

# The Mechanism of Inhibition of HIV Entry in MAGI Cells by Procaine Based Benzamide Derivative SP-10

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## Abstract

We studied the effect of the benzamide SP-10 on HIV-1 IIBB replication in HeLa cells transfected with CD4 and chemokines receptors CCR5 and CXCR4. In cells pre-incubated with SP-10 for 48 hrs prior the virus infection, SP-10 inhibited viral replication by 75% with an IC50 of 36 µM compared to AZT (IC50=27.4 nM). The effect of SP-10 was dependent on the duration of the pre-incubation, suggesting that SP-10 targets the host cell rather than the virus itself. The confocal microscopy and immunoprecipitation studies indicated that MAGI HeLa cells treated with SP10 for 48 hrs displayed a decrease of CD4 and CCR5 receptors at the cell surface. As a consequence gp120 binding on the cell membrane was also decreased. Env engagement of HIV receptors has been reported to be actin-dependent, as the polymerization of this cytoskeletal protein is required for virus entry in cells. SP-10 pre-medicated MAGI cells contained reduced levels of polymerized F-actin that could explain the decreased expression of CD4 and CCR5 receptors on the cell surface and the inability of gp120 to bind to the cells. SP-10 also inhibited the replication of the multidrug resistant viral strain MDR769 by 50% with concentrations in the µM range and displayed very low in vitro toxicity compared to the reference compounds AZT and DDI. These data suggest that SP-10 is a promising lead compound for HIV therapy.

## Background

The efficacy of the anti-HIV drugs currently available on the market is too often hampered by the emergence of viral resistance. Even Fuzeon<sup>®</sup>, the last anti-retroviral released on the market, induced the mutation of gp41 generating resistant strains. Therefore there is a critical need for new anti-HIV therapeutic.

We report herein the anti-retroviral properties and the mechanism of action of a benzamide derivatives, SP-10, studied against HIV-1 IIBB and the multi-drugs resistant strain MDR769.

## Methods

### Materials

MAGI cell were obtained from NIH AIDS Research & Reference reagent Program (Rockville, MD). Fluorescence rgp 120 HIV-1 IIBB was from Immuno Diagnostics, Inc., (Woburn, MA) HIV-1MN gp120 was obtained from Advanced Biotechnologies (Columbia, MD). Alexa Fluor 488 phalloidin and Alexa Fluor 594 Deoxyribonuclease I were from Molecular Probes, Inc. (Eugene, OR). Antisera or antibodies used: anti-CD4 and anti-CCR5 from Santa Cruz Biotechnology (Santa Cruz, CA). SP-10 was synthesized by Comgenex (Budapest, Hungary) Cell culture plasticware was from BD Falcon (Franklin Lakes, NJ). All other chemicals used were of analytical grade and were obtained from various commercial sources.

### SP-10 synthesis

Boc-L-Tryptophan (A) (4.556 g; 15 mmol) was dissolved in DCM (60 ml), CDI (2.513 g; 15.5 mmol) was added and then the reaction mixture was stirred at RT for 100 min. 2-methylpiperazine (1.502 g; 15 mmol) was added and stirring was continued at RT for 6 more hours. DCE (15 ml) was added and the organic solution was washed with 5% aq. Na2CO3, 3% aq. HCl and water, respectively. The organic phase was dried over Na2SO4, filtered and evaporated to dryness. The residue was solidified with diethyl ether-hexane mixture to obtain the title product (B) as a white crystalline solid (3.021 g; 52%). The piperazine derivative obtained in the previous step (B) (3.021 g; 7.82 mmol) was dissolved in DCE (30 ml), TEA (15.64 mmol; 2.18 ml) was added followed by the addition of cyclopropanecarbonyl chloride (0.77 g; 7.43 mmol; 0.674 ml). The reaction mixture was stirred at RT for 5 hours. The organic solution was washed with 3% aq. HCl, 3% aq. Na2CO3 and with water, respectively. The organic phase was dried over Na2SO4, filtered and evaporated to dryness to obtain the desired product as a white solid (C) (3.245 g; 91%) The Boc-protected aminoamide derivative (C) prepared in the previous step (3.254 g; 7.16 mmol) was dissolved in DCM (5 ml). TFA (8 ml) was added while cooling in an ice-water bath. The cooling bath was removed and the reaction mixture was stirred at RT for 5 hours. The mixture was evaporated to dryness, then 10% aq. NaOH (20 ml) was added to the residue while cooling in an ice-water bath. The aqueous solution was extracted with DCE (2x30 ml) and then the combined organic phase was washed with water to neutral. The organic solution was dried over Na2SO4, filtered and evaporated to dryness to obtain the free amine as a light yellow solid (D) (0.787 g; 32%).

The amino-compound obtained in the previous step (D) (0.763 g; 1.62 mmol) was dissolved in DCE (30 ml), TEA (4.05 mmol; 0.565 ml) was added followed by the addition of 4-nitrobenzoyl chloride (0.256 g; 1.54 mmol). The reaction mixture was stirred at RT for 5 hours. The organic solution was extracted with 3% aq. HCl, 3% aq. Na2CO3 and water respectively. The organic phase was dried over Na2SO4, filtered and evaporated to dryness to obtain the desired product as a yellow solid (SP10) (0.79 g; 96%).

### Cell culture

MAGI cells were cultured under 5% CO2 in DMEM medium with 2mM L-glutamine containing 10% FBS, 100µg/ml penicillin, 100mg/ml streptomycin, 0.25mg/ml fungizone, 0.2mg/ml G418, 0.1mg/ml hygromycin B, and 1mg/ml puromycin as provider's instruction.

### Viral Replication

The GenPhar AV-Finder™-HIV Drug Discovery Assay was used *in vitro* for viral replication study. HeLa cells transfected with CD4, CCR5 & CXCR4 (3000/well) seeded to 96-well plates with the adenovirus AD-3R in DMEM were pre-treated with the corresponding concentration of the compounds of interest for 48h, and then incubated with HIV-1 IIBB (2000i.u/ml) for overnight. After that, the medium was replaced by fresh medium containing the corresponding concentration of compounds of interest and the infectivity was assessed by measuring the fluorescence on each well 3 days later (λexc=485 nm; λexc=520 nm). For multi-drugs resistant strain MDR-769, without pre-medication, at day 3, MDR-769 (200IP/well) and increasing concentrations of SP-10 or reference compounds (AZT) were added and incubated overnight. At day 4, the medium was replaced by fresh medium containing the corresponding concentration of the compounds of interest. The infectivity was assessed by measuring the fluorescence on each well at day 7.

### Cell surface CD4 and CCR5 receptors expression

MAGI cells cultured on coverslips were pre-treated with SP-10 for 48h, then labeled with cell membrane impermeable EZ-Link<sup>®</sup> Sulfo-NHS-LC-Biotin. After wash three times in cold PBS containing 100mM glycine, cells were labeled with Alexa Fluor<sup>®</sup> 555 streptavidin for 15 min on ice, then fixed with 4% paraformaldehyde. After fixation, cells were washed and incubated with anti-CD4 receptor or anti-CCR5 receptor antibody at room temperature for 1 hour, and labeled with Alexa Fluor<sup>®</sup> 488 donkey anti-rabbit IgG for another 1 hour. Cells were mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA), and visualized under confocal laser scanning microscopy (CLSM, Olympus FV500 Series, Japan).

### Fluorescence rgp120 binding

MAGI cells cultured on chamber slide were pre-treated with SP-10 for 48h, then incubated with 1µg/ml Fluorescence rgp 120 HIV-1 IIBB (Immuno Diagnostics, Inc., Woburn, MA) for overnight. After wash with PBS with 0.2% BSA, cells were fixed in 3.8% formaldehyde, then wash with PBS and mounted by VECTASHIELD mounting medium with DAPI and visualized under CLSM.

### Immunoprecipitation and Immunoblotting

MAGI cell cultured on 100mm dishes were pre-treated with SP-10 for 48h, then harvested and labeled with Biotin as described above. At the end of the labeling, cells were lysated with ice-cold lysis buffer (20-mmol/L Tris-HCl, 150-mmol/L NaCl, 1-mmol/L EDTA, 1-mmol/L EGTA, 1% Triton X-100, 0.1% SDS, and 0.25% Deoxycholic acid with protease inhibitor cocktail). The cell suspension was sonicated for 15s, lysated on ice for 1 hour and then centrifuged at 22,500g × 30 min, 4°C. After centrifugation the supernatant was incubated with CD4 or CCR5 antibody and immunoprecipitated by Protein A/G Plus-agarose. The samples were boiled for 5 min in 2X Tris-Glycine SDS sample buffer, and then subjected to 4-20% gradient SDS-polyacrylamide gel (SDS-PAGE) and electrotransferred to nitrocellulose membranes. The blots were probed with HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) and developed by enhanced chemiluminescence (ECL, Amersham Life, Arlington Heights, IL). The densities of the appropriate bands were determined using the OptQuant Acquisition & Analysis software (Packard BioScience).

### Confocal laser scanning microscopy for actin staining

MAGI cells grown on coverslips were pre-treated with SP-10 for 48h, then wash with PBS and fixed in 4% paraformaldehyde. The cell membranes were permeabilized with 0.1% Triton X-100, stained for 20 min with Alexa Fluor 488 phalloidin for F-actin and Alexa Fluor 594 Deoxyribonuclease I for G-actin. The coverslips were mounted with VECTASHIELD mounting medium, cells were observed under CLSM.

### Protein measurement

Protein was measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) based on the method of Bradford. Bovine serum albumin was used as a standard.

### Statistics

Statistical analysis was performed by one-way analysis of variance (ANOVA) and unpaired Student's t test using the INSTAT 3.00 package from GraphPad (San Diego, CA).

## Results

Figure 1 Synthesis of 1-(4-cyclopropanecarbonyl-3-methylpiperazin-1-yl)-2-(1H-indol-3-yl-methyl)-4-(4-nitrophenyl)-butane-1,4-dione (SP-10)

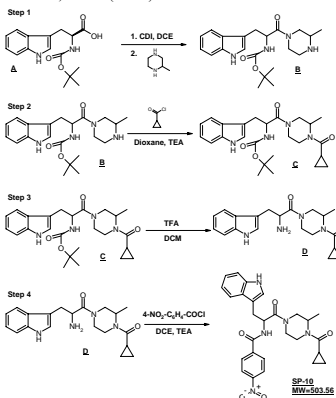


Figure 3 Inhibitory effect of SP-10 pre-medication on the HIV-1 MDR769 strain replication in HeLa cells

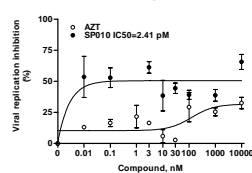


Figure 4 Effect of 48h pre-treatment with 1 µM SP-10 on biotin (Red) and CCR5 receptor antibody (green) labeling in MAGI cells

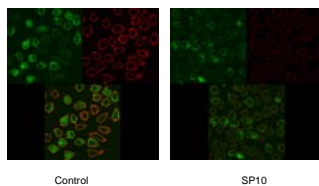


Figure 2 Inhibitory effect of 6, 12, 24 or 48 hours SP-10 pre-medication on the HIV-1 IIBB strain replication in HeLa cells

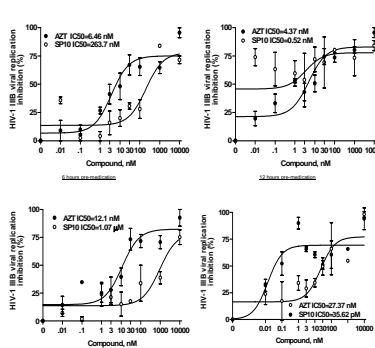


Figure 4 Effect of 48h pre-treatment with 1 µM SP-10 on biotin (Red) and CD4 receptor antibody (green) labeling in MAGI cells

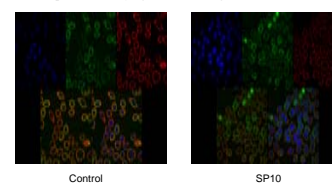


Figure 6 Effect of 48h pre-treatment with 1 µM SP-10 on gp120 binding in MAGI cells

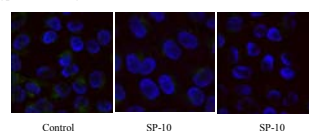


Figure 7 Effect of 48h pre-treatment with SP-10 on the immunoprecipitation of biotinylated cell surface proteins with anti-gp120 and anti-CD4 antibodies in MAGI cell

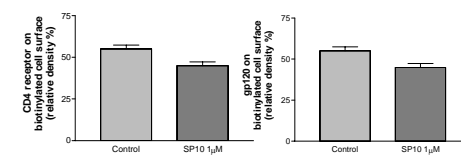
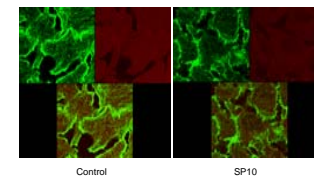


Figure 8 Effect of 48h pre-treatment with 1 µM SP-10 on F-actin (green) and G-actin (red) immunolabeling in MAGI cells



## Conclusion

1. After 48h pre-incubation, SP-10 inhibited the HIV-1 IIBB viral replication by 75% with IC50 of 36 µM.
2. SP-10 inhibited the multidrug resistant viral strain MDR769 replication by 50% with an IC50=2.41µM.
3. SP-10 decreased CD4 receptor and CCR5 receptor expression on surface of MAGI cells.
4. SP-10 inhibited gp120 binding to MAGI cells and decreased the gp120 amounts on surface of MAGI cells.
5. SP-10 blocked the formation of actin filaments (F-actin) and altered actin accumulation near the cell membrane.
6. SP-10 displayed very low toxicity *in vitro*.

SP-10 might represent the lead compound of a new class of anti-HIV therapy