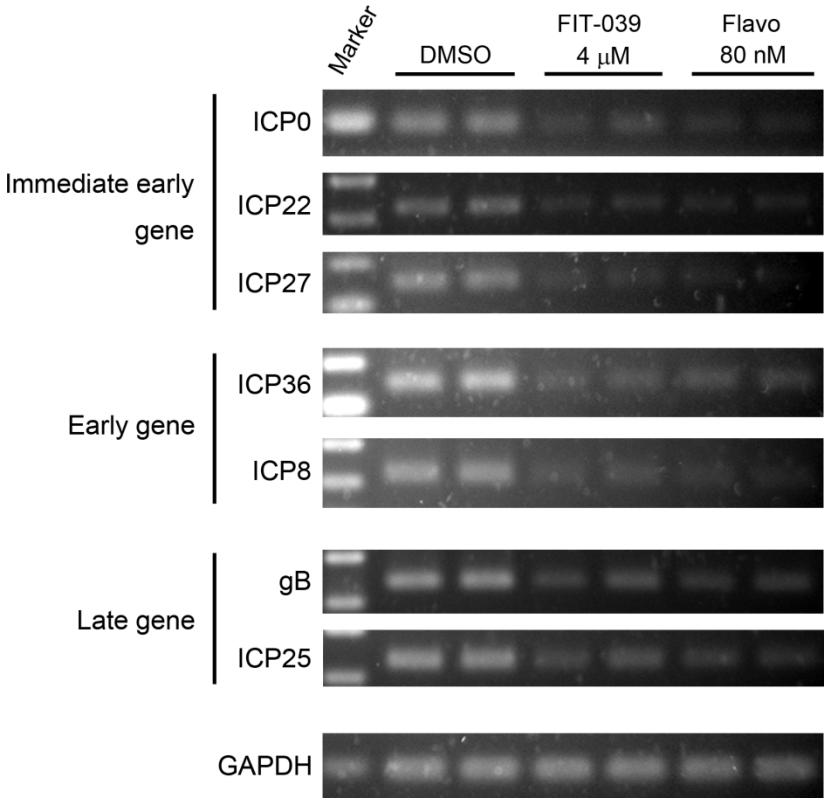
Supplementary Figure 3



Double-reciprocal plots of FIT-039 against CDK9/CycT1

Double-reciprocal plots showing the competitive inhibition of ATP by FIT-039. CDK9/CycT1 activity was measured at the indicated concentration of FIT-039 and ATP. Reciprocal velocity was plotted *versus* 1/[ATP]. K*m* = 36.85 μM, V_{max} = 5.78 pmol/min, and K*i* = 5.23 μM.

Supplementary Figure 4

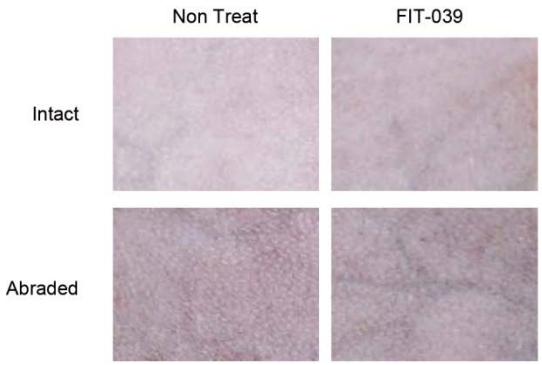


FIT-039 and flavopiridol inhibited the transcription of HSV-1 genes

FIT-039 and flavopiridol suppressed the transcription of the HSV-1 immediate-early genes (ICP0, ICP22, and ICP27), early genes (ICP36 and ICP8), and late gene (gB and ICP25). Attachment of HSV-1 to HeLa cells was allowed at 4 ºC for 15 minutes, and the cells were then incubated at 37 ºC for 24 hours with the indicated compounds. The cells were subjected to RT-PCR. Flavo: Flavopiridol.

Supplementary Figure 5

A



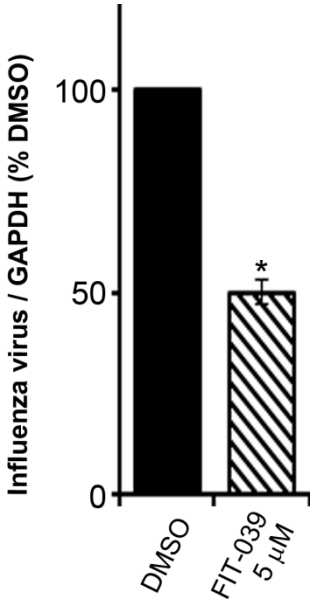
B

Rabbit No.	Reaction	27 hours		72 hours	
		Intact	Abraded	Intact	Abraded
1	Erythema	0	0	0	0
	Edema	0	0	0	0
2	Erythema	0	0	0	0
	Edema	0	0	0	0
3	Erythema	0	0	0	0
	Edema	0	0	0	0
Mean		0.0	0.0	0.0	0.0
P.I.I.		0.00			

Skin irritation test in rabbits

(A, B) The backs of rabbits were clipped and epidermal abrasions were performed with a sterile needle at one test site, while the opposite site remained intact. A total of 0.5 g of FIT-039 was then applied to each site, which was then covered with a non-reactive cloth. Three rabbits were subjected to each experimental group. The test sites were examined for dermal reactions 27 and 72 hours after the test article application (A) in accordance with the FHSA-recommended Draize scoring criteria. The Primary Irritation Index (P.I.I.) of FIT-039 was calculated to be 0.00; No irritation was observed on the skins of rabbits (B).

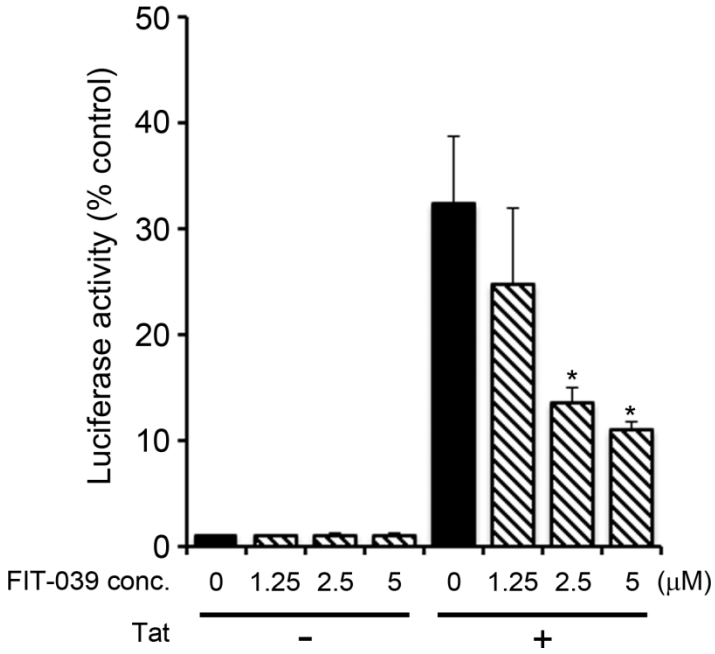
Supplementary Figure 6



FIT-039 suppressed the replication of influenza virus H1N1

MDCK cells were infected with influenza virus H1N1 PR8 strain and treated with 5 μM FIT-039 for 48 hrs. Influenza virus H1N1 replication was analyzed by real-time PCR at 5 μM of FIT-039. Each bar represents the average ± standard deviation of the results from three experiments preformed in duplicate. Asterisks indicate significant differences (* $P < 0.005$) versus the DMSO treatment as determined by the Student's t test.

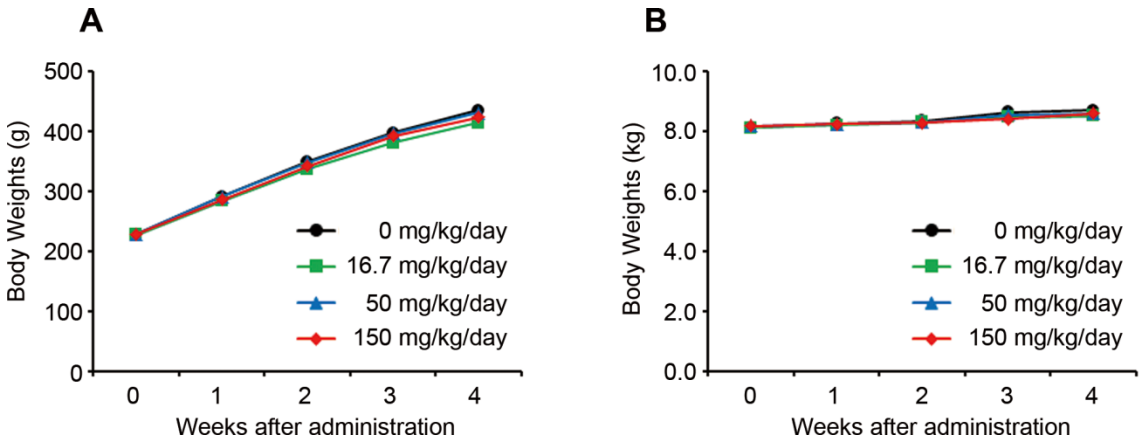
Supplementary Figure 7



FIT-039 inhibited HIV-TAT induced transcription in a dose-dependent manner.

CV1 cells were co-transfected with hRL-tk, LTR-Luc, EGFP-C1, herring sperm DNA and 3μg CMV4-Tat or CMV4-(no insert). hRL-tk (Promega) as an internal control. Medium was changed to each compound medium at the indicated concentrations 24 hr after transfection. These cells were harvested 48 hr post-treatment. Luciferase and renilla luciferase activity were measured by Luciferase Assay System (Promega). Each bar represents the average ± standard deviation of the results from three experiments preformed in duplicate. Asterisks indicate significant differences (* $P < 0.005$) versus the DMSO treatment as determined by the Student's t test.

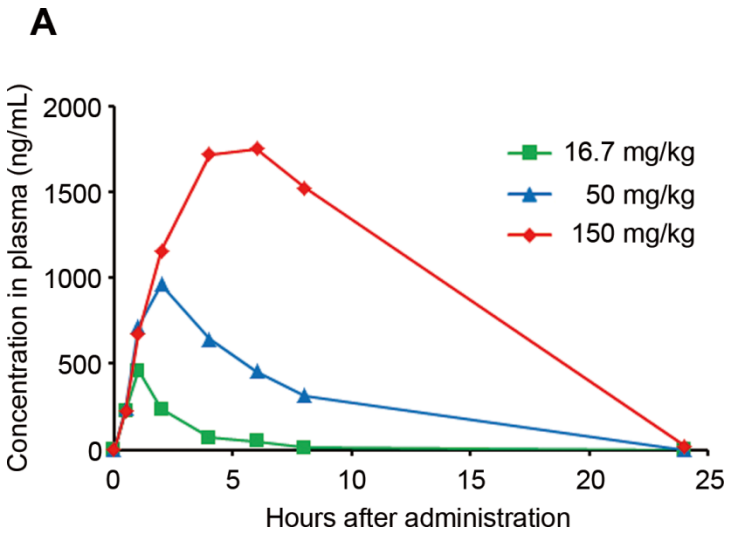
Supplementary Figure 8



Four weeks repeated-dose oral toxicity studies in rats and dogs.

FIT-039 (16.7, 50 or 150 mg/kg) or the solvent (0.5% methylcellulose) were orally administrated to male rats or dogs once a day for 28 days. Body weights and general conditions were determined for 28 days from the first administration. Rats body weights (A), dogs body weights (B).

Supplementary Figure 9



Oral absorbability of FIT-039 in rats.

FIT-039 (16.7, 50 or 150 mg/kg) were orally administrated to male rats. Oral absorbability was determined at 0, 0.5, 1, 2, 4, 6, 8 and 24 hrs from the first administration by LC/MS/MS.

Supplementary Tables

Supplementary Table 1

Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition
Abl	−2.0	EphA3	1.0	MAPKAP-K2	−6.0	PKCε	25.0
Abl (E255K)	−5.6	EphA4	2.0	MAPKAP-K3	3.0	PKCζ	−4.0
Abl (H396P)	10.0	EphA5	9.0	MARK1	2.0	PKCη	33.0
Abl (M351T)	−1.0	EphA6	2.4	MARK2	2.9	PKCθ	18.0
Abl (Q252H)	−1.0	EphA7	27.0	MARK3	3.2	PKCι	3.0
Abl(T315I)	−29.0	EphA8	1.0	MARK4	3.1	PKD1	0.0
Abl(Y253F)	1.0	EphB1	17.0	MEK1	−5.0	PKD2	−10.0
ACK1	5.0	EphB2	14.0	MELK	39.0	PKD3	3.8
ALK	39.0	EphB3	9.0	Mer	25.0	PKG1α	44.0
ALK4	23.0	EphB4	11.0	Met	6.0	PKG1β	41.0
AMPK	10.0	ErbB4	−8.0	MET(Y1235D)	11.3	PKN1	77.4
AMPKa2/b1/g1	3.7	Erk1	−2.1	MGC42105	6.9	PKR	−1.7
Arg	2.0	Erk5	4.7	MINK	8.0	PLK1	17.9
ARK5	11.0	FAK	27.0	MKK6	−1.0	Plk3	5.0
ASK1	0.0	Fer	32.0	MKK7β	−26.0	PLK4	4.2
AurA/TPX2	−3.0	Fes	23.0	MLCK	13.0	PRAK	28.0
AurC	0.8	FGFR1	−8.0	MLK1	16.0	PRK2	17.0
Aurora-A	1.0	FGFR1(V561M)	2.0	MLK2	8.8	PrKX	12.0
Axl	6.0	FGFR2	4.0	MLK3	5.9	PTK5	11.0
BMPR1A	1.1	FGFR2(N549H)	−3.0	MNK1	6.2	Pyk2	11.0
Bmx	−12.0	FGFR3	−11.0	Mnk2	15.0	QIK	−3.3
BRAF	6.5	FGFR3(K650E)	2.6	MOS	6.7	Ret	−4.0
BRK	12.0	FGFR3(K650M)	0.6	MRCKα	2.0	Ret (V804L)	11.0
BRSK1	3.2	FGFR4	−15.0	MRCKβ	1.0	RET(M918T)	0.3
BRSK2	2.0	Fgr	24.0	MSK1	63.0	Ret(V804M)	6.0
BTK	2.0	Flt1	9.0	MSK2	49.0	RIPK2	20.0
BTK(R28H)	3.0	Flt3	63.0	MSSK1	13.0	ROCK-I	6.0
CaMKI	−5.0	Flt3(D835Y)	66.0	MST1	9.0	ROCK-II	16.0
CaMKIIβ	40.0	Flt4	26.0	MST2	13.0	Ron	4.0
CaMKIIγ	30.0	Fms	6.0	MST3	2.0	Ros	22.0
CaMKIIδ	30.0	FRK	1.3	MST4	5.1	Rse	−32.0
CaMKIV	4.0	Fyn	1.0	MuSK	17.0	Rsk1	12.0
CaMKIδ	−4.0	GCK	4.0	NDR1	−0.1	Rsk2	5.0
CDC7	2.5	GRK5	2.0	NEK1	−4.3	Rsk3	39.0
CDK3/cyclinE	12.0	GRK6	3.0	NEK11	30.0	Rsk4	−26.0
CDK4	1.8	GRK7	−1.0	NEK2	−55.0	SAPK2a	1.0
CGK2	37.3	GSK3a	74.9	NEK3	6.0	SAPK2a(T106M)	−4.0
CHK1	−3.0	GSK3β	79.0	NEK4	−0.5	SAPK2b	0.0
CHK2	6.0	Haspin	77.0	NEK6	−1.0	SAPK3	−10.0
CHK2(I157T)	6.0	Hck	14.0	NEK7	−1.0	SAPK4	−7.0
CHK2(R145W)	0.0	HER2	−12.6	NEK9	1.0	SGK	3.0
CK1a	−3.5	HER4	−1.0	NLK	30.0	SGK2	−1.0
CK1e	−1.2	HGK	13.2	NuaK1	7.2	SGK3	4.0
CK1γ1	1.0	HIPK1	2.0	p70S6K	77.0	SIK	4.0
CK1γ2	3.0	HIPK2	4.0	p70S6Kb	48.7	SLK	1.6
CK1γ3	8.0	HIPK3	6.0	PAK1	0.7	Snk	8.0
CK1δ	−10.0	HIPK4	35.1	PAK2	13.0	SPHK1	−7.7
CK2	2.0	IGF-1R	17.0	PAK3	50.0	Src(1-530)	3.0
CK2a1/b	0.0	IKKe	−5.2	PAK4	8.0	Src(T341M)	11.0
CK2α2	−11.0	IKKα	−28.0	PAK5	0.0	SRM	−17.4
cKit	5.0	IKKβ	−4.0	PAK6	−7.0	SRPK1	6.0
cKit(D816H)	7.0	IR	17.0	PAR-1Bα	13.0	SRPK2	−1.0
cKit(D816V)	0.0	IRAK1	28.0	PASK	15.0	STK33	14.0
cKit(V560G)	−1.0	IRAK4	4.0	PBK	9.0	Syk	6.0
cKit(V654A)	0.0	IRR	74.0	PDGFRa(T674I)	9.4	TAK1	−13.0
CLK2	34.8	ITK	−2.2	PDGFRα	−7.0	TAK1-TAB1	7.1
CLK3	18.0	Itk	10.0	PDGFRα(D842V)	12.0	TAO1	−2.0
COT	7.0	JAK2	−5.0	PDGFRα(V561D)	36.0	TAO2	1.0
c-RAF	5.0	JAK3	0.0	PDGFRβ	−2.0	TAO3	14.0
CRIK	3.5	JNK1α1	0.0	PDHK2	11.5	TBK1	9.0
CSK	20.0	JNK2α2	3.0	PDHK4	−0.5	TEC	1.6
cSRC	−6.0	JNK3	1.0	PDK1	−8.0	Tie2	−8.0
CTK	2.1	KDR	1.0	PEK	−1.3	Tie2(R849W)	12.0
DAPK1	4.0	KIT(T670I)	4.3	PGK	10.8	Tie2(Y897S)	15.0
DAPK2	17.0	Lck	18.0	PHKG1	2.1	TLK2	−7.0
DCAMKL2	−4.0	LIMK1	12.0	PHKG2	4.9	TNK1	3.2
DDR1	7.2	LKB1	−3.0	PhKγ2	−1.0	TrkA	38.0
DDR2	18.0	LOK	34.0	PIK3CA/PIK3R1	1.6	TrkB	41.0
DLK	−1.4	LTK	6.8	Pim-1	64.0	TRKC	13.7
DMPK	−1.0	Lyn	−1.0	Pim-2	43.0	TSSK1	6.0
DRAK1	−16.0	LYNb	1.7	Pim-3	27.0	TSSK2	5.0
DYRK1B	74.9	MAP2K2	4.9	PKA	28.0	TTK	−16.1
DYRK2	46.0	MAP2K3	7.3	PKBα	8.0	Txk	12.0
DYRK3	72.1	MAP2K4	10.0	PKBβ	4.0	TYK2	1.2
eEF-2K	13.0	MAP2K5	7.9	PKBγ	7.0	ULK3	−7.0
EGFR	−4.0	MAP2K6	5.6	PKCμ	12.0	VRK2	3.0
EGFR(L858R)	4.0	MAP2K7	10.8	PKCb1	5.7	WEE1	−2.7
EGFR(L861Q)	3.0	MAP3K1	6.4	PKCb2	−7.1	WNK1	0.8
EGFR(T790M)	14.0	MAP3K2	4.2	PKCα	21.0	WNK2	22.0
EGFR(T790M,L858R)	4.0	MAP3K3	8.4	PKCβI	15.0	WNK3	13.0
EML4-ALK	2.6	MAP3K4	−3.8	PKCβII	13.0	Yes	−4.0
EphA1	30.0	MAPK1	24.0	PKCγ	19.0	ZAP-70	−1.0
EphA2	12.0	MAPK2	−17.0	PKCδ	9.0	ZIPK	−6.0

Large panel of kinase screening of FIT-039

Kinase inhibitory activity of FIT-039 (10 μM) against kinome were screened by Merck Millipore's KinaseProfiler service (Merck Millipore) and Profiling Srevices (Carmabiosciences).

Supplementary Table 2

Item	Dose (mg/kg/day, 14 days)		Item	Dose (mg/kg/day, 14 days)	
	0	1000		0	1000
AST (U/L)	78.8 ± 4.5	72.0 ± 5.7	WBC (x 10^2/μL)	106.0 ± 7.1	124.5 ± 4.9
ALT (U/L)	36.0 ± 4.2	34.0 ± 11.3	RBC (x 10^4/mL)	618.5 ± 12.0	663.0 ± 91.9
γGTP (U/L)	ND	ND	Hb (g/dL)	13.7 ± 0.2	13.0 ± 0.0
T-BIL (mg/dL)	0.04 ± 0.01	0.03 ± 0.00	HT (%)	41.6 ± 0.3	41.7 ± 1.8
CRE (mg/dL)	0.21 ± 0.02	0.20 ± 0.01	MCV (fL)	67.5 ± 2.1	63.5 ± 6.4
BUN (mg/dL)	14.6 ± 0.1	15.8 ± 1.4	MCH (pg)	22.5 ± 0.7	20.0 ± 2.8
GLU (mg/dL)	187.6 ± 7.6	182.0 ± 22.6	MCHC (%)	33.0 ± 0.0	31.0 ± 1.4
TP (g/dL)	5.6 ± 0.4	6.4 ± 0.1	PLT (x 10^4/mL)	98.7 ± 8.3	123.9 ± 11.0
ALB (g/dL)	4.1 ± 0.3	4.4 ± 0.2			
Ca (mEq/L)	10.7 ± 0.6	11.0 ± 0.4			
Na (mEq/L)	141.4 ± 0.6	141.5 ± 3.5			
K (mEq/L)	5.0 ± 0.3	5.1 ± 0.7			
Cl (mEq/L)	102.0 ± 1.5	101.0 ± 1.4			

Hematology tests in the 2-week repeated-dose oral toxicity study in rats

Hematology tests were performed on day 28 in the 2-week repeated-dose oral toxicity study (Fig. 3G). No significant difference was observed in any testing item between FIT-039 and the solvent.

Supplementary Table 3: Knockout phenotypes of target kinases of FIT-039

Kinase	Inhibitory effect (%)	Phenotypes	Reference
GSK3b	79.0	embryonic lethal	(1)
PKN1	77.4	autoantibody production glomerulonephritis	(2)
Haspin	77.0	not reported	
p70s6k	77.0	growth retardation	(3)
DYRK1B	74.9	no abnormal phenotype detected	(4)
GSK3a	74.9	decreased percent body fat incerased lean body mass improved glucose tolerance increaced liver glucogen level increaced insulin sensitivity	(5)
IRR	74.0	no abnormal phenotype detected	(6)
DYRK3	72.1	no abnormal phenotype detected	(7)

Supplementary Table 4

		Dose	Bace - pair substitution type			Frameshift type	
		(μg/plate)	TA100	TA1535	WP2 uvrA	TA98	TA1537
S9 mix (+)	FIT-039	0	143	14	27	28	11
		313	123	17	26	24	10
		625	116	12	24	26	7
		1250	124	13	28	24	7
		2500	124	17	23	25	11
		5000	116	12	22	23	6
	Positive control	Chemical	AF-2	SA	AF-2	AF-2	9AA
		Dose (μg/plate)	0.01	0.5	0.01	0.1	80
		Number of colonies/plate	462	456	98	503	358
S9 mix (-)	FIT-039	0	116	12	33	40	17
		313	134	11	40	40	10
		625	130	12	33	35	10
		1250	130	15	39	39	9
		2500	135	12	36	32	9
		5000	156	9	37	32	11
	Positive control	Chemical	2AA	2AA	2AA	2AA	2AA
		Dose (μg/plate)	1	2	10	0.5	2
		Number of colonies/plate	1027	364	739	401	292

Mutagenicity test in bacteria (Ames test)

Bacterium Salmonella strains and Escherichia coli strain were pre-incubated with FIT-039 or positive control compounds with or without S9mix. These mixtures were spread on agar plates and incubated for 48 hours. The number of colonies were counted, and mutagenicity was assessed. No increases in mutated colony counts were recognized in any strain, regardless of the presence or absence of the S9mix. These results indicate that FIT-039 did not cause any chromosomal aberrations.

Supplementary Table 5

Item	Dose (mg/kg/day)		
	0	500	2000
PCE (%)	48.90 ± 1.83	49.50 ± 1.20	49.37 ± 2.37
MNPCE (%)	0.02 ± 0.03	0.04 ± 0.04	0.05 ± 0.06

Mutagenicity test in mice (Micronucleus test)

FIT-039 (500 or 2000 mg/kg) or the solvent (polyethylenegrycol #400) were orally administrated to male CD1 mice once a day for 2 days. Sis mice were assigned to each experimental group. Their bone marrow cells were collected at the femur 24 hours after the final administration, and the emergence of micronucleated polychromatic erythrocytes (MNPCE) and ratio of polychromatic erythrocytes in erythrocytes (PCE%) were examined. No significant differences were observed in the percentages of PCE or MNPCE between the FIT-039 or the solvent. These results indicate that FIT-039 does not exhibit any genotoxicity or bone marrow cell toxicity.

Supplementary Table 6: Quantitative PCR primer sequences

RNA	Sequence		
	Forward Primer	Reverse Primer	Probe
HSV-1	5' CGCATCAAGACCACCTCCTC 3'	5' GCTCGCACCACGCGA 3'	5' TGGCAACGCGGCCCAAC 3'
HSV-2	5' CGCATCAAGACCACCTCCTC 3'	5' GCTCGCACCACGCGA 3'	5' CGGCGATGCGCCCCAG 3'
HAdV-5	5' GACATGACTTTTGAGGTGGA 3'	5' TCGATGATGCCGCGGTG 3'	5' CCCATGGAYGAGCCCACCCT 3'
HAdV-19	5' GCCGAGAAGGGCGTGCGCAGGTA 3'	5' TACGCCAACTCCGCCCACGCGCT 3'	
Infulenza	5' GGACTGCAGCGTAGACGCTT 3	5' CATCCTGTTGTATATGAGGCCCAT 3	5' CTCAGTTATTCTGCTGGTGCACTTGCCA 3
GAPDH	5' CTCCCCACACATGCACTTA 3'	5' CCTAGTCCCAGGGCTTTGATT 3'	5' AAAAGAGCTAGGAAGGACAGGCAACTTGGC 3'

Supplementary Table 7: RT- PCR primer sequences

RNA	Sequence	
	Forward Primer	Reverse Primer
HSV-1 ICP0	5' ATACACATGGCCCCTTTGAC 3'	5' GTCCCTGTGTGTTTGTTGTG 3'
HSV-1 ICP22	5' CAGCCTTGGAGTCTGAGGTC 3'	5' GTGGGGGAATGTCGTCATAA 3'
HSV-1 ICP27	5' GGCGACTGACATTGATATGC 3'	5' GGGTCTTCCATGTCCTCGT 3'
HSV-1 ICP36	5' TACCCGAGCCGATGACTTAC 3'	5' AAGGCATGCCCATTGTTATC 3'
HSV-1 ICP8	5' AGCTCGTCCGTGTACGTCTT 3'	5' CCCTCGGTAACGACCAGATA 3'
HSV-1 gB	5' GGACACGAAACCGAAGAAGA 3'	5' ATGCCCTCCGTGTAGTTCTG 3'
HSV-1 ICP25	5' CTCGATACCTGGAACGAGGA 3'	5' CGTGGAAGAAACGAGAGAGC 3'
HAdV-5 E1A	5' TACGGGGGACCCAGATATTA 3'	5' CAGGCTCAGGTTCAGACACA 3'
GAPDH	5' ACGGATTTGGTCGTATTGGG 3'	5' GTAGTTGAGGTCAATGAAGGGGTC 3'

Supplementary Notes

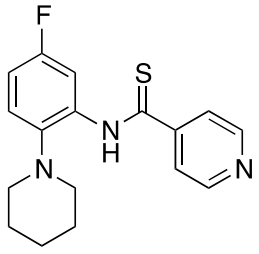
Synthesis FIT-039, FIA-348, FIA-002, FIT-047, and FIA-017

Synthesis of small chemicals

All chemical reagents used were commercial grade and were used as received. Amides (FIT-039, FIA-348, FIA-002, FIT-047, and FIA-017) were prepared from the corresponding amines as described previously (H. Onogi, M. Hagiwara, T. Hosoya, M. Yamamoto, Y. Nonaka, T. Hiramatsu, Aniline derivative having anti-DNA virus activity, WO 2009/020198). Analytical thin-layer chromatography (TLC) was performed on precoated (0.25 mm) silica gel plates (Merck Chemicals, Silica Gel 60 F_{254}, Cat. No. 1.05715). Column chromatography was conducted using silica gel (Kanto Chemical Co., Inc., Silica Gel 60N, spherical neutral, particle size 40–50 μm, Cat. No. 37563-85 or particle size 63–210 μm, Cat. No. 37565-85). Melting points (Mp) were measured with a Opti Melt MPA100 (Stanford Research Systems) and were uncorrected. ^{1}H spectra were obtained with a Bruker AVANCE 400 spectrometer or Bruker AVANCE 500 spectrometer at 400 or 500 MHz, respectively. ^{13}C NMR spectra were obtained with a Bruker AVANCE 500 spectrometer at 126 MHz. ^{19}F NMR spectrum was obtained with

a Bruker AVANCE 400 spectrometer at 376 MHz. $CDCl_3$ (Acros Organics, Cat. No. 368651000) was used as a solvent to obtain NMR spectra. Chemical shifts (δ) were given in parts per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^1H NMR in $CDCl_3$) as an internal reference, or α,α,α-trifluorotoluene (δ 63.0 ppm for ^{19}F NMR in $CDCl_3$) as an external standard with coupling constants (*J*) in hertz (Hz). The abbreviations s, d, t, q, m, and br signify singlet, doublet, triplet, quartet, multiplet, and broad, respectively. IR spectra were measured by diffuse reflectance method on a Shimadzu IRPrestige-21 spectrometer attached to a DRS-8000A with absorption bands given in cm^{-1}. High-resolution mass spectra (HRMS) were measured on a Bruker micrOTOF mass spectrometer under positive electrospray ionization (ESI^+) conditions at Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University.

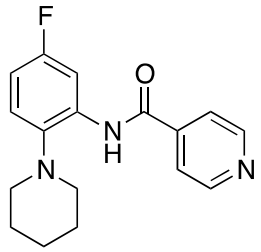
_N_-[5-Fluoro-2-(1-piperidinyl)phenyl]isonicotinthioamide (FIT-039)



Mp 181–184 °C (decomp.); TLC R_f 0.49 (*n*-hexane/dichloromethane/ethyl acetate = 3/5/2); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.58–1.76 (m, 6H, $3CH_2$), 2.83 (t, 4H, *J* = 5.0 Hz,

2CH_2), 6.95 (ddd, 1H, *J* = 2.8, 8.0, 8.0 Hz, aromatic), 7.24 (dd, 1H, *J* = 5.6, 8.0 Hz, aromatic), 7.70–7.74 (AA'BB', 2H, aromatic), 8.74–8.78 (AA'BB', 2H, aromatic), 9.28 (dd, 1H, *J* = 2.8, 10.8 Hz, aromatic), 11.13 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 23.7 (1C), 27.0 (2C), 54.3 (2C), 107.0 (d, 1C, $J^2_{C–F}$ = 29.9 Hz), 112.7 (d, 1C, $J^2_{C–F}$ = 22.8 Hz), 120.2 (2C), 122.2 (d, 1C, $J^3_{C–F}$ = 9.5 Hz), 135.6 (d, 1C, $J^3_{C–F}$ = 12.0 Hz), 140.3 (d, 1C, $J^4_{C–F}$ = 2.8 Hz), 149.5 (1C), 150.5 (2C), 159.2 (d, 1C, $J^1_{C–F}$ = 243 Hz), 191.8 (1C); ^{19}F NMR ($CDCl_3$, 376 MHz) δ –113.4 (ddd, *J* = 5.6, 8.0, 10.8 Hz); IR (KBr, cm^{-1}) 733, 760, 937, 1229, 1449, 1517, 1599, 2826, 3206; HRMS (ESI^+) *m*/z 338.10981 ($[M+Na]^+$, $C_{17}H_{18}FN_3NaS^+$ requires 338.10977).

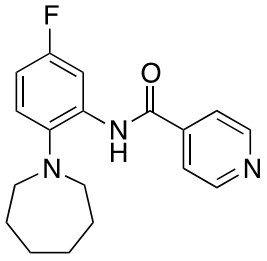
_N_-[5-Fluoro-2-(1-piperidinyl)phenyl]isonicotinamide (FIA-348)



Mp 115–116 °C; TLC R_f 0.40 (*n*-hexane/ethyl acetate = 1/1); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.62–1.69 (m, 2H, CH_2), 1.70–1.78 (br, 4H, 2CH_2), 2.75–2.83 (br, 4H, 2CH_2), 6.81 (ddd, 1H, *J* = 2.8, 8.8, 10.8 Hz, aromatic), 7.18 (dd, 1H, *J* = 5.6, 8.8 Hz, aromatic), 7.71–7.74 (AA'BB', 2H, aromatic), 8.34 (dd, 1H, *J* = 2.8, 10.8 Hz, aromatic), 9.14–9.17

(AA'BB', 2H, aromatic), 9.83 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 23.8 (1C), 27.2 (2C), 54.2 (2C), 106.7 (d, 1C, $J^2_{C–F}$ = 29.0 Hz), 110.5 (d, 1C, $J^2_{C–F}$ = 22.7 Hz), 120.7 (2C), 122.0 (d, 1C, $J^3_{C–F}$ = 8.8 Hz), 134.3 (d, 1C, $J^3_{C–F}$ = 12.6 Hz), 138.6 (d, 1C, $J^4_{C–F}$ = 2.5 Hz), 141.8 (1C), 150.9 (2C), 160.0 (d, 1C, $J^1_{C–F}$ = 243 Hz), 162.7 (1C); ^{19}F NMR ($CDCl_3$, 376 MHz) δ –114.5 (ddd, *J* = 5.6, 10.8, 10.8 Hz); IR (KBr, cm^{-1}) 681, 1159, 1265, 1495, 1524, 1605, 1682, 2936, 3306; HRMS (ESI^+) *m*/z 322.13150 ($[M+Na]^+$, $C_{17}H_{18}FN_3NaO^+$ requires 322.13261).

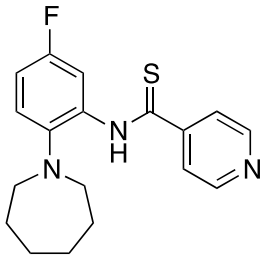
_N_-[5-Fluoro-2-(1-hexahydro-1_H_-azepinyl)phenyl]isonicotinamide (FIA-002)



Mp 135–136 °C; TLC R_f 0.50 (*n*-hexane/ethyl acetate = 1/2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.61–1.82 (br, 8H, $4CH_2$), 2.97–3.07 (br, 4H, $2CH_2$), 6.80 (ddd, 1H, *J* = 2.0, 8.1, 8.1 Hz, aromatic), 7.19 (dd, 1H, *J* = 5.8, 8.1 Hz, aromatic), 7.74–7.77 (AA'BB', 2H, aromatic), 8.33 (dd, 1H, J = 2.0, 10.4 Hz, aromatic), 8.82–8.85 (AA'BB', 2H, aromatic), 9.93 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 27.1 (2C), 30.1 (2C), 57.7 (2C), 106.8 (d, 1C, $J^2_{C–F}$ = 29.0 Hz), 111.1 (d, 1C, $J^2_{C–F}$ = 22.7 Hz), 121.0 (2C), 124.0 (d, 1C, $J^3_{C–F}$

= 10.0 Hz), 134.6 (d, 1C, $J^3_{C–F}$ = 11.3 Hz), 141.2 (d, 1C, $J^4_{C–F}$ = 2.5 Hz), 142.2 (1C), 151.1 (2C), 160.2 (d, 1C, $J^1_{C–F}$ = 242 Hz), 163.1 (1C); ^{19}F NMR ($CDCl_3$, 376 MHz) δ –114.5 (ddd, *J* = 5.8, 8.1, 10.4 Hz); IR (KBr, cm^{-1}) 681, 872, 1244, 1271, 1444, 1520, 1603, 1682, 2853, 2926, 3300; HRMS (ESI^+) *m*/z 336.14815 ($[M+Na]^+$, $C_{18}H_{20}FN_3NaO^+$ requires 336.14826).

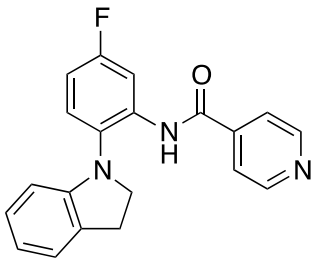
_N_-[5-Fluoro-2-(1-hexahydro-1_H_-azepinyl)phenyl]isonicotinthioamide (FIT-047)



Mp 131–133 °C; TLC R_f 0.46 (*n*-hexane/dichloromethane/ethyl acetate = 2/5/3); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.64–1.75 (m, 8H, $4CH_2$), 2.99–3.04 (m, 4H, $2CH_2$), 6.94 (ddd, 1H, *J* = 2.9, 8.7, 8.7 Hz, aromatic), 7.25 (dd, 1H, *J* = 5.5, 8.7 Hz, aromatic), 7.69–7.74 (AA'BB', 2H, aromatic), 8.73–8.78 (AA'BB', 2H, aromatic), 9.27 (dd, 1H, *J* = 2.9, 10.8 Hz, aromatic), 11.2 (s, 1H, NH); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 26.8 (2C), 29.9 (2C), 57.7 (2C), 106.8 (d, 1C, $J^2_{C–F}$ = 29.0 Hz), 113.1 (d, 1C, $J^2_{C–F}$ = 22.7 Hz), 120.5 (2C), 124.0 (d, 1C, $J^3_{C–F}$ = 10.0 Hz), 135.8 (d, 1C, $J^3_{C–F}$ = 12.6 Hz), 142.7 (d, 1C, $J^4_{C–F}$ = 2.5 Hz), 150.0 (1C), 150.7 (2C), 159.2 (d, 1C, $J^1_{C–F}$ = 243 Hz), 192.1 (1C); ^{19}F NMR

(CDCl$_3$, 376 MHz) δ –113.5 (ddd, J = 5.5, 8.7, 10.8 Hz); IR (KBr, cm^{-1}) 731, 812, 1155, 1364, 1354, 1447, 1514, 1597, 2926, 3175; HRMS (ESI$^+$) m/z 352.12365 ([M+Na]$^+$, $C_{18}H_{20}FN_3NaS^+$ requires 352.12542).

***N*-[5-Fluoro-2-(1-indolinyl)phenyl]isonicotinamide (FIA-017)**



Mp 150–151 °C; TLC R_f 0.28 (*n*-hexane/ethyl acetate = 1/1); ^{1}H NMR (CDCl$_3$, 500 MHz) δ 3.18–3.24 (m, 2H, CH$_2$), 3.57–3.68 (br, 1H, CH$_2$), 3.78–3.88 (br, 1H, CH$_2$), 6.30 (d, 1H, J = 7.5 Hz, aromatic), 6.87 (dd, 1H, J = 7.5, 7.5 Hz, aromatic), 6.91 (ddd, 1H, J = 1.0, 8.0, 8.0 Hz, aromatic), 7.06 (dd, 1H, J = 7.5, 7.5 Hz, aromatic), 7.26–7.29 (m, 2H, aromatic), 7.51–7.54 (AA'BB', 2H, aromatic), 8.46 (dd, 1H, J = 3.0, 10.5 Hz, aromatic), 8.73–8.75 (AA'BB', 2H, aromatic), 9.04 (br s, 1H, NH); ^{13}C NMR (CDCl$_3$, 126 MHz) δ 29.1 (1C), 56.1 (1C), 107.7 (d, 1C, $J^2_{C–F}$ = 29.0 Hz), 109.5 (1C), 111.9 (d, 1C, $J^2_{C–F}$ = 23.1 Hz), 120.4 (1C), 120.6 (2C), 125.1 (1C), 125.8 (d, 1C, $J^3_{C–F}$ = 9.6 Hz), 127.6 (1C), 130.5 (1C), 131.2 (d, 1C, $J^4_{C–F}$ = 2.1 Hz), 136.4 (d, 1C, $J^3_{C–F}$ = 12.5 Hz),

141.5 (1C), 150.5 (1C), 150.9 (2C), 161.0 (d, 1C, J^1_{C-F} = 246 Hz), 163.2 (1C); ^{19}F NMR ($CDCl_3$, 376 MHz) δ –111.8 (ddd, J = 6.0, 8.0, 10.5 Hz); IR (KBr, cm^{-1}) 748, 1258, 1454, 1524, 1605, 1682, 2932, 3348; HRMS (ESI^+) m/z 356.11645 ($[M+Na]^+$, $C_{20}H_{16}FN_3NaO^+$ requires 356.11696).

Methods

In vitro kinase assay for ATP competitive analysis

The ATP competitive analysis were assayed in a reaction mixture, containing CDK9/cyclinT1, 8 mM MOPS-NaOH (pH 7.0), 0.2 mM EDTA, 100 μM KTFCGTPEYLAPEVRREPRILSEEEQEMFRDFDYIADWC, 10 mM MgAcetate and [γ-33P-ATP]. The reaction is initiated by the addition of the MgATP mix. ATP and FIT-039 concentrations were 1-1000 μM and 0.003-30 μM, respectively. After incubation for 40 minutes at room temperature, the reaction is stopped by the addition of 3% phosphoric acid solution. 10 μL of the reaction is then spotted onto a P30 filtermat and washed three times for 5 minutes in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting. This assay was commissioned to Millipore.

In vitro kinase assay and IC_{50} determination

The assay protocols of each kinase (CDK2/CycA2, CDK2/CycE1, CDK4CycD3, CDK5/p25, CDK6/CycD3, CDK7/CycH/MAT1 and CDK9/CycT1) have been

published on the CarnaBiosciences website (http://www.carnabio.com/english/index.html). Kinase activities were measured by a mobility shift assay(8). Compounds were dissolved in DMSO and diluted in a half-log scale for use in IC_{50} determinations. The DMSO solution was diluted in assay buffer to yield a final concentration of 1% DMSO for each compound. Kinase assays were performed using ATP at concentrations of the *Km* values for each kinase. The inhibition of kinase activity by each compound was calculated as follows: inhibition (%) = [1–(A–B)/(C–B)) × 100, where A is the response with the compound, B is the background response with kinase, and C is the response with vehicle (1% DMSO). The IC_{50} value of each compound was calculated by interpolation on a log-concentration-response curve fitted with a four-parameter logistic equation. The pIC_{50} values were given as –log10 (IC_{50}) values(9).

Large panel of kinase screening

Kinase inhibitory activity of FIT-039 (10 μM) against kinome were screened by Merck Millipore's KinaseProfiler service (Merck Millipore) and Profiling Services

(Carnabiosciences). These assay protocols of each service have been published on the Merck Millipore website (http://www.millipore.com/techpublications/tech1/pf3036) and the Carnabiosciences website (http://www.carnabio.com/english/index.html), respectively.

Influenza H1N1 infectious assay

MDCK cells were infected with influenza H1N1 (PR8 strain) with chemical compounds. Influenza H1N1-infected cells were incubated for 48 hr, following which total RNA was extracted using Sepasol RNA-I Super (NACALAI TESQUE, INC.). Reverse transcription was performed with PrimeScript Reverse Transcriptase (Takara Bio, Inc.), using random primer. Influenza H1N1 and cellular GAPDH were quantitative by real-time PCR. Analyses were performed using FastStart Universal Probe Master (ROX) (Roche Applied Science). PCR was performed with an initial denaturation reaction at 95 ºC for 1 min, and then amplified with 40 cycles of 95 ºC for 30 sec, 60 ºC for 30 sec, 72 ºC for 30 sec. The amplification was monitored on Step One Plus (Applied Biosystems, Inc.). The primers used are shown in Supplementary Table 6.

HIV-TAT promoter assay

LTR-Luc consists of the long termimal repeat (LTR; 8454nt-(9000nt)-20nt) from HIV-1 clone NL43 (U26942) cloned into pGL3-basic (Promega). pCMV4-tat consist of the tat cDNA (5208-5422 jointed to 7747-7792) from HIV-1 clone NL43 cloned into insert to pCMV4(10).

CV1 cells were plated 2 x 10^5 cells / 6 cm dish and co-transfected with 0.5 μg of hRL-tk, 1μg LTR-Luc, 0.5μg EGFP-C1, 5μg herring sperm DNA and 3μg CMV4-Tat or CMV4-(no insert) by calcium phosphate transfection method. hRL-tk (Promega) as an internal control. Medium was changed to each compound medium 24 hr after transfection. These cells were harvested 48 hr post-treatment by passive lysis buffer. Luciferase and renilla luciferase activity were measured by Luciferase Assay System (Promega).

Skin irritation test

The rabbit skin irritation test was performed at Drug Safety Testing Center Co., Ltd.

The backs of rabbits were clipped and epidermal abrasions were performed with a sterile needle at one test site, while the opposite site remained intact. A total of 0.5 g of FIT-039 was then applied to each site, which was then covered with a non-reactive cloth. Three rabbits were assigned to each experimental group. The test sites were examined for dermal reactions 27 and 72 hours after the test article application in accordance with the FHSA-recommended Draize scoring criteria(11). The Primary Irritation Index (P.I.I.) of FIT-039 was calculated.

Ames test

The Ames test was performed at Hatano Research Institute, Food and Drug Safety Center. Bacterium Salmonella strains (TA100, TA1535, TA98, and TA1537) and Escherichia coli strain (WP2 uvrA) were pre-incubated with FIT-039 or 4 positive control compounds (AF2: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, SA: sodium azide, 9AA: 9-aminoacridine, and 2AA: 2-aminoanthracene), with or without rat liver extract (S9mix), which was used to examine the effect of the metabolized compounds. These mixtures were spread on agar plates and incubated for 48 hours. The colonies were

counted and mutagenicity was judged.

Micronucleus test

The micronucleus test was performed at New Drug Development Research Center, Inc. FIT-039 (500 or 2000 mg/kg) or the solvent (polyethylenegrycol #400) were orally administrated to male CD1 mice once a day for 2 days. Sis mice were assigned to each experimental group. Their bone marrow cells were collected at the femur 24 hours after the final administration, and the emergence of micronucleated polychromatic erythrocytes (MNPCE) and ratio of polychromatic erythrocytes in erythrocytes (PCE%) were examined.

Four-week repeat-dose oral toxicity study in rats and dogs

These studies were performed at Biotoxtech Co., Ltd.. Male SD rat (4 weeks old) and male beagle dogs (6 months old) were purchased from CHARLES RIVER LABORATORIES JAPAN, Inc and BEIJING MARSHALL BIOTECHNOLOGY Co., Ltd., China, respectively. FIT-039 (16.7, 50 or 150 mg/kg) or the solvent (0.5%

methylcellulose) was orally administrated to these animals once a day for 28 days. Ten rats were assigned to each experimental group. Body weights and general conditions were determined for 28 days from the first administration.

Oral absorbability study in rat

These studies were performed at Biotoxtech Co., Ltd.. Male SD rat (4 weeks old) were purchased from CHARLES RIVER LABORATORIES JAPAN, Inc. FIT-039 (16.7, 50 or 150 mg/kg) or the solvent (0.5% methylcellulose) was orally administrated to these animals. Three rat were assigned to each experimental group. Venous blood samples were collected at 0, 0.5, 1, 2, 4, 6, 8 and 24 hrs from the first administration. These blood samples were deproteinized, and then measured by LC/MS/MS (Prominence; SHIMADZU Co., Ltd, API4000; AB Sciex, Pte. Ltd.).

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