

# Development of Highly Potent Pyrimidinedione Inhibitors as Topical Microbicides

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## Abstract

Topical microbicides to prevent the sexual transmission of HIV are urgently needed, especially in developing countries. Efforts to develop topical microbicides have received increasing attention, recognizing the unmet need for a safe and effective, easy to use and inexpensive means to reduce rates of HIV transmission. At present a wide variety of therapeutic agents are being evaluated as topical microbicides, especially substances which act to prevent virus attachment to target cells. In addition to these entry inhibitors, highly potent nonnucleoside RT inhibitors have also been evaluated. We began development of pyrimidinediones as topical microbicides based on their unique dual mechanism of anti-HIV action which includes highly potent NNRTI activity as well as the ability to inhibit virus entry at a conformational target formed upon interaction of virus and cells. The series of compounds thus far been exemplified by the lead molecule SJ-3366 [1-(2-Cyclopenten-1-yl)methyl-6-(3,5-dimethylbenzoyl)-5-ethyl-2,4-pyrimidinedione] which inhibits replication of all tested clinical strains of HIV-1 and HIV-2 at sub-nanomolar concentrations in human PBMCs. A wide variety of congeners of SJ-3366 were evaluated for their ability to inhibit virus attachment and reverse transcription and the compounds were ranked based on their activity. Several highly active entry inhibitors with therapeutic indices ranging from 0.5 to 4 million were identified and evaluated as potential topical microbicides in various anti-HIV assays, including PBMCs, in CD4-dependent and CD4-independent cell based assays, combination anti-HIV activity assays, assays at various MOIs and in the presence of high concentrations of mucinopolysaccharides. The compounds were also evaluated in an *ex vivo* cervical epithelial cell-based tissue assay which measure the inhibitory activity of the SJ compounds in a relevant tissue-based assay. The results of the *in vitro* and *ex vivo* assays suggest that the pyrimidinediones have the potential to be highly effective topical microbicides which combine the ability to inhibit both virus entry and reverse transcription. SAR evaluations to define the molecular features responsible for the relative potency of the molecules in the inhibition of attachment and RT have been performed and will be presented.

## 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% CO<sub>2</sub>. 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 mL of test compound was added to wells containing 50 mL of 800 Cpm/Mol alpha 32P - dGTP solution and 20 mL of diluted enzyme. Following a 30 minute incubation at 37°C, 10 mL of HiB-grade fish sperm oil followed by 150 mL of 10% TCA was added to the wells to precipitate the samples. Contents were transferred to a filter plate and the plate was then washed with 10% TCA two times. 20 mL of Supermix 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 hyaque 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 X475 (CD4+) and ME180 (CD4-) cells were incubated with chronically infected 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.

### Epivaginal Tissue Infection Assays

Compound and/or virus were added to epivaginal tissues supplied by MatTek Corporation. For toxicity analysis tissues were washed and stained with MTM in order to determine cytotoxicity. For efficacy evaluations, the tissues were washed to remove compound and virus and fresh media was added to the well. Following another 24 hours, virus inhibition was evaluated by p24 in the supernatant.

### 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.

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

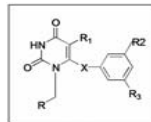
Compound	HIV-1 Clade B (U9/97/27) EC <sub>50</sub> (nM)	HIV-2 EC <sub>50</sub> (nM)	Therapeutic Index
SAR 16	0.24	<0.1	>2,083,300
SAR 17	0.23	<0.1	787,826
SAR 18	0.33	<0.1	>1,515,151
SAR 19	0.42	<0.1	>1,190,476
SAR 20	1.73	<0.1	76,127
SAR 22	>10	0.45	>50,000
SAR 40	2.44	<0.1	26,352
SAR 45	1.62	<0.1	70,802
SAR 49	0.54	<0.1	62,777
SAR 56	2.40	0.18	>208,353
SAR 62	0.13	<0.1	>3,845,154
SAR 63	0.36	<0.1	>1,388,888

NOTE: Similar results were observed with HIV-1 Clade C (ZA/97/003).

## Inhibition of HIV-1 (K103N) In Cell Based and Biochemical Assays

Compound	Site Directed NL4-3 <sub>K103N</sub> CEM-SS Cells EC <sub>50</sub> (µM)/Fold Resistance	K103N Enzymatic Assay IC <sub>50</sub> (µM)
SAR 16	0.0489	0.0489
SAR 17	0.0267	0.088/29
SAR 18	0.0047	0.45/10
SAR 19	0.0037/5	0.48/11
SAR 20	0.0525	0.93/2
SAR 22	0.45/13	2/5
SAR 40	0.1428	0.8/8
SAR 45	0.0770	>100/1150
SAR 49	0.0220	0.09/5
SAR 56	0.1137	0.09/5
SAR 62	0.0150	0.82/13
SAR 63	0.0120	85.6/243

## General 2, 4 (1H,3H)-Pyrimidinedione Structure



SAR	R1	R2	R3	Z	R
16	Et	Me	Me	O	Cyclopenten-1-yl
17	Et	Me	Me	O	Cyclopenten-1-yl
18	Et	Me	Me	CO	Cyclopenten-1-yl
19	Et	Me	Me	CO	Cyclopenten-1-yl
20	Et	Me	Me	S	Cyclopenten-1-yl
22	Et	Me	Me	O	Cyclopenten-1-yl
40	Et	Me	Me	O	Phenyl
45	Et	Me	Me	O	1-Cyclopenten-1-yl
49	Et	Me	Me	O	1-Cyclopenten-1-yl
56	Et	Me	Me	CO	1-Cyclopenten-1-yl
62	Et	Me	Me	CO	1-Cyclopenten-1-yl
63	Et	Me	Me	CO	1-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<sub>d</sub> and the V<sub>max</sub>; allosteric inhibition of RT with a therapeutic index of approximately 1,000,000.

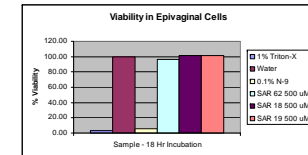
**Inhibition of virus attachment:** The compounds inhibit both HIV-1 and HIV-2 by binding to a conformational target formed following the incubation of target cells and virus at 4°C. With clinical strains of virus attachment inhibition yields a TI=100,000.

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

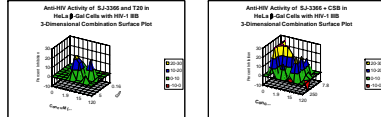
## Evaluation in Epivaginal Tissues

### Other Key Properties of SJ Compounds as Microbicides

- Nontoxic when evaluated against normal vaginal flora (*Lactobacillus crispatus* and *Lactobacillus jensenii*)
- Little to no reduction in efficacy when evaluated in presence of mucin or serum proteins (HSA, AAGP, AB Serum or BSA)
- Testing in progress against other STIs (HCV, HBV, chlamydia, gonorrhea, herpes, trichomoniasis)
- Evaluating efficacy when added to target cells at various pH to mimic vaginal environment



## Combination Efficacy in Cell-Based Attachment Inhibition Assay:



## Anti-HIV Activity in CEM-SS Cells

Compound	HIV-1 <sub>lab</sub> EC <sub>50</sub> (nM)	HIV-1 <sub>clin</sub> EC <sub>50</sub> (nM)	Therapeutic Index
SAR 16	1	280	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	63,000
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	2,087,500
SAR 63	0.4	20	218,250

## Inhibition of HIV-1<sub>lab</sub> Attachment

Compound	EC <sub>50</sub> (µM)	Therapeutic Index
SAR 16	0.073	>849
SAR 17	0.022	5485
SAR 18	0.021	>23,810
SAR 19	0.017	7371
SAR 20	0.21	1519
SAR 22	>1	>500
SAR 40	0.55	907
SAR 45	0.024	4646
SAR 49	0.027	3974
SAR 56	0.23	>2174
SAR 62	0.059	>8475
SAR 63	0.050	>10,000

## Virus Sterilization Concentration

Compound	EC <sub>50</sub> (µM) (Fold Increase over EC50) Presence of Compound	EC <sub>50</sub> (µM) (Fold Increase over EC50) Compound Removed
SAR 16	0.25 (250)	0.25 (250)
SAR 18	0.25 (1250)	0.25 (1250)
SAR 19	0.05 (250)	0.05 (250)
SAR 62	0.5 (2500)	12.5 (6250)
SAR 63	0.02 (50)	0.02 (50)
UC761	0.025 (50)	0.025 (50)

## Conclusions

- They pyrimidinediones represent excellent microbicide candidates because of their ability to inhibit two early steps in virus replication: entry and RT.
- Of 78 congeners, twelve molecules were determined to be highly active inhibitors of HIV-1 (TI > 100,000)
- Of these twelve congeners evaluated, five were defined to have significant microbicide potential and were evaluated in detail.
- Of these five, SAR 63 appeared to have the greatest efficacy in preventing virus infection and replication and is considered our lead microbicide compound.
- The pyrimidinediones may also offer the ability to select a combination of two SAR compounds with differing antiviral properties for development (i.e., high RT inhibition and high entry inhibition activities)