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Introduction

Microbicides are currently one of the main strategies to prevent HIV infection that is especially important in developing countries. The objective of this work is to study the anti-HIV in vitro activity and in vivo toxicity of 5-hydroxytyrosol (5-HT), an antiviral natural compound[1, 2], in order to develop an effective low-cost microbicide

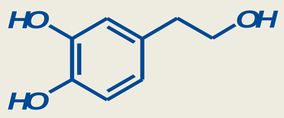


Figure 1. Chemical structure of 5-Hydroxytyrosol (5-HT)

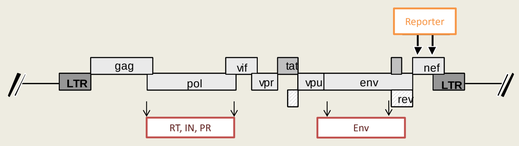


Figure 2. Recombinant viruses used in this work.

Materials and methods

The antiviral activity of 5-HT was assessed against SIV and several HIV-1 strains including founders viruses, strains resistant to other antiretrovirals using different experimental models: cell lines, lymphocytes and monocytes from human peripheral blood, autologous co-culture of DC-SIGN expressing cells and lymphocytes and infection through epithelial layers[3]. Anti-HIV activity of 5-HT was also assessed in combination with Tenofovir or Lamivudine. Synergism was analyzed according to T-C Chou and P. Talalay method. Mechanism of action was studied using VSV pseudotyped HIV-1, RT-PCR and transfection experiments. Toxicity was tested in vitro and in vivo, through evaluation of local tolerability at vaginal mucosa in rabbits (n=6) at two different concentrations (90 and 397mg/L) during 7 consecutive days by topical route

5HT IS ACTIVE AGAINST WILD-TYPE HIV, SEVERAL HIV SUBTYPES AND FOUNDER VIRUS *IN VITRO*

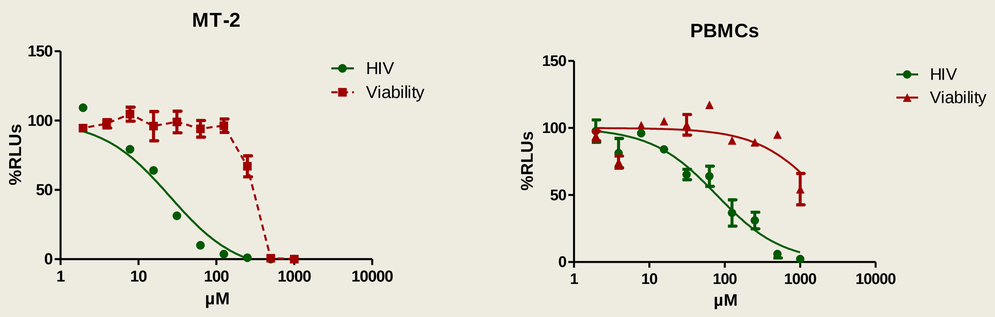


Figure 3. Anti-HIV activity of hydroxytyrosol (5-HT) in MT-2 cells (left graph) and PBMCs (Right graph) with wild type X4 tropic recombinant virus NL4.3-Renilla, with VSV-pseudotyped HIV NL4.3-VSV-Luc or with R5 tropic recombinant virus JR-Renilla. Cell viability was measured in the same conditions as infections, but without virus infection. IC_{50}: Inhibitory Concentration 50; ND: Not determined. NR: Not reached. IC_{50} MT-2: 35,65 μM; IC_{50} PBMCs: 55,58 μM. CC_{50} MT-2: >250 <1000

Figure 4. Evaluation of the anti-HIV activity of hydroxytyrosol (5-HT) in pre-activated PBMCs infections with viral strains of subtypes A, C, D, E, F and G (left panel) and with viral strains of founder HIV (right panel). IC50 values were all between 30 and 60 μM with no significant difference among them.

5-HT IS ACTIVE AGAINST HIV TRANSMISSION IN THE IMMUNE SYNAPSE

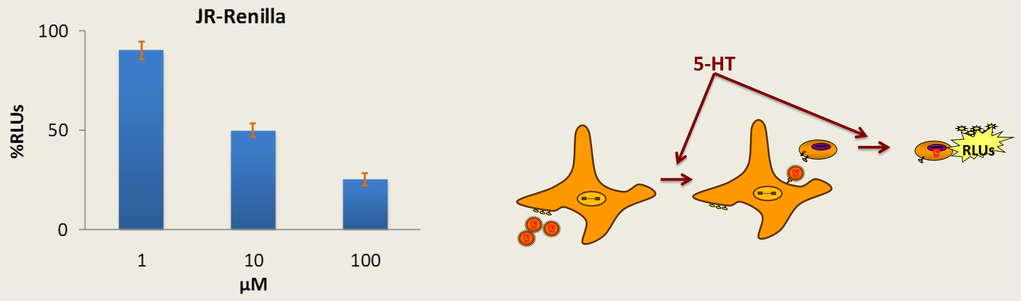
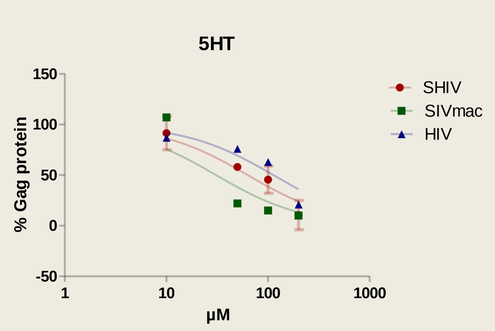


Figure 5. DC-SIGN mediated trans-infection inhibition of 5-HT. When DC-SIGN+ cells were present in cell culture, 5-HT anti-HIV potency was >10 fold higher, which would be essential for its microbicide activity, since mucosae infection is highly enhanced by APCs.

5-HT IS ACTIVE AGAINST SIV AND SHIV IN VITRO



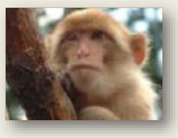
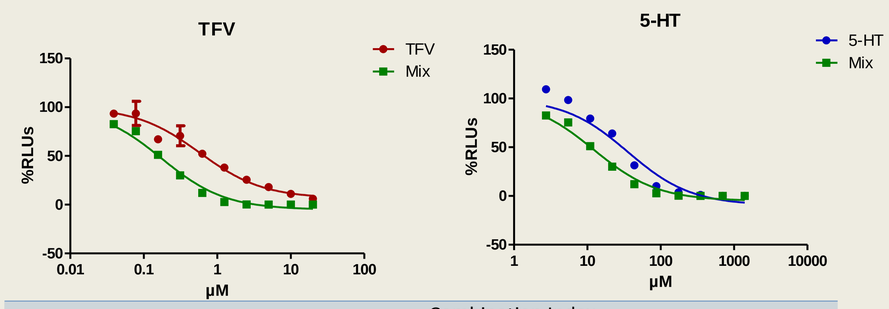


Figure 6. Evaluation of SIV, SHIV and wild type HIV inhibition by 5-HT by gag protein quantification. IC_{50} were 9,25 μM for SIVmac and 28 μM for SHIV.

5HT DISPLAYS A STRONG SYNERGSITIC EFFECT WITH TENOFOVIR *IN VITRO*



Combination Index			
	ED50	ED75	ED90
PBMCs IL-2	0,307	0,252	0,390
PBMCs PHA/IL-2	0,237	0,223	0,380
MT-2	0,558	0,490	0,469

CI: Combination Index.
CI >1.30 antagonism, 1.10–1.30 weak antagonism, 0.90–1.10 additive, 0.70–0.90 weak synergy, <0.70 strong synergy

Figure 7. Evaluation of the anti-HIV activity of 5-Hydroxytyrosol (5-HT) combined with TFV (ratio 70/2) in MT-2 cells and PBMCs pre-activated with IL-2 (ratio 1/30) or with PHA/IL-2 (ratio 1/40). Data obtained were analysed using CalcuSyn software. Figure shows MT-2 combinations experiments.

5HT INHIBITS HIV-1 INTEGRATION IN PREACTIVATED PBMCS

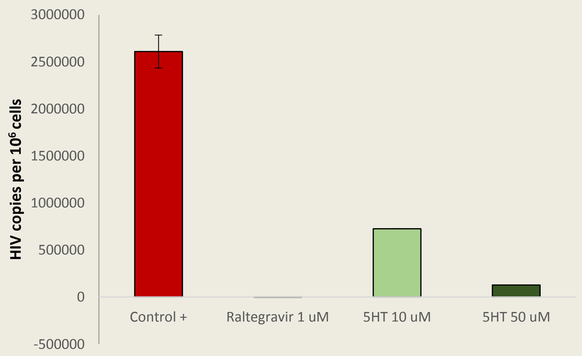


Figure 8. HIV-1 integration evaluation. Preactivated human PBMCs were pretreated with two different concentrations of 5HT and infected with HIV-1 (NL4.3). After 5 days in culture, genomic DNA was extracted and viral DNA integration was quantified by real time PCR.

5HT TARGETS VIRAL TRANSCRIPTION AND DOES NOT BLOCK VIRAL ENTRY

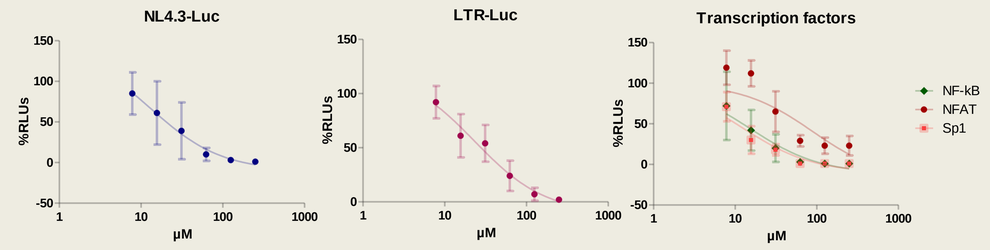


Figure 9. Inhibition of HIV transcription by 5HT. 5HT is able to inhibit transcription mediated through the whole HIV provirus (NL4.3-Luc) and the HIV promoter (LTR-Luc). This effect is mainly due to NF-kB and Sp1 inhibition.

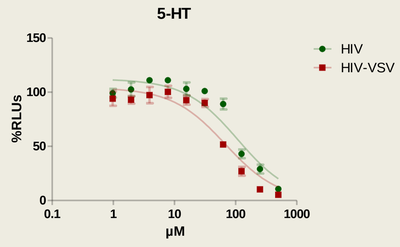


Figure 10. 5HT inhibits HIV and psedotyped HIV replication, suggesting that its effect is viral envelope independent and thus do not interfere with viral entry.

5HT IS NOT TOXIC *IN VIVO*

	Clinical Signs	Mortality / Viability	Macroscopic examination	Microscopic examination	pH (Mean ± SD)	Microbiological assessment: Vaginal exudate before treatments	Microbiological assessment: Vaginal mucosa after treatments
Group 1: Blank control n = 6	Swelling in the genital area for several days n = 1	0	n = 3	LI: Grade 1 or 2 in proximal, medial and/or distal parts (n = 4) VC: Grade 1 (n = 4) E: Grade 1 (n = 2)	7.46 ± 0.30	Streptococcus sp.: n = 6 Klebsiella pneumoniae: n = 0	Streptococcus sp.: n = 0 Klebsiella pneumoniae: n = 4
Group 2: Placebo HEC gel n = 6	_	0	n = 3	LI: Grade 1 or 2 in proximal or medial parts (n = 2) VC: Grade 1 (n = 3) E: Grade 1 (n = 2)	7.80 ± 0.48	Streptococcus sp.: n = 6 Klebsiella pneumoniae: n = 0	Streptococcus sp.: n = 0 Klebsiella pneumoniae: n = 1
Group 3: 5HT gel 30 mM n = 6	_	0	n = 4	LI: Grade 1 or 2 in proximal, medial and/or distal parts (n = 4) VC: Grade 1 (n = 4) E: Grade 1 (n = 2)	7.73 ± 0.27	Streptococcus sp.: n = 6 Klebsiella pneumoniae: n = 0	Streptococcus sp.: n = 0 Klebsiella pneumoniae: n = 2
Group 4: 5HT gel 100 mM n = 6	_	0	n = 6	VC: Grade 1 (n = 6) E: Grade 1 (n = 3)	7.66 ± 0.39	Streptococcus sp.: n = 5 Klebsiella pneumoniae: n = 1	Streptococcus sp.: n = 1 Klebsiella pneumoniae: n = 0
Group 5: 5HT gel 200 mM n = 6	_	0	n = 5	LI: Grade 1 or 2 in proximal and medial parts (n = 5) VC: Grade 1 (n = 5) E: Grade 1 (n = 3)	7.55 ± 0.35	Streptococcus sp.: n = 5 Klebsiella pneumoniae: n = 0	Streptococcus sp.: n = 1 Klebsiella pneumoniae: n = 3

Figure 11. Topical administration of 5HT GEL at three different concentrations (30, 100 and 200 mM) caused no morphological alterations in the rabbit vagina

Conclusions

5-HT was active against SIV and different HIV-1 strains in a variety of scenarios *in vitro*. A strong synergistic activity with Tenofovir was found being viral transcription the main target of 5-HT through NF-kB and SP-1 inhibition. In summary 5-hydroxytyrosol is a new class of microbicide combining both anti-inflammatory and anti-HIV properties and represents a potential candidate for clinical trials.

Acknowledgments

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