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The effect of gramicidin, a topical contraceptive and antimicrobial agent with anti-HIV activity, against herpes simplex viruses type 1 and 2 in vitro

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Summary. The effect of an anti-HIV compound, gramicidin, previously used as a topical antibiotic and vaginal contraceptive, on the replication of herpes simplex viruses (HSV) type 1 and 2 has been examined. Human WI-38 fibroblasts were inoculated with either HSV type in the presence of serial dilutions of gramicidin and reduction in viral yield was measured by ELISA. The 50% inhibitory dose (IC_{50}) of gramicidin against 3 HSV-1 and 4 HSV-2 isolates was equal to 0.3 μg/ml and was comparable to the efficacy of the anti-HSV agent acyclovir (ACV). The IC_{50} of gramicidin required to protect WI-38 from cytolytic effect of HSV was 10 μg/ml at day 5 postinfection, indicating that at this time point the activity of gramicidin was inferior than that of ACV. Nevertheless, gramicidin suppressed the replication of ACV-resistant thymidine kinase and DNA polymerase HSV mutants at doses effective against ACV-sensitive strains. The results suggest that the antimicrobial and spermostatic agent, gramicidin, has potential against sexually transmitted diseases (STDs) and for prophylaxis of sex-borne HIV and HSV infections.

Introduction

Symptomatic human herpes simplex virus type 1 (HSV-1) infections are fairly benign in immunocompetent individuals as HSV-caused oropharyngeal sores tend to disappear spontaneously. Primary clinical manifestations of HSV-2 infection, which is mainly transmitted sexually, are anogenital lesions [12, 24]. Genital herpes affects one third of the world's population, and among people with human immunodeficiency virus (HIV) the incidence may be as high as 80 percent [14]. HSV infections are particularly severe and even life-threatening to patients with acquired immune deficiency syndrome [22]. Only 20 percent of herpes seropositive persons have symptomatic infection. The rest of them are asymptomatic but nevertheless are able to shed the virus. In view of the high prevalence of genital herpes and its cofactor role in enhancing sex-borne HIV transmission the herpesviruses are of particular concern [7, 14].

HSV infections are usually treated with nucleoside analogs such as acyclovir (ACV) [11]. Although ACV is relatively safe and non-toxic, its prophylactic use in suppressing HSV shedding in the genital tract would require repeated exposure to the drug and may thus favor the spread of drug-resistant strains [23]. HSV becomes resistant to ACV due to the loss or mutation of the viral thymidine kinase (TK) or to the alteration in the viral DNA polymerase [10, 15, 18]. The need for alternative agents effective against ACV-resistant mutants and which can be used topically for prophylaxis of HSV is obvious. Ideally, one must identify an effective and safe compound which could afford barrier protection against both HSV and HIV. It would be preferable if such an agent were useful against common microbial STDs and played a contraceptive role as well.

Gramicidin has recently been identified by us as a potent non-toxic anti-HIV agent with activity three to five orders of magnitude higher than nonoxynol-9 – the most common spermicidal and anti-STD agent [2–4]. Unlike the detergent nonoxynol-9, gramicidin acts as an ionophore by triggering the efflux of cytoplasmic potassium (K^+) from target cells and causing the depolarization of the host cell membrane. On the other hand, infection by many enveloped viruses, including HIV and HSV, leads to a drastic increase of intracellular K^+ [1, 25, 26]. Two separate events, virus entry and viral budding, are dependent on the cell surface polarity maintained by transmembrane ionic gradients. Thus, the alteration of K^+ balance by channel-forming gramicidin would adversely affect the viral infection process requiring cellular cooperation. Although there are several classes of drugs that can regulate monovalent cation homeostasis very few are effective at low, non-toxic doses and most of them are used only for experimental purposes in vitro. Gramicidin appears to be the rare exception in this category of drugs.

Gramicidin was the first antibiotic ever to be isolated [9] and has broad spectrum activity against gram-positive bacteria, fungi, and protozoa. Gramicidin does not irritate mucous membranes, is poorly absorbed through the skin, and it is still used today as one of the active ingredients in ophthalmic antimicrobial solutions, e.g., Neosporin. Cyclic or gramicidin S is used in the former Soviet Union (FSU) as an active ingredient of a spermicidal vaginal contraceptive and for the therapy of genital ulcers caused by various STDs [21]. In this in vitro study we have investigated the activity of gramicidin on replication of herpesviruses. Since gramicidin exerts its action upon the host cell rather than by targeting the virus it was argued that it may also be effective against acyclovir-resistant HSV.

Materials and methods

Antiviral compounds

Gramicidin D is a mixture of three pairs of gramicidins A, B, and C, making up 80%, 6%, and 14% respectively. Each pair consists of 2 subspecies, one with valine in position one, comprising 80–95% of the component, and the other with isoleucine. Gramicidin D was

purchased from Sigma (St Louis, MO) dissolved in 95% ethanol at concentration 1 mg/ml as a stock solution and kept at room temperature until used. Acyclovir, 9-[2-hydroxy-ethoxymethyl]guanine (ACV), used as a reference drug, was obtained from Sigma and prepared in saline as 1 mg/ml stock solution.

Cells and viruses

Human embryonic lung fibroblasts WI-38 (CCL-75, ATCC, Rockville, MD) were grown in RPMI medium 1 640 with 10% FBS, L-glutamine, and penicillin/streptomycin (GIBCO, Grand Island, NY) at 37 °C with 5% CO_2 humidified air atmosphere. The commercial strain of HSV-2 (VR-734, strain G, Lot 12D, ATCC, Rockville, MD) was derived from a human with the genital infection. The battery of type 1 and 2 herpesviruses with various degrees of resistance to ACV was kindly provided by Dr. Jack Hill (Glaxo Wellcome, Research Triangle Park, NC). These viruses are well characterized mutants with deletion or alteration of thymidine kinase (TK) or alteration in DNA polymerase and have been described in detail in the literature. DM2.1 is a TK-deficient HSV-1 mutant reported by Efstathiou et al. [10]. TK altered phenotypes of HSV-2 are variants described by Kost et al. [18]. ACV resistant polymerase deficient PAAr was reported by Jofre et al. [15]. KOS (VL#13232) and 186 (VL#14875) are ACV-sensitive laboratory controls of HSV type 1 and 2 respectively. Prior to infection experiments these viruses were expanded in bulk in WI-38 monolayers as follows. Large T-75 flasks with fibroblast cultures were inoculated with above-described HSV strains and grown further for 3 days. The culture supernatants were then recovered, centrifuged, and stored at −70 °C. Virus titers were estimated on the basis of cytopathic effect by limiting dilution assay in WI-38 host cells and were used in subsequent infection experiments at 5 000 $TCID_{50}$ per well.

Anti-HSV assay by ELISA

The assay conditions were similar to those described previously [5]. One day prior to the assay WI-38 monolayers were trypsinized with 0.25% trypsin/EDTA (Sigma) and plated in 96-well microculture plates at seeding concentration 10^5 cells per well. The next day triplicate wells of WI-38 monolayers were exposed to serial 10-fold dilutions (range 10 μg/ml-1 ng/ml) of gramicidin or ACV. Thereafter, positive control wells and drug-exposed wells containing 180 μl of medium received 20 μl aliquots of HSV-1 or HSV-2 preparation at 5 000 $TCID_{50}$ per well and were incubated further for 24 h. Mock-infected controls received either 20 μl of culture medium or 20 μl of ethanol solution at final concentration equal to 0.95%. The supernatants from infected WI-38 fibroblasts were then collected and levels of HSV antigen were measured by ELISA (Premier HSV, Meridian Diagnostics Inc., Cincinnati, OH) according to the manufacturer's instructions. The primary antibody supplied with this ELISA kit recognizes HSV antigens of both virus types. The amount of HSV antigen, corresponding to levels in the inoculum, was subtracted as a blank value from experimental values reached upon virus propagation in host WI-38 cells. IC_{50} doses were calculated from dose-response curves plotted as a function of log concentrations of drugs.

Anti-HSV assay based on cell survival

Inoculated WI-38 cells were grown in the presence of ten-fold dilutions of gramicidin or ACV for five days until all untreated control cells were dead as a result of lytic HSV infection. The culture wells were then exposed for 4 h to MTS tetrasolium salt and phenazine methosulfate preparation (CellTiter^{96}, Promega, Madison, WI) and the percent

of surviving WI-38 cells in drug-protected cultures was estimated by measuring the optical density of formazan dye produced by live cells. The obtained values were then compared to both the absorbance values of dead cells and optical density of uninfected viable cells grown in the same plate. The percent values of surviving cells were then shown in relation to the final concentration of test drugs present for 5 continuous days in wells with HSV-infected WI-38. This end-point test is similar in its principle to dye-uptake or plaque reduction counting methods commonly used in HSV assays.

Cytotoxicity assays on uninfected, proliferating fibroblasts

The effect of test compounds on cell viability was measured by two independent colorimetric assays as follows. WI-38 cells were grown in the presence or absence of ten-fold dilutions of gramicidin or ACV for two days. These cells were then exposed for 4 h MTS/phenazine methosulfate (CellTiter^{96}, Promega, Madison, WI) and the optical density of formazan dye was measured in a plate reader at 450 nm with reference filter at 620 nm. The obtained values were then compared to the optical density of non-treated viable cells grown in the same plate. This assay measures the viability and growth rate of proliferating cells incubated with test drugs. This test is similar in its principle to ^{3}H-thymidine uptake assay. Both tests were used by us in the past to test the toxicity of gramicidin in other cell types such as lymphocytes and have generated identical results.

The conditions for the second test, the lactate dehydrogenase (LDH) release assay, are similar to the MTS test except that a different cell viability parameter was measured. This assay quantitatively measures the stable cytosolic enzyme, LDH, that is released upon cell lysis in much the same way as ^{51}Cr is released in radioactive assays. The results were evaluated using an enzymatic assay which measures the conversion of INT tetrazolium salt into red formazan product (CytoTox^{96}, Promega, Madison, WI). A standard 96-well plate reader was set to record wavelength absorbance at 490 nm. The residual LDH activity present in incubation medium was subtracted as a blank value. The amount of LDH released from cells grown for 2 continuous days in the presence of gramicidin or acyclovir was compared to untreated and detergent-lysed controls according to the equation provided by the manufacturer.

Results

Effect on de novo HSV production

As early as 24 h postinfection HSV-exposed WI-38 fibroblasts displayed typical signs of cytolytic infection characterized by rounding and clumping of dying cells. The severity of infection appeared to be in reverse correlation with the concentration of antivirals present in the well. In order to quantitate this observation the supernatants from infected cultures were collected and assayed by ELISA for HSV antigen. Based on three independent experiments with triplicates for each dose of antivirals it appeared that gramicidin, previously known for anti-HIV and antimicrobial activities, was capable of inhibiting de novo HSV production. Typical results as illustrated by the effect of antivirals on VR-734 G strain of HSV-2 are shown in Fig. 1. Under described assay conditions the average IC_{50} for gramicidin was 0.3 μg/ml which was comparable to IC_{50} of ACV tested under identical conditions (Table 1). In these ELISA-based tests against four representative ACV-resistant strains the activity

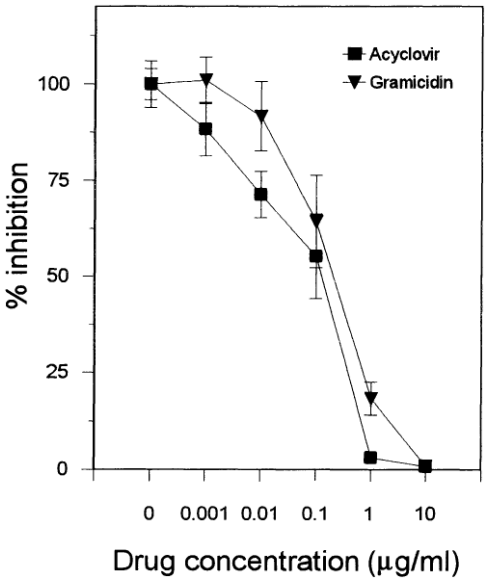

Fig. 1. The effect of ten-fold dilutions of gramicidin and acyclovir (shown on the horizontal axis as a range 0.001–10 µg/ml) on productive infection of herpes simplex virus type 2 (HSV-2) in WI-38 human fibroblasts as measured on day 1 postinfection by antigen ELISA. IC_{50} values (shown on the left Y axis) of gramicidin and acyclovir against VR-734 strain of HSV-2 (at $TCID_{50}$ 5,000 per well) were 0.3 and 0.2 µg/ml respectively. Each point represents the mean of three wells. Error bars represent standard deviations

Table 1. IC_{50} values of gramicidin and acyclovir as established by HSV antigen ELISA

No.	HSV type	Strain	Phenotype	IC_{50} (µg/ml)	
				Gramicidin	Acyclovir
1	1	KOS	ACV-sensitive	0.2 ± 0.05	0.4 ± 0.21
2	1	PAAr	Polymerase$^-$	0.4 ± 0.16	52.4 ± 17.9
3	1	DM2.1	TK$^-$	0.3 ± 0.29	77.3 ± 39.4
4	2	VR-734	ACV-sensitive	0.3 ± 0.09	0.2 ± 0.93
5	2	186	ACV-sensitive	0.1 ± 0.07	0.1 ± 0.08
6	2	Kost	TK altered	0.2 ± 0.13	15.8 ± 9.48
7	2	8708	TK altered	0.3 ± 0.24	9.9 ± 3.63

of gramicidin was not compromised and appeared to be the same as against ACV-sensitive wild type herpesviruses. ACV-resistant mutants either had deleted (DM2.1) or altered TK (Kost and 8708) or altered viral DNA polymerase (PAAr). In contrast, the activity of ACV against these mutant strains was significantly weaker (Table 1).

Protective effect against cytolytic HSV infection

In preliminary studies we observed that the viability of HSV-exposed, untreated WI-38 controls was gradually declining with time and at day 5 postinfection essentially all cells were killed due to the cytolytic nature of HSV infection [5].

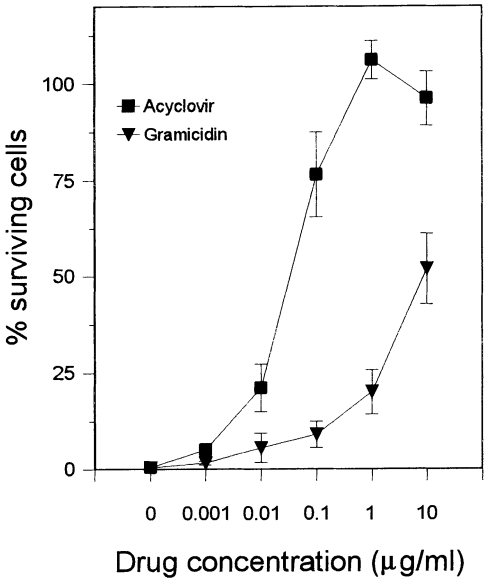

Fig. 2. The protective effect of serial dilutions of gramicidin and acyclovir against cell-killing action of HSV upon target WI-38 fibroblasts as measured on day 5 postinfection by MTS assay. The period of 5 days was selected as an end-point at which all control cells (at "0" concentration of drugs) were dead as a result of cytolytic HSV infection. IC_{50} values of gramicidin and acyclovir against VR-734 strain of HSV-2 ($TCID_{50}$ 5,000 per well) were 10 and 0.2 µg/ml respectively. Each point on the curve represents the mean percentage value SD of triplicate wells

This time period was thus selected as an end-point. It appeared that a single dose of gramicidin present for 5 continuous days was capable of protecting target cells from cytolytic HSV infection. However, in this assay, aimed at measuring the survival of drug-treated, HSV-exposed fibroblasts, gramicidin was less potent than ACV. The IC_{50} of gramicidin was equivalent to 10 µg/ml which is significantly lower than IC_{50} of ACV (Fig. 2). Despite inferior activity in this assay, gramicidin-treated cells appeared to be morphologically intact without showing any signs of decay. This indicates that under assay conditions, the effect exercised by gramicidin was not transient and does not merely delay viral replication. Both gramicidin and acyclovir were of little effect when they were added to already infected dying cells, suggesting that these drugs are more efficient at early stages of HSV replication. However, gramicidin was capable of significantly reducing HTLV-I and HIV production when it was incubated with chronically infected T cell lines (data not shown). Unfortunately, this type of experiment cannot be carried out with fast-replicating, cytolytic HSV, thus preventing verification of the possibility that gramicidin may be effective at later stages as well.

Lack of cytotoxicity toward proliferating, uninfected cells

Both drugs, gramicidin and ACV, were not toxic to proliferating WI-38 cells at concentrations that exceeded IC_{50} by at least two orders of magnitude. The non-isotopic MTS/phenazine assay, used routinely as a substitute for ^{3}H-thymidine proliferation assay, has not revealed any negative effect on the rate of cell growth (Fig. 3a).

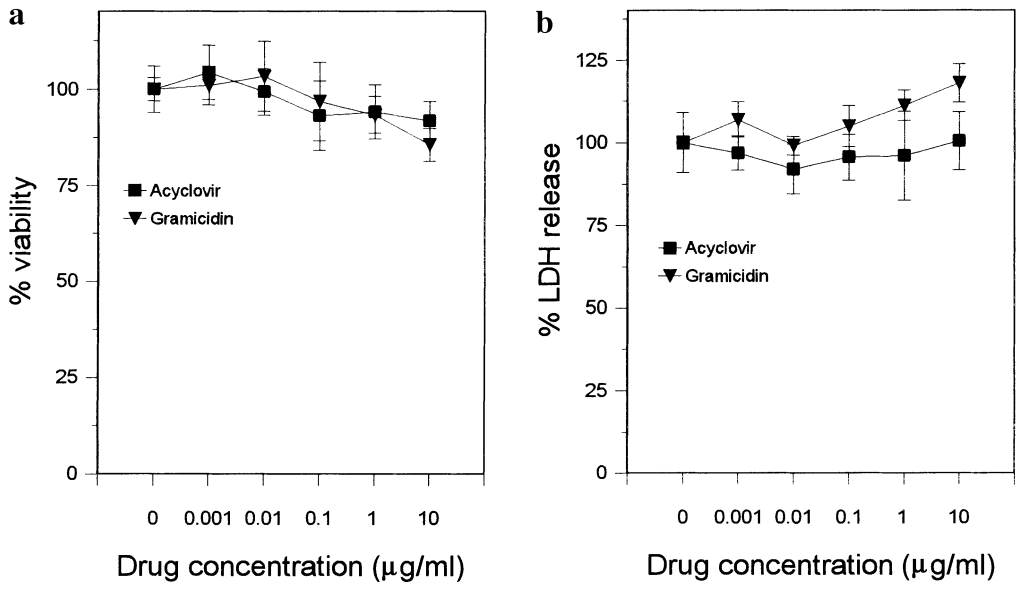

Fig. 3. a The effect of gramicidin and acyclovir on proliferating capacity and viability of WI-38 cells as determined on day 2 by MTS/phenazine assay which measures the activity of mitochondrial hydrogenases converting MTS into formazan dye. The highest tested 10 µg/ml dose of each compound had no significant effect in suppressing the metabolic activity of replicating host cells. **b** The effect of gramicidin and acyclovir on the release of intracellular lactate dehydrogenase (LDH) from drug-treated WI-38 as measured after 2 days of continuous exposure to test drugs. The highest levels of excess LDH were within 15% of baseline for untreated controls ($P < 0.05$ by Student's *t* test). The results are based on three independent determinations and mean values are shown as the percentage of control. Standard deviations are shown as error bars

The excess of LDH released from uninfected, replicating WI-38 cells, exposed for 2 days even to the highest 10 µg dose of gramicidin, was not above 15% baseline of untreated controls (Fig. 3b). This observation supports the prevailing opinion that gramicidin has a negligible effect on the integrity of the cell membrane and does not cause excessive leakage of intracellular constituents other than potassium.

Discussion

The results demonstrate that gramicidin is capable of inhibiting HSV replication at concentrations that are comparable to acyclovir. Although in cell protection experiments ACV was more effective, gramicidin has shown better efficacy against ACV-resistant variants with TK or DNA polymerase mutations. LDH and MTS assays revealed that gramicidin was not disrupting the cell membrane and had no adverse effect on cell proliferation and viability. The lack of toxicity seems to be in agreement with clinical evidence based on use of topical preparations containing 25 µg/ml of gramicidin.

The progress in understanding the replication of viruses has yielded a great number of mechanism-based antiviral agents. ACV, vidarabine, ganciclovir, and foscarnet are now routinely used for systemic and topical treatment of HSV infections [8]. However, clinical trials revealed an important problem which was particularly alarming among HIV-infected individuals. It became apparent that mutants resistant to current drugs are expanding and replacing sensitive strains in the host population [7, 23]. The new drugs interfering with cellular counterparts of viral replication, rather than with the virus itself, would offer better prospects of overcoming this hurdle. In the course of the past few years we have undertaken the task of investigating antiviral drugs acting upon host cells rather than on viruses directly. As a result, gramicidin has been identified as a promising, new anti-HIV compound with activity at nanogram doses [2, 3]. As gramicidin is effective against ACV-resistant HSV it is unlikely that it targets TK or DNA polymerase. Although the function of gramicidin remains to be defined, the dissimilarity in HSV and HIV replication mechanisms indicates that this drug affects the host cell rather than a specific viral enzyme.

Due to the lack of experimental evidence the mechanism of gramicidin action in the context of viral replication can only be inferred. The infectious capacity of many enveloped viruses, including HIV and HSV, depends on the specific alteration in intracellular content of monovalent cations [1, 25]. The entry of the virus into the target cell is accompanied by a concomitant rise in the cytosolic content of K^+, indicating that cellular cooperation is required for productive infection. Diuretic drugs, which affect $Na^+/K^+/2Cl^-$ cotransporter activity, were shown to block HIV infection [26]. Drugs inhibiting Na^+/K^+ ATPase pump, such as bee venom melittin or ouabain, have been reported to inhibit HSV-1-induced cell fusion and viral spread [1]. Based on this evidence, it is likely that the potassium ionophore, gramicidin, exerts its antiviral activity by reversing K^+ flux and causing rapid depolarization of the cell membrane [2, 3]. Anti-HSV doses of gramicidin were about 1 log higher than those required to block HIV infection. This discrepancy in activity possibly reflects the difference in antiviral assays involving unrelated viruses and host cell type. However, it is also possible that gramicidin-caused perturbation of the ionic gradient across the cell membrane may affect retroviruses more specifically than herpesviruses. Nevertheless, in anti-HSV assays the effective dose of gramicidin did not coincide with cytotoxicity, suggesting the selective activity of this compound.

Although numerous anti-HSV compounds have been demonstrated to be effective in vitro, very few have reached the clinical level [8]. The major reason for this is the lack of activity at pharmaceutically acceptable doses. The antibiotic neomycin is effective against HSV at 1 mM dose, which is four orders of magnitude higher than the effective concentration of gramicidin [13]. Another reason is the concern for the safety of an experimental drug. Gramicidin has been used as an antibiotic for more than fifty years and has an established record of safety. Gramicidin along with neomycin and polymyxin B (Neosporin), is used in the USA for the treatment of eye infections. Gramicidin

alone is employed in the FSU as a topical contraceptive. Many microbial STDs such as gonorrhea, syphilis, chlamydia, candidiasis, bacterial vaginosis, and trichomoniasis can cause genital ulcers and inflammations which may facilitate the spread of HIV [14, 20]. Contraceptive paste with gramicidin is also indicated in the FSU for healing cervicovaginal inflammations, meaning that it is not irritating even to damaged vaginal mucosa [21]. This advantage may greatly enhance the potential of gramicidin for clinical implementation.

Gramicidin is a remarkably stable peptide and can be stored at less-than-favorable temperature conditions for years. The 2% solution of gramicidin S in alcohol has a recommended shelf-life of 10 years and can retain its biological activity even after 30 min in the autoclave [21]. Moreover, the activity of gramicidin is not affected by pH nor by the nature of solvents [16]. Genital and body fluids such as semen, vaginal excretions, blood, saliva, tears, or pus do not decrease the potency of gramicidin [17]. Gramicidin does not smell or stain and hence suits ideally the requirements for a host-controlled barrier device. Although gramicidin can act as a spermicide at high doses, our observations indicate that doses as low as 1 ng/ml can suppress completely the motility of sperm [4]. Thus, antivirally active nanogram quantities of gramicidin are not by definition spermicidal, but can, nevertheless, exhibit contraceptive activity as a spermostatic agent. Due to emerging evidence that the spermicide nonoxynol-9 is less effective and more toxic than believed originally [6, 19], gramicidin has a fairly good chance to replace it as a better compound.

Clinical studies of gramicidin would help to identify its usefulness as a topical contraceptive agent with prophylactic potential against microbial and viral STDs.

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