# Efficacy, Resistance, Fitness and Replication Capacity Profiles of Novel Pyrimidinedione Nonnucleoside Inhibitors of HIV-1 and HIV-2

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## Abstract

SJ-3366 [1-(3-Cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione] is a highly active ($EC_{50}$ = 1 nM, TI approximately one million) novel inhibitor of HIV-1 with the ability to inhibit both HIV entry and reverse transcription. SJ-3366 inhibits both HIV-1 and HIV-2, a property that distinguishes this specific NNRTI and related molecules from other NNRTIs. The dual mechanism of action and extended therapeutic range render SJ-3366 an attractive therapeutic agent for further clinical development. In addition to SJ-3366, the pyrimidinedione SAR series includes 11 other highly potent compounds with therapeutic indices greater than 500,000 and antiviral properties similar, but not identical, to SJ-3336. Since effective treatment of HIV infection is critically dependent upon the potential of therapeutic agents to select for drug-resistant viruses, it is important to understand the resistance profile of any new anti-HIV agent prior to clinical use. As a NNRTI, common resistance engendering mutations in the viral RT, especially the Y181C, Y188C, K101E and K103N amino acid changes, significantly reduced the efficacy of SJ-3366. Our SAR evaluations defined four compounds active against viruses possessing the K103N amino acid change in the RT. The K103N-active molecules maintain high therapeutic efficacy against HIV-1, are active against HIV-2, and are similarly inhibitory to reverse transcription and virus entry. Sensitivity and cross-resistance testing provide a means to rationally prioritize potential drug combinations that will yield effective anti-HIV therapeutic strategies. Interestingly, our data demonstrate synergistic inhibition of HIV with ddI and fluoro-ddA only, and no evidence of synergistic toxicity or antagonism with any combination of agents. We have performed extensive resistance selection evaluations with the NNRTI SJ-3366 by serial passage of infectious virus in the presence of increasing concentrations of SJ-3366, resulting in viruses with complete resistance to the compound. At each passage, cell growth and viability, extent of virus productivity (intracellular and extracellular) and viral infectivity and cytopathic effect were quantified. Further characterization of the virus obtained at each passage included the evaluation of compound sensitivity, inhibition of virus attachment and reverse transcription, as well as sequencing of the viral reverse transcriptase and envelope. The resistant viruses obtained at each passage were also evaluated for their sensitivity to known attachment, fusion, reverse transcriptase and protease inhibitors. The results of these studies provide a highly extensive characterization of the properties of the SJ series of compounds, highlighting properties of the compounds important for resistance, virus replication capacity and viral fitness which will help determine their potential success as a an anti-HIV therapeutic agent.


## Methodology

**Resistance Selection**
Resistant virus was selected by serial passage of HIV-$1_{IIIB}$ and HIV-$2_{ROD}$ in CEM-SS cells in the presence of increasing concentrations of SJ-3366 starting with two times the $EC_{50}$. The peak day of virus production of each passage was determined by measuring the amount of reverse transcriptase for each day from the passage. The peak virus production day from a passage was used to initiate the subsequent passage.

**Sensitivity Assay**
Following a six day acute infection of CEM-SS cells with the virus strain to be tested in the presence of compound, cell viability was measured spectrophotometrically (450/650 nm absorbance) using XTT dye reduction.

**Attachment Assay**
Compound and virus (at a pre-determined titer) were added to HeLa-LTR-β-Gal cells that had been plated in a 96-well flat-bottomed plate 24 hours prior to assay initiation. Cells, compound and virus were allowed to incubate for 2 hours at 37°C/5% CO2. Following the incubation, the cells were washed to remove any unbound virus and compound. Following addition of media to the wells and a 48 hour incubation, the cells were lysed and evaluated for β-gal expression using Gal-Screen (Tropix).

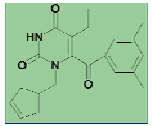
**Comparative Infection**
A sample of the virus pool with the greatest content of virus from each passage was used to infect CEM-SS cells. Standardization of the infections was based on quantity of p24 or reverse transcriptase in order to comparatively evaluate the relative growth kinetics of each virus pool. The culture was monitored until the cell viability dropped below 50% by trypan blue dye exclusion. Cell number and viability was monitored each day and viral supernatant was evaluated by quantifying the amount of p24 or viral reverse transcriptase.

**p24 Assay**
Sample and lysis buffer are added to coated wells to disrupt the HIV-1 p24 antigen and virus particles. The plate is washed and biotinylated human anti-HIV IgG is added to the wells in order to bind any HIV-1 p24 antigen that bound to the well. Streptavidin-horseradish peroxidase complexes with biotinylated antibodies before adding the substrate (TMB) and hydrogen peroxide (termination step). Absorbance is read at 450/570 nm. The intensity of color is directly proportional to the amount of uncomplexed p24 antigen in the sample.

**Reverse Transcriptase Assay**
Supernatant samples were mixed with a cocktail containing tritiated thymidine triphosphate and were incubated for 1 hour. 10 mL of the mixture was then transferred to a DEAE filtermat and was washed 5 times with buffer, 2 times with water and 1 time with alcohol. Once dry, the filtermat was transferred to a scintillation bag with approximately 4 mL of scintillation fluid. The samples were read on the Microbeta Trilux scintillation counter and reported as counts per minute (cpm).

## Structure of SJ-3366

**1-(3-Cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione**
**Molecular weight = 352.43**



## Inhibitory Activity of SJ-3366

| VIRUS DESCRIPTION | SJ-3366 vs HIV-1 | SJ-3366 vs HIV-2 |
|---|---|---|
| Lab Adapted Strains | Sub-nanomolar TI: 0.5-4 million | Submicromolar TI: 20-50,000 |
| Low Passaged Clinical Strains (T-Tropic and Monocyte Tropic) | Sub-nanomolar TI: 0.5- 4 million | Sub-nanomolar to Sub-micromolar TI: Up to 1 million |
| NNRTI – Resistant Strains | Submicromolar | ND |
| Protease Inhibitor Resistant Strains | Subnanomolar | ND |

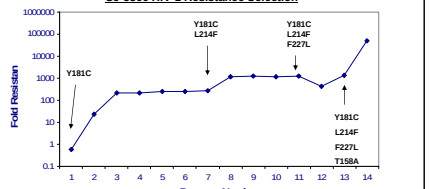
## Mechanism of Action

- Inhibits HIV-1 RT with a $K_i$ of 3.2 nM using a heteropolymeric template; affects both the $K_m$ and $V_{max}$
- Does not inhibit HIV-2 RT in enzymatic assays
- SJ3366 Inhibits HIV-1 attachment to target cells but not the fusion of HIV infected and uninfected cells
- SJ3366 inhibits attachment of HIV-1 and HIV-2 to target cells by recognition of a conformational target formed after virus attachment

## *In Vitro* HIV-1 Combination Evaluation and Activity vs NNRTI Resistance Mutations

- In two drug combination assays with nucleoside, nonnucleoside, protease and attachment inhibitors additive anti-HIV interactions were observed except in the case of ddI and fluoro-ddA
- In three drug combination assays with AZT, 3TC, ddI, nevirapine, nelfinavir or indinavir additive to slightly synergistic (SJ-3366+AZT+Nevirapine) interactions were observed
- No synergistic toxicity or antagonistic anti-HIV activity was observed in any combination assays
- Decreased activity was observed with all NNRTI resistance engendering mutations tested including Y181C, K103N and K101E

## SJ-3366 HIV-1 Resistance Selection




## SAR Evaluation of Distinct SJ Compounds

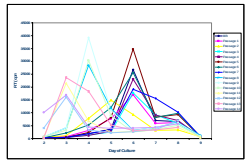
| SJ # | HIV-1 | HIV-2 | WT RT | K103N RT | ATTACHMENT |
|---|---|---|---|---|---|
| 16 | ++ | + | + | + | + |
| 17 | ++ | + | + | + | |
| 18 | ++ | + | | | + |
| 19 | ++ | + | | | + |
| 23 | + | | | + | |
| 30 | + | + | | | |
| 35 | + | | | | |
| 38 | + | + | | | |
| 39 | + | + | | | + |
| 40 | ++ | + | + | + | + |
| 49 | + | + | + | | + |
| 56 | ++ | | | | + |
| 61 | + | | | | |
| 62 | ++ | | | | + |
| 63 (SJ-3366) | ++ | | | | |

## HIV-1 Passage History Selection

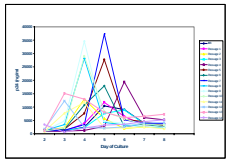
| Passage (SJ-3366 (μM) | Peak Day (RT) | RT Activity (cpm) | Extracellular p24 (ng/mL) | Intracellular p24 (ng/mL) | Infectious Units (TCID50) | CPE Observation (RT Peak Day) |
|---|---|---|---|---|---|---|
| 1 (0.008) | 5 | 57,986 | 7180 (D5) | 9650 (D7) | 4.3 (D6) | 3.5-4.0 |
| 2 (0.016) | 4 | 77,246 | 15,200 (D5) | 12,300 (D4) | 7.3 (D5) | 3.0 |
| 3 (0.032) | 5 | 23,441 | 3220 (D5) | 1300 (D4) | 5.7 (D5) | 3.0 |
| 4 (0.064) | 6 | 18,874 | 3530 (D7) | 9820 (D4) | 4.6 (D6) | 4.0 |
| 5 (0.128) | 7 | 13,689 | 129,000 (D9) | 1420 (D9) | ≥3.1 (D7) | 3.0-4.0 |
| 6 (0.256) | 8 | 55,353 | 27,900 (D8) | 4230 (D7) | 7.1 (D8) | ND |
| 7 (0.5) | 4 | 37,858 | 119,000 (D6) | 6340 (D5) | 6.8 (D6) | 3.0-3.5 |
| 8 (1) | 7 | 51,523 | 92,600 (D7) | 144 (D6) | 6.0 (D7) | 4.0-4.5 |
| 9 (2) | 9 | 37,547 | 82,400 (D10) | 1160 (D10) | 5.6 (D10) | 3.5-4.0 |
| 10 (4) | 7 | 54,549 | 56,000 (D7) | 1200 (D8) | 7.3 (D6) | 3.0 |
| 11 (8) | 8 | 37,289 | 712 (D7) | 1950 (D7) | 3.0 (D4) | 2.0 |
| 12 (16) | 5 | 24,319 | 110,000 (D6) | 977 (D6) | 2.5 (D4) | 2.5 |
| 13 (38) | 5 | 244,359 | 115,415(D5) | 4245 (D4) | ND | 2.5 |
| 14 (78) | 4 | 54,962 | 4862 (D4) | ND | ND | 2.5 |

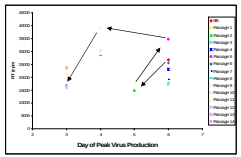
## Analysis of Comparative Infections

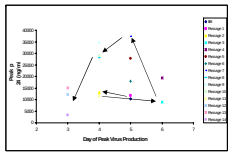
### Standardization by RT



### Standardization by p24






## Replication Capacity and Fitness of HIV-1 SJ-3366 Resistant Virus

| Passage | Peak Day of Infection | Level of Virus Replication | Replication Capacity | RT Fitness | Attachment Fitness (Titer in Assay) |
|---|---|---|---|---|---|
| WT | 6 | ---- | ---- | 286 (WT) | 15 |
| P1 | 6 | Low | Slower | 355 (WT) | 25 |
| P2 | 5 | Intermediate | WT | 309 (WT) | 25 |
| P3 | 7 | Low | Slower | 135 (Low) | 50->50 |
| P4 | 7 | Low | Slower | 172 (Low) | >50 |
| P5 | 5 | Intermediate | WT | 99 (Low) | 50->50 |
| P6 | 5 | Intermediate | WT | 432 (High) | 25 |
| P7 | 5 | Low | WT | 265 (WT) | 50 |
| P8 | 5 | High | WT | 392 (Int High) | 15 |
| P9 | 4 | High | Faster | 184 (Int Low) | 25 |
| P10 | 5 | High | WT | 226 (WT) | 50 |
| P11 | 4 | Intermediate | Faster | 178 (Low) | 50->50 |
| P12 | 3 | Intermediate | Faster | 322 (WT) | 50->50 |
| P13 | 3 | Low | Faster | 211 (WT) | >50 |
| P14 | 3 | Low | Faster | 408 (WT) | 25 |

## Summary

- SJ-3366 is a potent NNRTI of HIV-1 and HIV-2 with a secondary mechanism of action that targets virus attachment
- Although a loss of activity is observed with most of the NNRTI known mutant viruses, SJ-3366 remains active against these viruses due to its high therapeutic index
- Changes in viral fitness and replication capacity occur as HIV becomes resistant to the compound
- No synergistic toxicity or antagonistic anti-viral activity was observed in combination with other classes of HIV inhibitors rendering SJ-3366 a good candidate for combination therapy
- The oral bioavailability of SJ-3366 is 24% and has a half life of 5-6 hours
- IND-directed pharmacology and toxicology studies are currently in progress
- There is a panel of approximately 11 compounds in the SJ series that may be good potential clinical candidates