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Preclinical evaluation of docusate as protective agent from herpes simplex viruses

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Abstract

Prevention of sexually transmitted infections (STIs) is key to public health efforts to control these diseases. An effective vaginal microbicide could provide topical, broad-spectrum prevention against the transmission of several STI pathogens. Docusate is a sulfated surfactant and, as such, may inactivate viral pathogens by disrupting viral envelopes and/or denaturing/disassociating proteins. Accordingly, the in vitro efficacy and toxicity of docusate (dioctyl sodium sulfosuccinate; Zorex™; Meditech Pharmaceuticals, Inc., Scottsdale, Arizona) against herpes simplex viruses (HSV) were evaluated. Docusate was effective in vitro against wild type and drug-resistant strains of HSV type 1 and 2 with EC_{90-100} (effective concentration giving 90–100% virus yield reduction) of approximately 0.005% (w/v). Sodium dodecyl sulfate (SDS) was equipotent, however, docusate was somewhat less toxic to uninfected Vero cells compared with SDS after 2 days incubation (docusate CC_{50} $\sim$0.01% vs. SDS $\sim$0.005%). The cytotoxicity profiles of docusate were time- and dose-dependent and thus associated with the frequency of use. Kinetics of inactivation examined by pre-mixing virus and drug in a time-course experiment demonstrated that docusate could reach its EC_{90-100} within 30 min. Docusate pretreatment of cells was associated with a 45% reduction in infectivity of those cells, despite a triple washing procedure. Once infected, an approximate 30% plaque reduction was observed with treatment.

Keywords: Docusate; Herpes simplex virus; Preclinical evaluation

1. Introduction

Sexually transmitted infections (STIs), such as genital herpes, are a major public health problem worldwide. It is estimated that several hundred million individuals are infected with one or more STI (for reviews see Gerbase et al., 1998; Des-

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ormeaux et al., 1999). One desired approach for control of transmission has been the development of nontoxic, topical, female-controlled, broad-spectrum microbicides effective against the transmission of these pathogens. Several categories of vaginal microbicides have been developed or are currently in clinical trials (Harrison, 1999). These include detergent-type spermicides/microbicides, microbial adhesion inhibitors, antimicrobial drugs, and buffering agents and products that maintain normal vaginal environment (Desormeaux et al., 1999). One class of detergent-type spermicides/microbicides currently in use is surface-active agents, such as nonoxynol-9 (N-9), SDS, benzalkonium, or docusate. Such agents are known to inactivate pathogens by disrupting an organism's membrane or viral envelope, although this may not be the complete story, since SDS has also been shown to inactivate the nonenveloped virus HPV (human papillomavirus) (Howett et al., 1999). However, the major problem associated with the use of such spermicides as N-9 has been local inflammation and/or ulceration of vaginal and cervical mucosae (Desormeaux et al., 1999). One class of natural detergents, bile salts, was reported to be effective against HSV, HIV and *Neisseria gonorrhoeae* and less toxic than N-9 and benzalkonium (Herold et al., 1999). SDS and C31G have been studied precisely because of their broad-spectrum virucidal activities (Howett et al., 1999; Krebs et al., 1999, 2000; Piret et al., 2000) and because both of these agents are less toxic than N-9.

Docusate is a sulfated surfactant and, as such, it dissociates lipid membranes. It is also a chaotropic agent, causing denaturation of proteins. Spermicidal and microbicidal activities of docusate were also documented (Chantler et al., 1992; Jones and Willcox, 1991). Docusate sodium and calcium are also Over-The-Counter products for occasional constipation. To further examine the potential role of docusate as a topical microbicide, we evaluated the virucidal activities and cytotoxicity of docusate against HSV strains in vitro.

2. Materials and methods

2.1. Compounds

Docusate and SDS were purchased from Sigma. Stock solutions (1%, w/v) of docusate and SDS were prepared by dissolving them in sterile deionized distilled (Milli-Q) water and stored at room temperature.

2.2. Virus and cells

HSV strains G (HSV-2) and F (HSV-1) were used in the experiments. Delta 333 is a laboratory HSV-2 TK^- (thymidine kinase deleted) mutant strain with genotypic and phenotypic resistance to both penciclovir and acyclovir. HSV-1 drug-resistant strain 615.8 is a DNA polymerase mutant with phenotypic resistant to forscarnet (Sacks et al., 1989). A HSV susceptible cell line, Vero cells (African green monkey kidney cell), was used in the virus yield reduction assays. The culture medium for Vero cells was 5% minimum essential medium (MEM; GIBCO/BRL) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin).

2.3. Virus plaque reduction assay

Antiviral effects of docusate were determined by modified plaque reduction assays. Confluent cells were washed with PBS and subsequently infected with HSV for 1 h at 37 °C. After viral inoculum was removed, the infected cells were washed with PBS and overlaid with 0.5% methylcellulose in culture medium. Cells were incubated at 37 °C for 2 days for HSV-2 infection and 3 days for HSV-1. When plaque size was adequate, cells were fixed with 10% formalin and subsequently stained with 0.5% crystal violet.

All data were generated from duplicate or triplicate wells in two or three independent experiments. Mean plaque counts are shown in the tables and figures. Effects of compounds at varying concentrations were expressed as percentage of control (the mean plaque counts in drug treated wells/the mean plaque counts in control wells).

2.4. Inactivation of HSV by docusate

HSV G and F strains were diluted to 200 pfu/ml with 5% MEM, respectively. Docusate and SDS were diluted to 2× final concentrations (final concentrations: 0, 0.0005, 0.001, 0.0025, 0.005 and 0.01%, respectively) with 5% MEM. Equal volumes of diluted virus and drugs were mixed and incubated at 37 °C water-bath for 1 h. One milliliter of the mixture was then used to infect confluent Vero cells in 6-well plates at 37 °C for 1 h. After infection, viral inoculum was removed and the cells washed with PBS. The cells were subsequently overlaid with 1.5 ml of 0.5% methylcellulose in culture medium for plaque assay.

To examine the kinetics of inactivation of HSV by docusate, 200 pfu/ml of HSV-2 was pre-mixed with 2× final concentrations of docusate (final concentrations: 0, 0.001, 0.0025, 0.005 and 0.01%, respectively) in 5% MEM and incubated at 37 °C for 0, 15, 30, 60, 120, and 240 min. At each time point, the treated mixture was used to infect confluent Vero cells. After viral inoculum was removed, the cells were covered with methylcellulose for plaque assay.

2.5. Cytotoxicity of docusate

The cytotoxicity of docusate was examined using Vero cells with the neutral red uptake assay described by Schmidt and Korba (2000). Culture medium was removed from confluent Vero cells in 24-well plates. The cells were then washed once with PBS. One milliliter of culture medium containing docusate or SDS at concentrations of 0, 0.001, 0.0025, 0.005, and 0.01% were added to each well. Cells were incubated at 37 °C for 1 h, 6 h, 2 and 3 days. At each time point, the medium was removed and cells washed with PBS. Five hundred microlitres of 0.01% neutral red (in PBS) was added to each well, and the samples were incubated at 37 °C for 30 min. The dye was then removed and the cells washed twice with 1 ml PBS per well. The dye was extracted by addition of 500 μl of 50% ethanol/1% glacial acetic acid in PBS to each well and incubated at room temperature for 15 min with gentle shaking at 120–150 rpm. Then 200 μl of extracted dye from each well was put into 96-well plate and the absorbance at 550 nm was read on an ELISA reader.

Long-term cytotoxicity of docusate was also examined using trypan blue exclusion assay. Confluent Vero cells were washed with PBS and incubated with culture medium containing various concentrations of docusate or SDS at 37 °C for 3 days. After culture medium was removed, the cells were washed once with PBS, trypsinized with Versene/1× trypsin and resuspended in 1 ml of 5% MEM culture medium. One hundred microlitres of cell suspension was mixed with 500 μl trypan blue solution. The viable cells were counted using a hemacytometer.

2.6. Pre-treatment of cells

Confluent Vero cells in 6-well plates were washed with PBS. One milliliter culture medium containing different concentrations of docusate or SDS was added to each well and the cells were incubated at 37 °C for 1 h. Following pre-incubation of the cells with docusate, two treatments were performed, one set of plates was directly infected with 100 pfu per well of HSV-2; another set of plates was washed three times with PBS, and then infected with 100 pfu per well of HSV-2. Both infections were incubated at 37 °C for 1 h with tilting every 10 min. After viral inoculum was removed, the infected cells were covered with methylcellullose for plaque assay.

2.7. Effect of docusate on HSV-infected cells

Confluent Vero cells were washed with PBS and then infected with 100 pfu per well of HSV-2 at 37 °C for 1 h. Following removal of viral inoculum, infected cells were washed once with PBS and covered with 0.5% methylcellulose containing either docusate or SDS at various concentrations for plaque assay.

2.8. Effect of docusate on HSV drug-resistant strains

The antiviral effect of docusate on HSV-2 thymidine kinase mutant Delta 333 and HSV-1

DNA polymerase mutant 615.8 was performed in a similar way as above (see Section 2.4)

3. Results

3.1. Inactivation of HSV

The in vitro inactivations of HSV by docusate were compared with another surfactant, SDS. Various concentrations of testing drug were pre-mixed with HSV-1 or HSV-2 and incubated at 37 °C for 1 h. Antiviral effects were then determined by plaque reduction assays (Fig. 1). Inactivation of HSV-1 by docusate along with SDS is shown in Fig. 1A and that of HSV-2 in Fig. 1B. Results showed that at a concentration of 0.005%, docusate completely inactivated both HSV-1 and HSV-2 after pre-mixing for 1 h ($EC_{90-100} = 0.005\%$). In contrast, although this concentration was also effective for SDS, it caused 50% cell death (toxic to cells). At concentration of 0.01%, both docusate and SDS were shown to be toxic to the cells causing 50–100% cell death (data not shown). In these experiments, docusate was slightly more effective against HSV-1 than HSV-2, and slightly more potent and less toxic than SDS at other concentrations (Fig. 1).

3.2. The kinetics of inactivation of HSV by docusate

To examine the kinetics of inactivation of HSV, docusate at various concentrations was mixed with HSV-2 and incubated at 37 °C in a time-course, with endpoints determined by plaque reduction assay. Results (Fig. 2) showed that docusate had a slight inactivation effect upon HSV-2 at concentrations of 0.001 and 0.0025% after 4 h incubation. However, inactivation reached 50% at time 0, almost 90% (EC_{90}) after 30 min, and 100% after 1 h incubation at a concentration of 0.005%. A concentration of 0.01% of docusate was found to be toxic to cells.

3.3. Short and long incubation cytotoxicities

Microbicidal effectiveness of surface active agents such as docusate against enveloped viruses suggests a potentially disruptive effect on

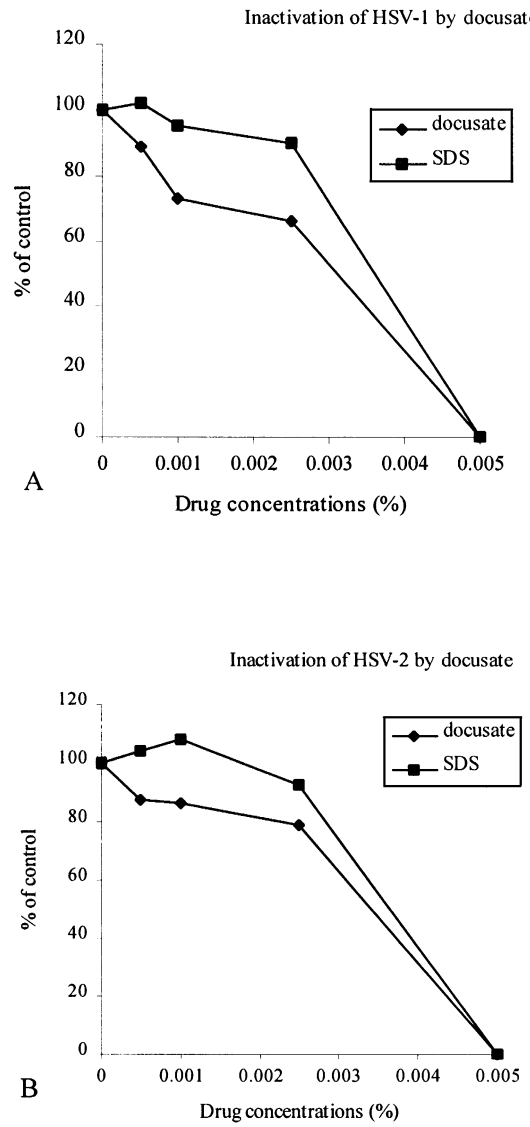


Fig. 1. Inactivation of HSV-1 (A) and HSV-2 (B) by docusate and comparison with SDS. Different concentrations of docusate or SDS were pre-mixed with HSV-1 or HSV-2 and incubated at 37 °C for 1 h. The mixture was then used to infect Vero cells. The antiviral effect was determined by plaque reduction assay and expressed as % of the control.

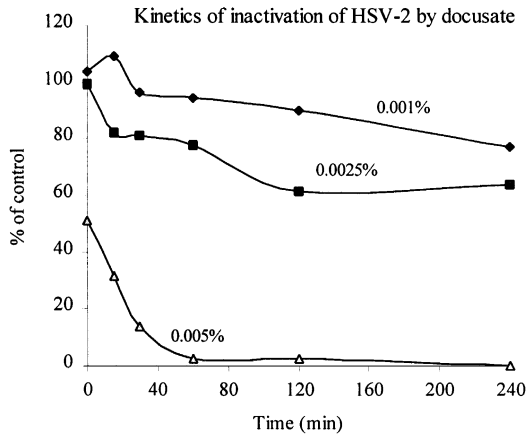
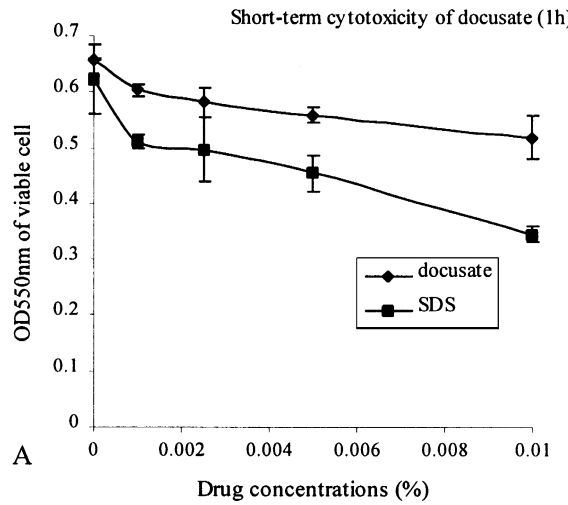


Fig. 2. Kinetics of inactivation of HSV-2 by docusate. Different concentrations of docusate were pre-mixed with HSV-2 and incubated at 37 °C in a time-course. At each time point, the treated mixture was used to infect confluent Vero cells for plaque reduction assay and the antiviral effect was expressed as % of the control.

cellular membranes. Accordingly, experiments were carried out to evaluate relative cytotoxicities of docusate over time. Cytotoxicities of docusate were compared with SDS and measured using Vero cells. Trypan blue exclusion (data not shown) and uptake of neutral red dye were then used to determine the viabilities of cells after incubations with different concentrations of docusate, and SDS. Results were consistent between assays (data not shown). For short term incubation, cells were exposed to docusate for 1 (Fig. 3A) and 6 h (data not shown), whereas for long term incubation, cells were exposed to docusate for 2 (data not shown) and 3 days (Fig. 3B). As can be seen from these figures, after 1 h exposure to docusate and SDS, minimal cytotoxicity of docusate to Vero cells was observed even at concentration of 0.01%. Cytotoxicity was increased after 6 h exposure (data not shown). Concentration at 0.01% was again found to be toxic to Vero cells after 3 days incubation. Thus, the cytotoxicity of docusate is time- and dose-dependent. Furthermore, docusate was slightly less cytotoxic than SDS. CC_{50} (cytotoxic concentration giving 50% of cell death) of docusate after 2 days incubation was approximately 0.01% and that of SDS was approximately 0.005%.

3.4. Antiviral activity of docusate determined by pre-treated cells

Some compounds can be internalized into cells or bound to the cellular membranes to exert antiviral effects. This experiment was designed to



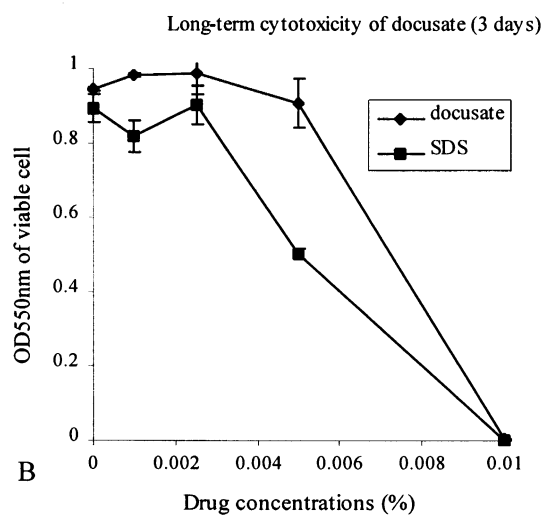


Fig. 3. Short term (1 h) (A) and long term cytotoxicity (3 days) (B) of docusate. Confluent Vero cells were incubated with culture medium containing different concentrations of docusate or SDS. The cytotoxicity was determined by neutral red uptake assay (Schmidt and Korba, 2000).

Table 1
Antiviral effect of docusate on pre-treated cells (% of control)

Concentrations (%)		0	0.0005	0.001	0.0025	0.005
Docusate	No wash	100	73.3	75.2	71.3	54.5
	Wash (three times)	100	89	85	69	64.5
SDS	No wash	100	110	91.8	112	89.4
	Wash (three times)	100	95.6	86.7	82.7	69.4

examine the effect of docusate on pre-treated cells. Two approaches were employed. First, we pre-incubated Vero cells with docusate and then infected these cells with HSV. In the second, we pre-incubated cells with docusate and then washed it off three times with PBS. The results, together with comparison of SDS, were summarized in Table 1. Cells pre-treated with docusate at a concentration of 0.005%, showed virus infection to be reduced by 45%, and 35% after docusate was removed by three washes. However, this concentration was not able to completely stop virus infection.

3.5. Effect of docusate on HSV-infected cells

To examine the effect of docusate on HSV-infected cells, cells were first infected with HSV-2 and then treated with different concentrations of docusate following adsorption by inclusion in the methylcellulose overlay. The results (Table 2) indicated that docusate only had slight inhibitory effect on HSV-infected cells, by approximately 30% plaque reduction at 0.005% of concentration.

3.6. Effect of docusate on HSV-drug-resistant mutants

To test the antiviral effect of docusate on the drug-resistant HSV strain, delta 333 virus was pre-mixed with different doses of docusate at 37 °C for 1 h. The antiviral effect was subsequently determined by plaque reduction assay. The results showed that docusate at concentration of 0.005% could completely inactivate delta 333 after incubation at 37 °C for 1 h (Table 3). Although SDS was slightly less efficacious in this assay, it was also capable of inactivating the resistant strain, delta 333.

The effect of docusate on the polymerase mutant virus was examined by pre-mixing docusate at various doses with 615.8 virus for 1 h at 37 °C. The antiviral activity against polymerase mutant virus was then determined by plaque reduction assay. The results showed that docusate at concentration of 0.005% could inactivate approximately 98% of polymerase mutant 615.8 after incubation at 37 °C for 1 h (Table 4), whereas SDS was less efficacious at this concentration.

4. Discussion

Surfactants/detergents are an important class of vaginal microbicides for the control of STIs. These products destroy microorganisms by dissolving their outer membrane and limiting infectivities. The studies described here were designed to examine the virucidal activity and potential role of docusate as a topical microbicide for the prevention of STIs or for the topical treatment herpes simplex virus-induced lesions. A recent report (van Damme L., Advances in topical microbicides, Presented at the XIII International AIDS Conference, 9–14 July, 2000, Durban, South Africa) stemming from a study of nonoxynol-9 as a potential vaginal microbicide has raised concerns about the clinical utility of surfactants because of an apparent increase in the rate of transmission of 50% in nonoxynol-9 users, with a direct correlation between frequency of use and risk of HIV transmission. Earlier analyses of this study did not suggest a significant clinical toxicity problem from this agent. However, the possibility that cytotoxicity potentiated HIV transmission has to be considered. SDS inactivates a broad-spectrum of viruses and STIs (Howett et al., 1999; Krebs et al., 1999, 2000), and

Table 2
Antiviral effect of docusate on HSV-2 infected cells (% of control)

Concentrations (%)	0	0.0005	0.001	0.0025	0.005
Docusate	100	102.8	86.5	98	70.3
SDS	100	106	98.1	102	78[a]

[a] Approximately 50% cell death observed under microscope.

has been proposed as a possible topical microbicide. Accordingly, the efficacy of SDS was studied in parallel.

We found that docusate was highly effective against both HSV-1 and HSV-2 infection at a dose of 0.005% (w/v), including acyclovir and forscarnet resistant mutants with EC_{90-100} approximately 0.005%. Although this concentration was also effective for SDS, SDS was toxic to cells, causing 50% cell death. This result indicates that docusate is less toxic than SDS. As shown in the kinetics of inactivation for HSV-2 (Fig. 2), this concentration achieves EC_{90-100} between 30 min and 1 h. Docusate was earlier observed to be effective against a number of other microbes, such as respiratory syncytial virus, influenza virus, bacteria, and fungi (unpublished data from Meditech Pharmaceuticals, Inc.). We speculate that it should be active against all enveloped viruses, and possibly nonenveloped viruses as well because surfactants can denature/disassociate proteins, as has been demonstrated for SDS, which can inactivate nonenveloped human papillomavirus (Howett et al., 1999).

We demonstrated that docusate was effective inactivating HSV-2 and blocking virus adsorption/or replication in pre-treated cells even after docusate was removed by three washes. Although the nature of the interaction between cells and drug is not known, we speculate that a portion of docusate was bound/adsorbed to the cell surface or taken up by the cells. Slight antiviral effect of docusate was observed on cells that were already infected with HSV, suggesting the possibility of limited cell uptake of docusate.

Cytotoxicity is a common problem for surfactants used as topical microbicides owing to indiscrimination of cellular membranes and viral envelopes. This is associated with frequency of use. We have shown with both docusate and SDS that this phenomenon is time- and dose-dependent. We demonstrated that the viability of cells cultured in vitro was dependent on microbicide concentrations and exposure duration. During short-term exposure (1 h) both docusate and SDS were minimally toxic at concentrations tested. During long-term exposure, docusate caused decreased cell viability with increased concentrations. A concentration of 0.01% was found to be 50–100% toxic to Vero cells after prolonged incubation for 3 days. The cytotoxicity of docusate was less toxic than SDS. CC_{50} of docusate for 2 days incubation was approximately 0.01% and that of SDS was $\sim$0.005%. Krebs et al. (1999) showed that SDS was the least toxic among N-9, C31G, and SDS. This may suggest that docusate is less toxic than N-9 and C31G.

Our studies demonstrate that docusate has favorable in vitro characteristics warranting consideration for use as a potent topical microbicide in the prevention of STIs. The clinical impact of toxicity potential of surfactants in this setting remains to be determined.

Table 3
Effect of docusate on HSV drug-resistant strain, Delta 333 (TK mutant) (% of control)

Concentrations (%)	0	0.001	0.0025	0.005
Docusate	100	84.3	72.5	0
SDS	100	114	102	33

Table 4
Effect of docusate on HSV polymerase mutant 615.8 (% of control)

Concentrations (%)	0	0.001	0.0025	0.005
Docusate	100	90.4	82.5	1.7
SDS	100	102.8	99.4	44

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