Punica granatum (Pomegranate) Juice Provides an HIV-1 Entry Inhibitor and Candidate Topical Microbicide

A. ROBERT NEURATH, NATHAN STRICK, YUN-YAO LI, AND ASIM K. DEBNATH

New York Blood Center, New York, New York 10021, USA

ABSTRACT: For ∼24 years the AIDS pandemic has claimed ∼30 million lives, causing ∼14,000 new HIV-1 infections daily worldwide in 2003. About 80% of infections occur by heterosexual transmission. In the absence of vaccines, topical microbicides, expected to block virus transmission, offer hope for controlling the pandemic. Antiretroviral chemotherapeutics have decreased AIDS mortality in industrialized countries, but only minimally in developing countries. To prevent an analogous dichotomy, microbicides should be acceptable, accessible, affordable, and accelerative in transition from development to marketing. Already marketed pharmaceutical excipients (inactive materials of drug dosage forms) or foods, with established safety records and adequate anti-HIV-1 activity, may provide this option. Therefore, fruit juices were screened for inhibitory activity against HIV-1 IIIB using CD4 and CXCR4 as cell receptors. The best juice was tested for inhibition of: (1) infection by HIV-1 BaL, utilizing CCR5 as the cellular coreceptor, and (2) binding of gp120 IIIB and gp120 BaL, respectively, to CXCR4 and CCR5. To remove most colored juice components, the adsorption of the effective ingredient(s) to dispersible excipients and other foods was investigated. A selected complex was assayed for inhibition of infection by primary HIV-1 isolates. The results indicate that HIV-1 entry inhibitors from pomegranate juice adsorb onto corn starch. The resulting complex blocks virus binding to CD4 and CXCR4/CCR5 and inhibits infection by primary virus clades A to G and group O. Therefore, these results suggest the possibility of producing an anti-HIV-1 microbicide from inexpensive, widely available sources, whose safety has been established throughout centuries, provided that its quality is adequately standardized and monitored.

KEYWORDS: pomegranate juice; Punica granatum; human immunodeficiency virus (HIV-1); virus entry inhibitors; CD4; CXCR4 CCR5; receptors for HIV-1; coreceptors for HIV-1; microbicides

BACKGROUND

The global acquired immunodeficiency syndrome (AIDS) epidemic has proceeded relentlessly for ∼24 years with no promising prophylactic intervention in sight. In

Address for correspondence: A. Robert Neurath, Biochemical Virology Laboratory, Lindsley F. Kimball Research Institute, 310 East 67th Street, New York, NY 10021, USA; Voice: 212-570-2275; fax: 212-570-3299.
arneurath@att.net

Ann. N.Y. Acad. Sci. 1056: 311–327 (2005). © 2005 New York Academy of Sciences.
doi: 10.1196/annals.1352.015
2004, there were 4.9 million new HIV infections and 3.1 million AIDS deaths.¹ To
date, the number of individuals living with human immunodeficiency virus type 1
(HIV-1) infection/AIDS has reached 39.4 million,¹ and ~28 million people have
already died from AIDS since the beginning of the pandemic.¹² Most new infections
have been acquired by the mucosal route, heterosexual transmission playing the
major (~80%) role. Although the incidence of transmission per unprotected coital
act is estimated to be low (0.0001–0.004), but strikingly increased when acutely
infected individuals are involved,³,⁴ the cumulative effect is overwhelming.

Anti-HIV-1 vaccines applicable to global immunization programs are not
expected to become available for many years. Therefore, other prevention strategies
are urgently needed. This includes educational efforts and the application of
mechanical and/or chemical barrier methods. The latter correspond to microbicides,
that is, topical formulations designed to block HIV-1 infection (and possibly trans-
mition of other sexually transmitted diseases) when applied vaginally (and possibly
rectally) before intercourse.³,⁵–⁷ Conceptually, it is preferred that the active ingredi-
ett(s) of microbicide formulations (1) block virus entry into susceptible cells by pre-
venting HIV-1 binding to the cellular receptor CD4, the coreceptors CXCR4/CCR5,
and to receptors on dendritic/migratory cells (capturing and transmitting virus to
cells that are directly involved in virus replication), respectively,³,⁸–¹¹ and/or (2) are
virucidal. The formulations must not adversely affect the target tissues and should
not cause them to become more susceptible to infection after microbicide
removal.¹²,¹³

Treatment with antiretroviral drugs has decreased mortality from AIDS in indus-
trialized countries but so far has had a minimal effect in developing countries.¹⁴ To
avoid a similar dichotomy with respect to microbicides, they should be designed and
selected to become affordable and widely accessible, while shortening the time
between research and development and their marketing and distribution as much as
possible. This would be facilitated if mass manufactured products with established
safety records were found to have anti-HIV-1 activity. Qualifying candidates to be
considered for microbicide development may possibly be discovered by screening
pharmaceutical excipients (“inactive” ingredients of pharmaceutical dosage forms)
and foods, respectively, for antiviral properties.

While exploring the possibility that chemical modification of food proteins may
lead to the generation of compounds with anti-HIV-1 activity, we discovered in 1994
that bovine β-lactoglobulin (the major protein of whey) modified by 3-hydroxy-
phthalic anhydride (3HP-β-LG) blocked infection by HIV-1 and herpesviruses, both
in vitro and in animal model systems.¹⁵–²⁴ Considering its antiviral potency, ease of
preparation, and practically unlimited and inexpensive source (the worldwide pro-
duction of whey is approximately 86 billion kg annually), 3HP-β-LG appeared to
represent an excellent candidate microbicide for prevention of the sexual transmis-
sion of HIV-1. By coincidence, an epidemic of bovine spongiform encephalopathy
(BSE) was ongoing at the same time in the United Kingdom and considered to cause
a new variant of Creutzfeldt-Jakob disease (vCJD) in humans. This raised questions
related to the safety of bovine milk. However, scientific research results indicate that
BSE cannot be transmitted by cow’s milk even if the milk comes from a cow with
BSE, because no detectable infectivity in milk from BSE-infected animals could be
demonstrated. Evidence from other animal and human transmissible spongiform
encephalopathy (TSE) studies suggests that milk does not transmit these diseases.
Milk and milk products, even from countries with a high incidence of BSE, are therefore considered safe. Consequently, the United States Food and Drug Administration has exempted milk-derived products from restrictions applied to their use as pharmaceutical ingredients. In accordance with this, some bovine milk-derived products are being generally recognized as safe (GRAS). In addition, the current risk of acquiring vCJD from eating beef and beef products is approximately 1 case per 10 billion servings in the United Kingdom and likely to be smaller in other countries. Notwithstanding these unequivocal conclusions, the World Health Organization recommends that the pharmaceutical industry should avoid the use of materials from animal species in which TSEs naturally occur. Based on these negative recommendations from the WHO, further development of 3HP-β-LG as a topical microbicide had to be abandoned.

We initiated the screening of pharmaceutical excipients for compounds with anti-HIV activity. This led to the discovery that cellulose acetate 1,2-benzenedicarboxylate used for coating enteric tablets and capsules has anti-HIV activity and represents a promising candidate microbicide. Here we report the outcome of screening fruit juices, many of which have been reported to provide health benefits. All juices were neutralized to pH ≈ 7 to discount nonspecific effects caused by acidity.

The results presented here and the corresponding methods are freely available online at http://www.biomedcentral.com/content/pdf/1471-2334-4-41.pdf.

RESULTS

Anti-HIV-1 Activity of Pomegranate Juice

Serial twofold dilutions of juices (apple, black cherry, blueberry, coconut milk, cranberry, elderberry, grape [red], grapefruit, honey, lemon, lime, pineapple, pomegranate, and red beet [10% reconstituted dry powder]) were assayed for inhibition of infection by HIV-1 IIIB of cells expressing the CD4 and CXCR4 receptors and coreceptors. Most juices (diluted fourfold) had no inhibitory activity, except blueberry, cranberry, grape, and lime juice, respectively (endpoints for 50% inhibition of infection [ED50] between 1/16 and 1/64). Consistently, pomegranate juice (PJ) from distinct geographic areas had the highest inhibitory activity (FIG. 1; vertically shaded area). Since HIV-1 viruses utilizing CCR5 as coreceptor (= R5 viruses) are predominantly transmitted sexually, it was important to test whether PJ can inhibit not only infection by HIV-1 IIIB, a virus utilizing CXCR4 as coreceptor (= X4 virus), but also infection by an R5 virus, HIV-1 BaL. Results in FIGURE 1 (horizontally shaded area) show that infection by the latter virus is also inhibited, albeit less effectively, than that by HIV-1 IIIB.

Blocking virus entry is a primary target for microbicide development. Therefore, it was of interest to determine whether PJ inhibited the binding of the HIV-1 envelope glycoprotein gp120 to CD4, the common receptor for both X4 and R5 viruses. Pretreatment of both gp120 IIIB and BaL by PJ inhibited subsequent binding of soluble labeled CD4 (FIG. 2). This suggested that one or more PJ ingredients bound strongly or irreversibly to the CD4 binding site on gp120. These results, obtained in an enzyme-linked immunosorbent assay (ELISA) using gp120 immobilized on polystyrene plates, were confirmed in another assay in which both
FIGURE 1. Inhibition of HIV-1 infection of HeLa-CD4-LTR-β-gal and U373-MAGI-CCR5E cells, respectively, by pomegranate juice (PJ). LTR = long terminal repeat; vertically shaded area = HIV-1 IIIB; horizontally shaded area = HIV-1 BaL. Four distinct PJs (PJ1 to PJ4) were tested. Infection was monitored by measuring β-galactosidase.

FIGURE 2. Inhibition of CD4 binding to recombinant gp120 IIIB and BaL, respectively, by pomegranate juice (PJ). Recombinant gp120 coated wells were incubated with dilutions of the PJ for 1 h at 37°C. After removal of the juice and washing the wells, biotinyl-CD4 was added, and its binding to the wells was measured by ELISA.
gp120 and CD4 were in soluble form (data not shown). In reverse experiments, pre-treatment of CD4 with PJ failed to block subsequent gp120 binding. Other juices having anti-HIV-1 activity (blueberry, cranberry, grape, and lime) failed to block gp120 binding.

To delineate sites on gp120 blocked by the PJ inhibitor(s), the inhibitory effect of PJ on binding to gp120 IIB of antibodies to peptides derived from the amino acid sequence of gp120 was studied. The binding of antibodies to peptides (102-126), (303-338), (306-392), (386-417), (391-425), (411-445), and (477-508) was significantly (≥50%) inhibited (Fig. 3). The binding to gp120 IIB of monoclonal antibodies 9284 and 588D, specific for the gp120 V3 loop (residues 303–338) and the CD4 binding site, respectively, was each inhibited by 97%. Some of the relevant peptides contain residues involved in CD4 binding, whereas all discerned peptides include residues involved in coreceptor binding. The locations of the peptides and of residues involved in receptor/coreceptor binding on the X-ray crystallographic structure of gp120 are shown in Figure 4. These results suggest that the PJ inhibitor(s) may also block gp120–coreceptor binding. This will be addressed subsequently.

**Separation of Anti-HIV-1 Inhibitor(S) from Pomegranate Juice**

Pomegranate juice is intensely colored; therefore, it cannot be directly formulated into a microbicide because it would stain clothing, which is unacceptable. Attempts were made to separate or isolate the active ingredient(s) from PJ. After striving
intermittently for over 4 years to accomplish this, it was discovered that the inhibitor(s) of gp120–CD4 binding can be adsorbed effectively (≥99%) onto a selected brand of corn starch, PURITY® 21 corn starch NF grade (National Starch and Chemical Company, Bridgewater, NJ; S21) (FIG. 5), resulting in a nearly colorless product, designated as PJ-S21. PJ-S21 suspended in water or unbuffered 0.14 M NaCl had a pH of 3.2 (due to adsorbed PJ ingredients because starch provides a neutral pH) compatible with the acidic vaginal environment in which it would remain stable after application (see below). Inhibitors of gp120–CD4 binding could be eluted from PJ-S21 by extraction with ethanol/acetone 6:4. Drying of the extract followed by gravimetry indicated that the extract contained 3.17 mg solids per gram of PJ-S21.

PJ-S21, to the same extent as the original PJ, inhibited the binding of gp120 IIIB–CD4 complexes to cells expressing CXCR4, as determined by flow cytometry (Fig. 6). Similarly, binding of a gp120 BAL–CD4 fusion protein to cells expressing CCR5 was blocked by PJ and PJ-S21, as determined by a cell-based ELISA67 (Fig. 7). Therefore, PJ-S21 is an inhibitor of both X4 and R5 virus binding to the cellular receptor CD4 and coreceptors CXCR4/CCR5. PJ-S21 also inhibited gp120 binding to peripheral blood mononuclear cells as determined by flow cytometry (Fig. 8). To confirm that
FIGURE 5. Adsorption onto corn starch of gp120–CD4 binding inhibitor(s) from pomegranate juice (PJ). Corn starch (PURITY® 21, NF grade; 200 mg/ml) was added to PJ prefiltred to remove particulates. After mixing for 1 h at −20°C, the starch was allowed to settle and the supernatant fluid was removed by aspiration. The pellets, resuspended (200 mg/ml) in phosphate-buffered saline, and the supernatant fluids were tested at serial dilutions for inhibition of CD4 binding to gp120 IIIB as described in the legend for FIGURE 2. The inhibitory activity of the resuspended pellet against gp120 BaL–CD4 binding was then confirmed. Control starch did not inhibit gp120–CD4 binding.

FIGURE 6. Inhibition by pomegranate juice (PJ) and PJ-S21, respectively, of gp120 IIIB–CD4 complex binding to cells expressing CXCR4 coreceptors. HIV-1 IIIB gp120 (5 µg) and biotinyl-CD4 (2.5 µg) were added to 100 µl phosphate-buffered saline (PBS) containing 100 µg bovine serum albumin (BSA) (PBS-BSA) and PJ (final threefold dilution) or PJ-S21 (67 mg corresponding to 212 µg solids from PJ adsorbed onto starch). After 1 h at 20°C, the respective mixtures were added to 10⁶ MT-2 cells. After 30 min, the cells were washed 3 times with PBS-BSA and PE-streptavidin (a fluorescent label specific for biotin; 0.1 µg) was added. After 20 min, the cells were washed and fixed by 1% formaldehyde in PBS. Flow cytometry analysis was performed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometric Systems, San Jose, CA). The median relative fluorescence values for cells exposed to gp120–CD4; gp120–CD4 + PJ; gp120–CD4 + PJ-S21; and control cells were: 13.7, 4.0, 4.3, and 2.1, respectively.
FIGURE 7. Inhibition by pomegranate juice (PJ) and PJ-S21, respectively, of FLSC binding to CCR5 expressing Cf2Th/synCCR5 cells. FLSC is a full-length single chain protein consisting of BaL gp120 linked with the D1D2 domains of CD4 by a 20 amino acid linker. The inhibitory effect was quantitated using a cell-based ELISA. The starting concentration of PJ-S21 was 200 mg/ml, corresponding to 634 µg/ml solids adsorbed onto starch from PJ.

FIGURE 8. Inhibition by PJ-S21 of biotinyl-gp120 IIIB binding to peripheral blood mononuclear cells (PBMCs). HIV-1 IIIB biotinyl−gp120 (5 µg) was added to 100 µl of PBS-BSA containing graded quantities of PJ-S21. After 1 h at 20°C, the respective mixtures were added to 10⁶ PBMCs. After 30 min, the cells were washed 3× with PBS-BSA and PE-streptavidin (0.1 µg was added). Subsequently, the procedures described in the legend to Figure 6 were used. The median relative fluorescence values for control cells and cells exposed to biotinyl-gp120 in the absence and presence of PJ-S21 (100, 6.25, and 3.12 mg/ml) were 4.1, 81.31, 12.2, 35.2, and 50.0, respectively. 100 mg of PJ-S21 corresponds to ~320 µg solids adsorbed from PJ onto starch.
PJ-S21 functions as a virus entry inhibitor, the complex was added to cells at time intervals before and after infection of cells by HIV-1 IIIB and BaL, respectively. Results shown in Figure 9 demonstrate that PJ-S21 interferes with early steps of the virus replicative cycle.

To be considered as a topical microbicide, PJ-S21 must be formulated to withstand storage in a tropical environment. Accelerated thermal stability studies revealed that a water suspension of PJ-S21 maintained only 4, 11, and 33%, respectively, of its original activity (measured by inhibition of gp120–CD4 binding) when stored for 30 minutes at 60°C and 1 week at 50°C or 40°C. However, a dried PJ-S21 powder remained fully active after storage at 50°C for 12 weeks (the longest time used in the evaluation). Consequently, anhydrous formulations should be preferred for further development.

Three such formulations were prepared: two kinds of suppositories, melting at 37°C, and a tablet. The inhibitory activity of PJ-S21 was fully preserved after 12 weeks of storage at 50°C within tablets and at 30°C within the suppositories (the highest temperature considered to prevent melting). Data showing the inhibition of infection by HIV-1 IIIB and BaL, respectively, by PJ-S21 and its formulations (except the tablets that also contain anti-HIV-1 inhibitors other than PJ-S21, that is, Carbopol 974P33) are summarized in Figure 10. Their inhibitory activities against HIV-1 IIIB and BaL were similar, unlike the inhibitory activities of the original PJs (Fig. 1). These formulations were also virucidal, albeit at concentrations higher than those sufficient for inhibition of infection. These experiments also revealed that PJ-S21 was not cytotoxic under the experimental conditions used. The inhibitory/virucidal activities were maintained in the presence of seminal fluid at a 1:1 (w/w) ratio of seminal fluid to PJ-S21 (data not shown).

A microbicide can be considered potentially successful only if it displays antiviral activity against primary virus isolates belonging to distinct virus clades and

![FIGURE 9. Inhibition of HIV-1 IIIB or BaL replication depends on the time of PJ-S21 addition pre- or postinfection. For comparison, the inhibition of infection by the nonnucleoside reverse transcriptase inhibitor TMC-120, added to cells at distinct intervals after HIV-1, was determined (dotted lines). Virus infection was measured by quantitation of β-galactosidase.](image-url)
phenotypes. PJ-S21 meets this requirement, because it inhibited infection by primary HIV-1 strains of all clades tested having R5 and X4R5 (dual-tropic) phenotypes (TABLE 1).

DISCUSSION

Pomegranates have been venerated for millennia for their medicinal properties and considered sacred by many of the world’s major religions. In deference to pomegranates, the British Medical Association and several British Royal Colleges feature the pomegranate in their coat of arms. The Royal College of Physicians of London had adopted the pomegranate in their coat of arms by the middle of the sixteenth century.68 The best known literary reference to the contraceptive power of pomegranate seeds is classical Greek mythology. Ironically, this report shows that

pomegranate juice contains HIV-1 entry inhibitors targeted to the virus envelope corresponding to a class of antiretroviral drugs still scarce in development.\(^{74}\)

Pomegranate juice contains several ingredients\(^{75,76}\) that, isolated from natural products other than PJ, were reported to have anti-HIV activity, such as caffeic acid,\(^{77}\) ursolic acid,\(^{78}\) catechin, and quercetin\(^{79,80}\) and also anti-herpes simplex virus (HSV) activity.\(^{81,82}\) However, these compounds, in purified form, obtained commercially, did not block (at 200 µg/ml) gp120–CD4 binding as measured by the ELISA as just described and did not adsorb to corn starch, unlike the entry inhibitor(s) from PJ. In fact, the supernatant after treatment of PJ with starch and removal of the entry inhibitors retained anti-HIV-1 activity and also inhibited HSV-1, whereas the HIV-1 entry inhibitors that adsorbed onto starch did not inhibit HSV. Thus, the antiviral activities in the supernatant appeared to be nonspecific and probably similar to those of extracts from pomegranate rind\(^{83,84}\) and were not characterized further. Additional information\(^{85–88}\) has revealed that the findings apply to crude extracts from pomegranate rind prepared at elevated temperatures under conditions that destroy the HIV-1 entry inhibitor described here.

The inhibitor(s) interfering with gp120 binding to CD4 (Figs. 2 and 5) blocked additional sites on gp120 (Fig. 3) involved in interaction with the CXCR4/CCR5 coreceptors (Figs. 4, 6, and 7). This was not completely expected and can be explained either by the presence of multiple inhibitors with distinct or overlapping specificities in PJ-S21 or by induction of gp120 conformational changes\(^{89}\) resulting in blockade of both CD4 and CXCR4/CCR5 binding sites on gp120. Similar effects have been noted for other small molecule inhibitors.\(^{90}\) Simultaneous blocking of more than a single site on HIV-1 involved in virus entry is expected to increase the effectiveness of candidate microbicides.\(^{11}\) The target sites for the inhibitor(s) are likely to be located within the protein moiety of gp120, because binding of labeled Galanthus nivalis lectin (specific for terminal mannose residues\(^{91}\)) and other lectins to gp120 oligosaccharides was not diminished in the presence of PJ or PJ-S21 (data not shown).

### TABLE 1. Inhibitory activity of PJ-S21 on infection by primary HIV-1 strains

| Primary strain | Subtype, Coreceptor use | \(ED_{50}\) (mg/ml)\(^a\) | \(ED_{90}\) (mg/ml)\(^a\) |
|----------------|--------------------------|--------------------------|--------------------------|
| 92RW008        | A, R5                    | 0.50 ± 0.05              | 2.76 ± 0.28              |
| 94UG103        | A, X4R5                  | 1.42 ± 0.54              | 3.42 ± 0.98              |
| 92US657        | B, R5                    | 0.62 ± 0.11              | 2.86 ± 0.33              |
| 93IN101        | C, R5                    | 3.56 ± 1.10              | 8.87 ± 2.55              |
| 93MW959        | C, R5                    | 1.02 ± 0.19              | 3.54 ± 0.90              |
| 92UG001        | D, X4R5                  | 0.62 ± 0.17              | 2.94 ± 0.85              |
| 93THA051       | E, X4R5                  | 0.86 ± 0.01              | 4.09 ± 0.08              |
| 93BR020        | F, X4R5                  | 4.25 ± 0.78              | 8.31 ± 1.04              |
| RU570          | G, R5                    | 0.42 ± 0.09              | 1.54 ± 0.16              |
| BCF02          | Group O, R5              | 0.59 ± 0.29              | 3.92 ± 0.27              |

\(\text{ED}_{50(90)}\) = effective dose(s) of PJ-S21 for 50% (90%) inhibition of infection. One gram of PJ-S21 contains approximately 3.2 mg of the inhibitors adsorbed to starch from pomegranate juice.
Blocking of CD4 binding sites on HIV-1 gp120 by monoclonal antibodies or a CD4-IgG2 recombinant protein has been shown to be sufficient to inhibit HIV-1 infection of human cervical tissue \textit{ex vivo}\textsuperscript{11} and in preventing virus transmission to macaque monkeys when applied vaginally.\textsuperscript{92} Therefore, it seems likely that PJ-S21 will be similarly effective, an expectation that remains to be confirmed in an \textit{in vivo} macaque model system and in human clinical trials, as a candidate topical microbicide. This anticipation would be strengthened if drinking of PJ decreases the HIV-1 viral load in already infected individuals, an issue to be explored.

The application of PJ-S21 as a topical anti-HIV-1 microbicide requires reasonable uniformity among batches produced at distinct times and locations. Similarities in gp120–CD4 binding inhibitory activity among distinct freshly prepared and commercial juices stored for unknown periods (Fig. 2) suggest that this should be feasible. Pasteurization of juice for 30 seconds at 85°C resulted in complete loss of inhibitory activity. A commercial PJ concentrate exposed to 61°C and two other concentrates, presumably prepared by evaporation at elevated temperatures, had no or drastically diminished activity. The gp120–CD4 inhibitory activity from PJ3 (juice with fructose and citric acid added) failed to bind to starch. Separate experiments revealed that these compounds interfere with inhibitor binding to corn starch. Therefore, PJs intended for production of the PJ-S21 complex must be sterilized by filtration and be free of additives.

Particular attention must be devoted to the selection of starch, a pharmaceutical excipient generally used in vaginal formulations,\textsuperscript{93} for effective binding of the virus entry inhibitors from PJ. Among a dozen starches tested, the best results were obtained with S21. With other brands, the adsorption of the inhibitors was either incomplete or their binding did not result in a complex having activity in the ELISA measuring gp120–CD4 binding inhibition (ARGO® corn starch), presumably, because of irreversible binding of the PJ inhibitors. Interestingly, only a few references are available regarding the use of starch as an adsorbent for different compounds: flavors,\textsuperscript{94,95} dyes,\textsuperscript{96–98} low-molecular mass saccharides,\textsuperscript{99} lipids,\textsuperscript{100,101} proteins,\textsuperscript{102} and iodine.\textsuperscript{103}

The intended dose of PJ-S21 for vaginal application is 1.0 to 1.5 g (= 3.17 – 4.76 mg solids from PJ adsorbed onto starch), that is, ≥100-fold higher than the dose needed for blocking HIV-1 infection \textit{in vitro} (Fig. 10, TABLE 1) and therefore expected to meet requirements for likely \textit{in vivo} protection against vaginal challenge.\textsuperscript{104} This quantity of PJ-S21 is produced from 5 to 7.5 ml of PJ, that is, ≤5% of a single (150 ml) serving of juice, attesting to the safety, feasibility, and economy of this proposed candidate topical microbicide.

In an alternative approach to formulation development, PJ-S21 can be incorporated into a water-dispersible film (similar to the widely available “breath control” strips) or into water-dispersible sponges,\textsuperscript{105} which are converted into a gel following topical application.\textsuperscript{34} Each of the aforementioned formulations would meet the following requirements: (1) minimization of waste disposal problems associated with the use of applicators needed for delivery of microbicidal gels/creams; (2) simplicity; (3) small packaging and discretion related to purchase, portability, and storage; (4) low production costs; (5) amenability to industrial mass production at multiple sites globally; and (6) potential application as rectal microbicides. Furthermore, it would remain possible to produce for local use PJ-S21–based gel formulations with a limited shelf life, avoiding the costs of producing dry PJ-S21 powders.
via appropriate low temperature drying processes. Whichever of these formulations is selected, adequate quality control will be needed to assure uniform anti-HIV-1 activity of the final product(s) as well as to establish reproducible conditions for manufacture.

CONCLUSIONS

PJ-S21 can be classified as an AAAA candidate microbicide: acceptable; accessible; affordable; and accelerative in transition from development to marketing. Therefore, PJ-S21 would be expected to circumvent some problems associated with antiretroviral drugs and possibly some of the other candidate microbicides, that is, uncertainty related to potential side effects, investment and time needed to establish inexpensive large scale production, and monopoly of supply.

REFERENCES

1. UNAIDS/WHO. 2004. Global summary of the HIV and AIDS epidemic. December 2004. [http://www.unaids.org/EN/resources/epidemiology/epicore.asp].
2. WHO/SEARO CDS HIV/AIDS. 2004. HIV/AIDS Facts and Figures: Facts about HIV/AIDS Global. [http://w3.who.se/en/Section10/Section18/Section348.htm].
3. SHATTOCK, R.J. & J.P. MOORE. 2003. Inhibiting sexual transmission of HIV-1 infection. Nat. Rev. Microbiol. 1: 25–34.
4. PILCHER, C.D. et al. 2004. Brief but efficient: acute HIV infection and the sexual transmission of HIV. J. Infect. Dis. 189: 1785–1792.
5. STONE, A. 2002. Microbicides: a new approach to preventing HIV and other sexually transmitted infections. Nat. Rev. Drug. Discovery 1: 977–985.
6. SHATTOCK, R. & S. SOLOMON. 2004. Microbicides— aids to safer sex. Lancet 363: 1002–1003.
7. BROWN, H. 2004. Marvellous microbicides. Intravaginal gels could save millions of lives, but first someone has to prove that they work. Lancet 363: 1042–1043.
8. MOORE, J.P. & R.W. DOMS. 2003. The entry of entry inhibitors: a fusion of science and medicine. Proc. Natl. Acad. Sci. USA 100: 10598–10602.
9. PIERSON, T.C. & R.W. DOMS. 2003. HIV-1 entry inhibitors: new targets, novel therapies. Immunol. Lett. 85: 113–118.
10. DAVIS, C.W. & R.W. DOMS. 2004. HIV Transmission: closing all the doors. J. Exp. Med. 199: 1037–1040.
11. HU, Q. et al. 2004. Blockade of attachment and fusion receptors inhibits HIV-1 infection of human cervical tissue. J. Exp. Med. 199: 1065–1075.
12. FICHOROVA, R.N., L.D. TUCKER & D.J. ANDERSON. 2001. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. J. Infect. Dis. 184: 418–428.
13. FICHOROVA, R.N. et al. 2004. Interleukin (IL)-1, IL-6 and IL-8 predict mucosal toxicity of vaginal microbicidal contraceptives. Biol. Reprod. 71: 761–769.
14. WEISS, R. 2001. AIDS: unbeatable 20 years on. Lancet 357: 2073–2074.
15. NEURATH, A.R. et al. 1995. Blocking of CD4 cell receptors for the human immunodeficiency virus type 1 (HIV-1) by chemically modified bovine milk proteins: potential for AIDS prophylaxis. J. Mol. Recognition 8: 304–316.
16. JIANG, S. et al. 1996. Chemically modified bovine-lactoglobulin blocks uptake of HIV-1 by colon- and cervix-derived epithelial cell lines. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 13: 461–462.
17. NEURATH, A.R. et al. 1996. A herpesvirus inhibitor from bovine whey. Lancet 347: 1703.
18. NEURATH, A.R. et al. 1997. 3-Hydroxyphthaloyl-lactoglobulin: I. Optimization of production and comparison with other compounds considered for chemoprophylaxis of mucosally transmitted human immunodeficiency virus type 1. Antivir. Chem. Chemother. 8: 131–139.

19. NEURATH, A.R. et al. 1996. Bovine-lactoglobulin modified by 3-hydroxyphthalic anhydride blocks the CD4 cell receptor for HIV. Nat. Med. 2: 230–234.

20. JIANG, S. et al. 1997. Virucidal and antibacterial activities of 3-HP-LG. In Vaccines 97: Molecular Approaches to the Control of Infectious Diseases. F. Brown et al. Eds. :327–330. Cold Spring Harbor Laboratory Press. New York.

21. NEURATH, A.R. et al. 1997. 3-Hydroxyphthaloyl-lactoglobulin: II. Anti-human immunodeficiency virus type 1 activity in in vitro environments relevant to prevention of sexual transmission of the virus. Antivir. Chem. Chemother. 8: 141–148.

22. NEURATH, A.R., N. STRICK & Y.-Y. LI. 1998. 3-Hydroxyphthaloyl-lactoglobulin: III. Antiviral activity against herpesviruses. Antivir. Chem. Chemother. 9: 177–184.

23. KOKUBA, H., L. AURELIAN & A.R. NEURATH. 1998. 3-Hydroxyphthaloyl-lactoglobulin: IV. Antiviral activity in the mouse model of genital herpesvirus infection. Antivir. Chem. Chemother. 9: 353–357.

24. WYAND, M.S. et al. 1999. Effect of 3-hydroxyphthaloyl-lactoglobulin on vaginal transmission of simian immunodeficiency virus in rhesus monkeys. Antimicrob. Agents Chemother. 43: 978–980.

25. FDA'S CENTER FOR FOOD SAFETY AND APPLIED NUTRITION (CFSCAN). 2004. Commonly asked questions about BSE in products regulated by FDA's Center for Food Safety and Applied Nutrition (CFSAN) [http://vm.cfsan.fda.gov/~comm/bse-faq.html%20].

26. CHIU, Y.-Y. 2002. An Update CDER Biotechnology BSE Activities: BSE in manufacturing FDA regulatory actions. [http://www.temple.edu/pharmacy_QARA/fda_conf_Chiu.ppt].

27. U.S. FOOD AND DRUG ADMINISTRATION CfFSaAN. 2001. Agency response letter GRAS Notice No. GRN 000077. [http://vm.cfsan.fda.gov/~rbd/opa-g077.html].

28. CDC NCID. 2004. Update 2002: Bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease. [http://www.cdc.gov/ncidod/diseases/cjd/bse_cjd.htm].

29. WORLD HEALTH ORGANIZATION. 2002. Bovine spongiform encephalopathy (Fact Sheet No. 113). [http://www.who.int/mediacentre/factsheets/fs113/en/].

30. NEURATH, A.R. et al. 1999. Design of a “microbicide” for prevention of sexually transmitted diseases using "inactive" pharmaceutical excipients. Biologicals 27: 11–21.

31. NEURATH, A.R. et al. 2001. Cellulose acetate phthalate, a common pharmaceutical excipient, inactivates HIV-1 and blocks the coreceptor binding site on the virus envelope glycoprotein gp120. BMC Infect. Dis. 1: 17. [http://www.biomedcentral.com/content/pdf/1471-2334-1-17.pdf].

32. NEURATH, A.R. et al. 2002. Anti-HIV-1 activity of cellulose acetate phthalate: Synergy with soluble CD4 and induction of “dead-end” gp41 six-helix bundles. BMC Infect. Dis. 2: 6. [http://www.biomedcentral.com/content/pdf/1471-2334-2-6.pdf].

33. NEURATH, A.R., N. STRICK & Y.-Y. LI. 2002. Anti-HIV-1 activity of anionic polymers: a comparative study of candidate microbicides. BMC Infect. Dis. 2: 27. [http://www.biomedcentral.com/content/pdf/1471-2334-2-27.pdf].

34. NEURATH, A.R., N. STRICK & Y.-Y. LI. 2003. Water dispersible microbicidal cellulose acetate phthalate film. BMC Infect. Dis. 3: 27. [http://www.biomedcentral.com/content/pdf/1471-2334-3-27.pdf].

35. AVIRAM, M. et al. 2000. Pomegranate juice consumption reduces oxidative stress, athrogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. Am. J. Clin. Nutr. 71: 1062–1076.

36. PEHOWICH, D.J., A.V. GOMES & J.A. BARNES. 2000. Fatty acid composition and possible health effects of coconut constituents. West Indian Med. J. 49: 128–133.

37. HAMMERSTONE, J.F., S.A. LAZARUS & H.H. SCHMITZ. 2000. Procyanidin content and variation in some commonly consumed foods. J. Nutr. 130: 2086S–2092S.

38. AVIRAM, M. & L. DORNFIELD. 2001. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Atherosclerosis 158: 195–198.
39. JOSHI, S.S., C.A. KUSZYNSKI & D. BAGCHI. 2001. The cellular and molecular basis of health benefits of grape seed proanthocyanidin extract. Curr. Pharm. Biotechnol. 2: 187–200.
40. LIU, Y. et al. 2001. Citrus pectin: characterization and inhibitory effect on fibroblast growth factor-receptor interaction. J. Agric. Food Chem. 49: 3051–3057.
41. KAPLAN, M. et al. 2001. Pomegranate juice supplementation to atherosclerotic mice reduces macropaque lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis. J. Nutr. 131: 2082–2089.
42. HOWELL, A.B. 2002. Cranberry proanthocyanidins and the maintenance of urinary tract health. Crit. Rev. Food Sci. Nutr. 42: 273–278.
43. MILBURY, P.E. et al. 2002. Bioavailability of elderberry anthocyanins. Mech. Ageing Dev. 123: 997–1006.
44. WANG, X.H., L. ANDRAE & N. J. ENGESETH. 2002. Antimutagenic effect of various honeys and sugars against Trp-p-1. J. Agric. Food Chem. 50: 6923–6928.
45. SUN, J. et al. 2002. Antioxidant and antiproliferative activities of common fruits. J. Agric. Food Chem. 50: 7449–7454.
46. CAVANAGH, H.M., M. HIPWELL & J.M. WILKINSON. 2003. Antibacterial activity of berry fruits used for culinary purposes. J. Med. Food 6: 57–61.
47. SANCHEZ-MORENO, C. et al. 2003. Anthocyanin and proanthocyanidin content in selected white and red wines. Oxygen radical absorbance capacity comparison with nontraditional wines obtained from highbush blueberry. J. Agric. Food Chem. 51: 4889–4896.
48. POLAGRUT, J.A. et al. 2003. Effects of flavonoid-rich beverages on prostacyclin synthesis in humans and human aortic endothelial cells: association with ex vivo platelet function. J. Med. Food. 6: 301–308.
49. GHELDOF, N., X.H. WANG & N. J. ENGESETH. 2003. Buckwheat honey increases serum antioxidant capacity in humans. J. Agric. Food Chem. 51: 1500–1505.
50. AVIRAM, M. et al. 2004. Pomegranate juice consumption for 3 years with patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. Clin. Nutr. 23: 423–433.
51. JIRATANAN, T. & R.H. LIU. 2004. Antioxidant activity of processed table beets (Beta vulgaris var, conditiva) and green beans (Phaseolus vulgaris L.). J. Agric. Food Chem. 52: 2659–2670.
52. HUANG, H.Y. et al. 2004. Antioxidant activities of various fruits and vegetables produced in Taiwan. Int. J. Food Sci. Nutr. 55: 423–429.
53. NISFALI, P. et al. 2005. Antioxidant capacity of vegetables, spices and dressings relevant to nutrition. Br. J. Nutr. 93: 257–266.
54. NEURATH, A.R. et al. 2004. Punica granatum (pomegranate) juice provides an HIV-1 entry inhibitor and candidate topical microbicide. BMC Infect. Dis. 4: 41 [http://www.biomedcentral.com/content/pdf/1471-2334-4-41.pdf].
55. SHATTOCK, R.J. & R.W. DOMS. 2002. AIDS models: microbicides could learn from vaccines. Nat. Med. 8: 425.
56. SKINNER, M.A. et al. 1988. Characteristics of a neutralizing monoclonal antibody to the HIV envelope glycoprotein. AIDS Res. Hum. Retrovir. 4: 187–197.
57. LAAL, S. & S. ZOLLA-PAZNER. 1993. Epitopes of HIV-1 glycoproteins recognized by the human immune system. In Immunochemistry of AIDS, Chemical Immunology, Vol. 56. E. Norrby, Ed. :91-111. Karger. Basel.
58. KWONG, P.D. et al. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature 393: 648–659.
59. XIANG, S.H. et al. 2002. Mutagenic stabilization and/or disruption of a CD4-bound state reveals distinct conformations of the human immunodeficiency virus type 1 gp120 envelope glycoprotein. J. Virol. 76: 9888–9899.
60. PANTOPHLET, R. et al. 2003. Fine mapping of the interaction of neutralizing and non-neutralizing monoclonal antibodies with the CD4 binding site of human immunodeficiency virus type 1 gp120. J. Virol. 77: 642–658.
61. WESTERVELT, P., H.E. GENDelman & L. RATNER. 1991. Identification of a determinant within the human immunodeficiency virus 1 surface envelope glycoprotein critical for productive infection of primary monocytes. Proc. Natl. Acad. Sci. USA 88: 3097–3101.
62. Westervelt, P. et al. 1992. Macrophage tropism determinants of human immunodeficiency virus type 1 in vivo. J. Virol. 66: 2577–2582.
63. Rizzuto, C.D. et al. 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science 280: 1949–1953.
64. Cormier, E. G. & T. Dragic. 2002. The crown and stem of the V3 loop play distinct roles in human immunodeficiency virus type 1 envelope glycoprotein interactions with the CCR5 coreceptor. J. Virol. 76: 8953–8957.
65. Supaphiphat, P. et al. 2003. Effect of amino acid substitution of the V3 and bridging sheet residues in human immunodeficiency virus type 1 subtype C gp120 on CCR5 utilization. J. Virol. 77: 3832–3837.
66. Liu, S., S. Fan & Z. Sun. 2003. Structural and functional characterization of the human CCR5 receptor in complex with HIV gp120 envelope glycoprotein and CD4 receptor by molecular modeling studies. J. Mol. Model 9: 329–336.
67. Zhao, Q., G. Alespeti & A.K. Debnath. 2004. A novel assay to identify entry inhibitors that block binding of HIV-1 gp120 to CCR5. Virology 326: 299–309.
68. Langley, P. 2000. Why a pomegranate? BMJ 321: 1153–1154.
69. Navarro, V. et al. 1996. Antimicrobial evaluation of some plants used in Mexican traditional medicine for the treatment of infectious diseases. J. Ethnopharmacol. 53: 143–147.
70. Lee, J. & R.R. Watson. 1988. Pomegranate: a role in health promotion and AIDS? In Nutrients and Foods in AIDS. R.R. Watson RR. Ed.:213–216. CRC. Boca Raton.
71. Prashanthi, D., M.K. Asha & A. Amit. 2001. Antibacterial activity of Punica granatum. Fitoterapia 72: 171–173.
72. Mouhajir, F. et al. 2001. Multiple antiviral activities of endemic medicinal plants used by Berber peoples of Morocco. Pharm. Biol. 39: 364–374.
73. Negi, P.S., G.K. Jayaprakash & B.S. Jena. 2003. Antioxidant and antimutagenic activities of pomegranate peel extracts. Food Chem. 80: 393–397.
74. Greene, W.C. 2004. The brightening future of HIV therapeutics. Nat. Immunol. 5: 867–871.
75. Poyrazoglu, E., V. Goekmen & N. Artik. 2002. Organic acids and phenolic compounds in pomegranates (Punica granatum L.) grown in Turkey. J. Food Composition and Analysis 15: 567–575.
76. Module 2: Phytochemicals (minerals, phytamins, and vitamins). 2003. [http://www.ars-grin.gov/duke/syllabus/module2.htm].
77. Mahmoud, N. et al. 1993. Inhibition of HIV infection by caffeoylquinic acid derivatives. Antivir. Chem. Chemother. 4: 235–240.
78. Ma, C. et al. 1998. Inhibitory effects of ursolic acid derivatives from cynomorium sonoragmicum, and related triterpenes on human immunodeficiency virus protease. Phytother. Res. 12: S138–S142.
79. Mahmoud, N. et al. 1996. The anti-HIV activity and mechanisms of action of pure compounds isolated from Rosa damascena. Biochem. Biophys. Res. Commun. 229: 73–79.
80. DeTommasi, N. et al. 1998. Anti-HIV activity directed fractionation of the extracts of Margyricarpus setosus. Pharma. Biol. 36: 29–32.
81. Zhang, J. et al. 1995. Antiviral activity of tannin from the pericarp of Punica granatum L. against genital Herpes virus in vitro. Zhongguo Zhong Yao Za Zhi 20: 556–8, 576.
82. Li, Y. et al. 2004. Antiviral activities of medicinal herbs traditionally used in Southern Mainland China. Phytother. Res. 18: 718–722.
83. Pomegranates could help in battle against AIDS. 1996. Reuters NewMedia, Inc. [http://www.aegis.com/news/re/1996/RE960310.html].
84. Medical breakthrough. 1996. British Muslims Monthly Survey IV (3),6. [http://artsweb.bham.ac.uk/bmms/1996/03March96.html#Medical%20breakthrough].
85. Jassim, S.A.A., S.P. Denyer & G.S.A.B. Stewart. 1998. Antiviral or antifungal composition comprising an extract of pomegranate rind or other plants and method of use. US Patent 5,840,308. November 24, 1998.
86. Shehadeh, A.A. 2000. Herbal extract composition and method with immune-boosting capability. US Patent 6,030,622. February 29, 2000.
87. JASSIM, S.A.A., S.P. DENYER & G.S.A.B. STEWART. 2001. Antiviral or antifungal composition and method. US Patent 6,187,316. February 2, 2001.
88. JASSIM, S.A.A. & S.P. DENYER. 2002. Antiviral or antifungal composition and method. US Patent Application 20020064567. May 30, 2002.
89. HSU, S.-T. & A.M.J.J. BONVIN. 2004. Atomic insight into the CD4 binding-induced conformational changes in HIV-1 gp120. Proteins 55: 582–593.
90. NEURATH, A.R. et al. 1994. Tin protoporphyrin IX used in control of heme metabolism in humans effectively inhibits HIV-1 infection. Antivir. Chem. Chemother. 5: 322–330.
91. HAMMAR, L. et al. 1995. Lectin-mediated effects of HIV type 1 infection in vitro. AIDS Res. Hum. Retrovir. 11: 87–95.
92. VEAEZY, R.S. et al. 2003. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat. Med. 9: 343–346.
93. GARG, S. et al. 2001. Compendium of pharmaceutical excipients for vaginal formulations. Pharma. Techn. Drug Deliv. Sept. 14–24.
94. YAO, W.H. 2002. Adsorbent characteristics of porous starch. Starch/Starke 54: 260–263.
95. WHISTLER, R.L. 1991. Microporous granular starch matrix compositions. US Patent 4,985,082. January 15, 1991.
96. BERSET, C., H. CLERMONT & S. CHEVAL. 1995. Natural red colorant effectiveness as influenced by absorptive supports. J. Food Sci. 60: 858–861, 879.
97. STUTE, R. & H.U. WOELK. 1974. Interaction between starch and reactive dyes. New technique for the investigation of starch. II. Influence on fixation reaction of starch. Starch/Starke 26: 1–9.
98. SEGUCHI, M. 1986. Dye binding to the surface of wheat starch granules. Cereal Chem. 63: 518–520.
99. TOMASIK, P., Y.-J. WANG & J.L. JANE. 1995. Complexes of starch with low-molecular saccharides. Starch/Starke 47: 185–191.
100. ZHANG, G., M.D. MALADEN & B.R. HAMAKER. 2003. Detection of a novel three component complex consisting of starch, protein, and free fatty acids. J. Agric. Food Chem. 51: 2801–2805.
101. JOHNSON, J.M., E.A. DAVIS & J. GORDON. 1990. Lipid binding of modified corn starches studies by electron spin resonance. Cereal Chem. 67: 236–240.
102. TOMAZIC-JEZIC, V.J., A.D. LUCAS & B.A. SANCHEZ. 2004. Binding and measuring natural rubber latex proteins on glove powder. J. Immunoassay Immunochem. 25: 109–123.
103. CONDE-PETIT, B. et al. 1998. Comparative characterization of aqueous starch dispersions by light microscopy, rheometry, and iodine binding behavior. Starch/Starke 50: 184–192.
104. MOORE, J. et al. 2004. Development of fusion/entry inhibitors as topical microbicides. Presented at Microbicides 2004. London. March 28–31 2004. [http://www.microbicides2004.org.uk/progteue.html].
105. NEURATH, A.R. & N. STRICK. 2003. Biodegradable micbicidal vaginal barrier device. US Patent 6,572,875. June 3, 2003.
106. NEURATH, A.R., N. STRICK & S. JIANG. 1992. Synthetic peptides and anti-peptide antibodies as probes to study interdomain interactions involved in virus assembly: The envelope of the human immunodeficiency virus (HIV-1). Virology 188: 1–13.
107. KRAULIS, P. J. 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. 24: 946–950.
108. BACON, D.J. & W.F. ANDERSON. 1988. A fast algorithm for rendering space-filling molecule pictures. J. Mol. Graphics 6: 219–220.
109. MERRITT, E.A. & D.J. BACON. 1997. Raster3D: photorealistic molecular graphics. Meth. Enzymol. 277: 505–524.