

Comparative Evaluation of Twelve Pyrimidinedione Inhibitors of HIV-1 For Further Preclinical Development

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Abstract

We have previously reported the anti-HIV activity of SJ-3366 [1-(3-Cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione], which is a highly potent inhibitor of both HIV-1 and HIV-2. SJ-3366 inhibits the replication of HIV-1 by two mechanisms, acting as a nonnucleoside RT inhibitor and an entry inhibitor recognizing a conformational target formed by the association of virus with target cells. SJ-3366 inhibits HIV-1 at sub-nanomolar concentrations and HIV-2 at sub-nanomolar to sub-micromolar concentrations. Comparative evaluation of 78 congeners of SJ-3366 resulted in the definition of twelve highly active inhibitors with therapeutic indices ranging from 0.5 to 4 million. Additional studies have been performed to define the anti-viral properties of the twelve molecules in order to define the optimal clinical candidate for further development. The rank order of each of these twelve molecules in inhibitory bioassays measuring activity against HIV-1, HIV-2, virus attachment and reverse transcription was defined. Four of the molecules were highly inhibitory against viruses and RT possessing the K103N mutation in the RT. Further evaluation of the series was performed by defining the relative oral bioavailability of the candidate compounds in mice dosed with equivalent amounts of material solubilized as colloidal suspension, defining compounds which would be expected to achieve the highest plasma concentrations. Further evaluations focused on the relative ability of the most active of the twelve test molecules to prevent the emergence of resistant virus strains. The concentration of each compound which was required to sterilize virus infected cultures was defined. Finally highly sensitive comparative resistance selection assays were performed to quantitatively compare the kinetics of resistant virus emergence under identical selection conditions. The fold-resistance of the virus obtained at each passage and the resistance engendering mutations in the RT and env were defined. The complete preclinical characterization of the twelve molecules will be presented along with results of initial pharmacokinetic profile of SJ-3366 in mice.

METHODOLOGY

Cytoprotection 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-beta-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 beta-galactosidase expression using Gal-Screen (Tropix).

RT Inhibition Assay

In a 96-well round bottom plate, 30 μ L of test compound was added to wells containing 50 μ L of 800 Ci/Mol alpha 32P : dGTP solution and 20 μ L of diluted enzyme. Following a 30 minute incubation at 37°C, 10 μ L of MB-grade fish sperm followed by 150 μ L of 10% TCA was added to the wells to precipitate the samples. Contents were transferred to a filter plate and the plate was washed with 10% TCA two times. 20 μ L of Supersmix Scintillation fluid (Wallac) was added to each well and activity was assessed using the Microbeta TriLux.

PBMC Assay

Peripheral blood mononuclear cells were isolated by ficoll hypaque gradient centrifugation from whole blood and activated with PHA. Monocytes were further purified by adherence to plastic and washing to remove lymphocytes. Following a seven day incubation, supernatant reverse transcriptase (RT) activity or p24 antigen expression by ELISA was measured to quantify virus replication. Cell viability was determined by XTT dye reduction.

Transmission Assays

Ghost X4/R5 (CD4+) and ME180 (CD4-) cells were incubated with chronically infecting H9 cells for 4 hours. The cultures were washed following the incubation and at 24 and 48 hours following infection. On day 6 post infection, virus replication was measured by evaluating RT and p24.

Sterilization Assay

CEM-SS cells were infected with HIV-1 and incubated with 6 concentrations of compound. Every three to four days the cells were passaged by adding 1 mL of the infected culture with 4 mL of fresh CEM-SS cells. At each passage microscopic observations of syncytium formation and quantitation of cell-free virus by RT assay was monitored. Following 10 passages in the presence of compound, the cells were cultured in the same fashion without the addition of test compound.

Bioavailability Assay

Balb/c mice were dosed via oral gavage with test compounds and blood was removed from triplicate test animals at various time points following administration. The bioactive concentration of test material was defined by evaluation of the activity of the serum in the cytoprotection assay.

Anti-HIV Activity in Fresh Human PMBCs Infected with a Clinical Strain of HIV-1 and HIV-2

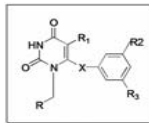
Compound	HIV-1 Clade B (US92/727) EC ₅₀ (nM)	HIV-2 EC ₅₀ (nM)	Therapeutic Index
SAR 16	0.24	<0.1	>2,083,300
SAR 17	0.23	<0.1	787,826
SAR 18	0.33	<0.1	>1,515,151
SAR 19	0.42	<0.1	>1,190,476
SAR 20	1.73	<0.1	76,127
SAR 22	>10	0.45	>50,000
SAR 40	2.44	<0.1	26,352
SAR 45	1.62	<0.1	70,902
SAR 49	0.54	<0.1	62,777
SAR 56	2.40	0.18	>208,353
SAR 62	0.13	<0.1	>3,846,154
SAR 63	0.36	<0.1	>1,388,888

NOTE: Similar results were observed with HIV-1 Clade C (ZA97/003).

Inhibition of HIV-1 (K103N) in Cell based and Biochemical Assay

Compound	Site Directed N4-3 _{302A} CEM-SS Cells EC ₅₀ (μ M)/Fold Resistance	K103N Enzymatic Assay IC ₅₀ (μ M)
SAR 16	0.0490	0.0935
SAR 17	0.0267	0.08829
SAR 18	0.0047	0.4510
SAR 19	0.00375	0.4911
SAR 20	0.0525	0.9372
SAR 22	0.45113	2/5
SAR 40	0.1428	0.985
SAR 45	0.0770	>100/1150
SAR 49	0.0220	0.095
SAR 56	0.1137	0.099
SAR 62	0.0190	0.9213
SAR 63	0.0120	85.6243

General 2.4 (1H,3H)-Pyrimidinedione Structure



SAR	R1	R2	R3	X	R
16	Et	Me	Me	O	Cyclohexenyl
17	Et	Me	Me	O	Cyclohexenyl
18	Et	Me	Me	C=O	Cyclohexenyl
19	Et	Me	Me	C=O	Cyclohexenyl
20	Et	Me	Me	S	Cyclohexenyl
22	Et	Me	Me	O	Cyclohexenyl
40	Et	Me	Me	O	Cyclohexenyl
45	Et	Me	Me	C=O	Cyclohexenyl
49	Et	Me	Me	Et	1-Cyclopenten-1-yl
56	Et	Me	Me	C=O	1-Cyclopenten-1-yl
62	Et	Me	Me	C=O	3-Cyclopenten-1-yl
63	Et	Me	Me	C=O	3-Cyclopenten-1-yl

Mechanisms of action of the Pyrimidinedione Series of Inhibitors

Inhibition of RT: Like typical NNRTIs, the compounds are mixed type inhibitors of HIV-1 which affect both the K_i and the V_{max}; allosteric inhibition of RT with a therapeutic index of approximately 1,000,000

Inhibition of virus attachment: The compounds inhibit both HIV-1 and HIV-2 by binding to a conformational target formed following the incubation of target cells and virus at 4°C. With clinical strains of virus attachment inhibition yields a 1E10,000

Anti-HIV Activity in CEM-SS Cells

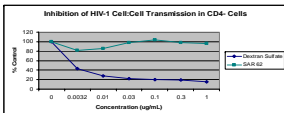
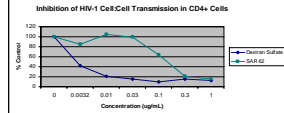
Compound	HIV-1 _{lab} EC ₅₀ (nM)	HIV-2 ₁₀₀₀ EC ₅₀ (nM)	Therapeutic Index
SAR 16	1	290	444,000
SAR 17	0.2	50	119,500
SAR 18	0.2	40	>2,500,000
SAR 19	0.2	20	730,000
SAR 20	2.0	250	61,000
SAR 22	10	>1000	22,300
SAR 40	4	110	12,850
SAR 45	2	160	12,100
SAR 49	0.5	30	48,600
SAR 56	2	110	155,000
SAR 62	0.2	40	2,097,500
SAR 63	0.4	20	218,250

Inhibition of HIV-1_{lab} Attachment

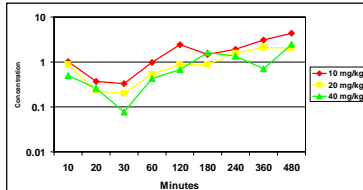
Compound	EC ₅₀ (μ M)	Therapeutic Index
SAR 16	0.073	>6849
SAR 17	0.022	5495
SAR 18	0.021	>23,810
SAR 19	0.017	7371
SAR 20	0.21	1519
SAR 22	>1	>500
SAR 40	0.55	9077
SAR 45	0.024	4616
SAR 49	0.027	3974
SAR 56	0.2374	>2174
SAR 62	0.059	>8475
SAR 63	0.050	>10,000

Preclinical Development and Evaluation of Most Active Compounds SAR 16, 18, 19, 62, and 63

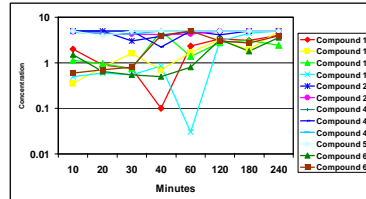
Transmission in CD4+ and CD4- Cells



Oral Bioavailability: Compound 62



Oral Bioavailability: All Test Compounds



Virus Sterilization Concentration

Compound	EC ₅₀ (μ M) (Fold Increase over EC ₅₀)	EC ₅₀ (μ M) (Compound Removed)
SAR 16	0.25 (250)	0.25 (250)
SAR 18	0.25 (1250)	0.25 (250)
SAR 19	0.05 (250)	0.05 (250)
SAR 62	0.5 (2500)	12.5 (6250)
SAR 63	0.02 (50)	0.02 (50)
UC781	0.025 (80)	0.025 (80)

CONCLUSIONS FROM STUDY

78 congeners were evaluated, yielding 12 potential clinical candidates. Five highly potent inhibitors were defined based on efficacy and toxicity profiles [16, 18, 19, 62, 63]. Three inhibitors exhibited higher levels of oral bioavailability in mice [16, 18, 19, 62]. Compound 63 performed exceptionally well in the virus outgrowth assay and had reasonably high oral bioavailability.