Investigation of Griffithsin’s Interactions with Human Cells Confirms Its Outstanding Safety and Efficacy Profile as a Microbicide Candidate

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Abstract

Many natural product-derived lectins such as the red algal lectin griffithsin (GRFT) have potent in vitro activity against viruses that display dense clusters of oligomannose N-linked glycans (NLG) on their surface envelope glycoproteins. However, since oligomannose NLG are also found on some host proteins it is possible that treatment with antiviral lectins may trigger undesirable side effects. For other antiviral lectins such as concanavalin A, banana lectin and cyanovirin-N (CV-N), interactions between the lectin and as yet undescribed cellular moieties have been reported to induce undesirable side effects including secretion of inflammatory cytokines and activation of host T-cells. We show that GRFT, unlike CV-N, binds the surface of human epithelial and peripheral blood mononuclear cells (PBMC) through an exclusively oligosaccharide-dependent interaction. In contrast to several other antiviral lectins however, GRFT treatment induces only minimal changes in secretion of inflammatory cytokines and chemokines by epithelial cells or human PBMC, has no measurable effect on cell viability and does not significantly upregulate markers of T-cell activation. In addition, GRFT appears to retain antiviral activity once bound to the surface of PBMC. Finally, RNA microarray studies show that, while CV-N and ConA regulate expression of a multitude of cellular genes, GRFT treatment effects only minimal alterations in the gene expression profile of a human ectocervical cell line. These studies indicate that GRFT has an outstanding safety profile with little evidence of induced toxicity, T-cell activation or deleterious immunological consequence, unique attributes for a natural product-derived lectin.

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Introduction

HIV-1 is the prototype example of a virus that utilizes an oligomannose-rich “glycan shield” to occlude functionally important domains of the envelope glycoproteins from antibodies, and evade the immune response [1,2]. Recently Doorens et al. [3] showed that previous measurement of the proportion of oligomannose NLG relative to complex NLG on recombinant HIV envelope glycoproteins underestimated the representation of oligomannose NLG on the native envelope spikes of HIV-1, which appear to display NLG that are almost exclusively mannose-terminal Man5–9-GlcNAc2 structures. It is likely that limited access to the high density of NLG presented on the HIV-1 trimeric glycoprotein spike by Golgi and endoplasmic reticulum (ER) α1–2 mannosidases results in an atypical preponderance of oligomannose glycans rather than complex NLG on HIV-1 surface glycoproteins [3,4,5]. Given that Man6–9-GlcNAc2 structures are present on less than 4% of the normal human N-glycome [6,7], dense clusters of oligomannose NLG appear to be a feature specific to viral envelope glycoproteins, particularly those of HIV-1 and other immunodeficiency lentiviruses [3]. Consequently, clusters of oligomannose NLG may be attractive molecular targets for antiviral drugs and vaccines that act to interrupt HIV-1 infection of target cells by: (i) binding on the virus envelope and thereby interfering with the structural transitions involved in receptor and co-receptor docking and virus entry into T-cells and (ii) blocking access to viral envelope oligomannose NLG targeted...
by C-type lectin receptors DC-SIGN and MMR on dendritic cells and macrophages [5,8,9].

It has long been known that a variety of oligomannose-specific lectins have potent in vitro HIV-1 inhibitory activities, and therefore have been proposed as microbicide candidates for topical prophylaxis of HIV-1 infection, and as potential therapeutics [8,9]. However many lectins possess lymphocyte mitogenic activities incompatible with their use as pharmaceuticals, and some are known human and animal toxins, although the pharmacological basis for their toxicity is poorly characterized [10,11]. The antiviral potency of lectins has been correlated to their capacity to bind multiple glycans simultaneously, often facilitated by their ability to form dimers and higher order multimers [12,13,14].

The most extensively characterized antiviral lectin is CV-N, a small protein that exists in both monomeric and homodimeric configurations and has exceptionally potent anti-HIV activity, in the low nanomolar range [15]. CV-N targets the Man9\(\alpha\)-2Man terminating glycans displayed in the Man6–9GlcNAc2 structures on the surface of many viral envelope glycoproteins. Each monomer of CV-N has the capacity to bind two oligomannose structures [16]. When formulated into a carboxyethylcellulose gel matrix, CV-N provided almost complete protection against a single high dose intrarectal or intravaginal challenge with a pathogenic simian-human immunodeficiency virus (SHIV) [17,18]. However, subsequent in vitro toxicity studies have raised concerns about the safety of CV-N based microbicides, finding that in vitro, CV-N has the capacity to promote secretion of pro-inflammatory cytokines and chemokines from human peripheral blood mononuclear cells (PBMC), activate quiescent CD4+ T-cells, and promote T-cell proliferation [19,20,21,22]. Similar results were also reported for other lectins such as microvirin (MVN) (17) and concanavalin A (Con A) (18). It should be noted that the toxicities of CV-N were much milder in treated cervical explants in comparison with PBMC [20,21]. The possible pathogenic consequences associated with these off-target activities have raised concerns about all other members of the natural product-based class of antiviral lectins [19,20,21].

GRFT has the most potent and broad spectrum HIV-1 inhibitory activity described for any antiviral lectin [15,23,24,25]. It is a 25 kDa domain-swapped homodimer, with the first 16 amino acids of each 12.7 kDa monomer completing the \(\beta\)-prism fold of the other [26]. The homodimer has six carbohydrate binding pockets, 3 located at each of the opposite ends of the double-prism homodimer. Atomic resolution crystal structures of an engineered monomeric GRFT showed that each monomer can bind to two different nonamannoside molecules through all three carbohydrate binding sites [13,26]. The antiviral activity of monomeric GRFT is substantially lower than that of the homodimeric form, confirming that the GRFT potency is dependent on its ability to bind multiple oligomannose structures simultaneously, with strong avidity [13]. We showed recently that GRFT causes no mitogenic stimulation of PBMC exposed to the drug [23]. GRFT is fully active in the presence of macaque vaginal secretions [25], and was shown to have a good safety profile in the rabbit vaginal irritation model, the Gold Standard preclinical safety test for vaginal products [23]. Moreover, treatment of human cervical explants with GRFT induced minimal alterations in the expression profile of a panel of proinflammatory chemokines and cytokines. GRFT also strongly inhibited HIV-1 infection of the cervical explants, and dissemination of HIV-1 infection from cells resident in the explants to donor T-cells [23]. In the present study, we performed a comprehensive set of experiments to interrogate the molecular response of cultured human cervico-vaginal cells and PBMC to GRFT exposure. Our investigations employed comparisons between the biological activities of GRFT, which binds mannose-terminal Man9\(\alpha\)-2GlcNAc2, with other lectins of well-defined carbohydrate binding specificity: (1) CV-N, which binds Man9\(\alpha\)-2Man terminating glycans on Man9\(\alpha\)-2GlcNAc2 structures; (2) phytohaemagglutinin A (PHA), targeting D-galactose and N-acetyl-D-galactosamine on glycan structures; (3) ConA, specific for terminal (tri) mannose on high mannose glycans and (4) Pokeweed agglutinin (PKM), which binds N-acetylgalactosamine. Our studies reveal clear distinctions in biological and toxicological properties of these lectins, and confirm GRFT’s superior safety profile for use as a topical microbicide.

**Results**

GRFT and CV-N binding to human cervical epithelium, cultured human cervico-vaginal cells, and PBMC

We used paraffin-embedded cervical epithelial sections from a 21-year old donor to evaluate the binding pattern of fluorescently labeled GRFT and CV-N to human mucosal epithelia. GRFT\(^{Lec-}\), a mutant form of GRFT where we eliminated the lectin activity through mutation of all six mannose binding sites, was used as a control to help distinguish binding mediated by the GRFT carbohydrate binding pockets versus binding associated with other GRFT structures. The light micrograph in Figure 1A shows an H&E stained cervical tissue section to orient the observer to the microanatomy of the human cervical epithelium. Different layers of the squamous epithelium starting at the basement membrane (basal, parabasal, intermediate, superficial) are evident; cervical connective tissue or stroma is beneath the basement membrane. Tissues incubated with labeled GRFT, GRFT\(^{Lec-}\) and CV-N are shown in Fig. 1B, C and D, respectively. Minimal fluorescence seen in tissues exposed to GRFT\(^{Lec-}\) (Fig. 1C) compared with GRFT-stained tissues (Fig. 1B) confirmed that the binding of GRFT to the outermost layer of the squamous epithelium was via its carbohydrate binding activity. There were distinct differences evident in the binding pattern of GRFT (Fig. 1C) relative to CV-N (Fig. 1D), which bound far more extensively than GRFT throughout all layers of the squamous epithelium, basement membrane and underlying stromal tissue. Additional fluorescence micrographs are provided in Fig. S1. In cultured cervico-vaginal epithelial cells we also observed binding of both GRFT and CV-N, but not GRFT\(^{Lec-}\) to Ect1/E6E7 cells (compare Fig. 1E through H), as well as End1/E6E7 and VK2/E6E7 cells (data not shown). We used flow cytometry to evaluate binding of fluorescently labeled GRFT, GRFT\(^{Lec-}\) and CV-N to human PBMC. Clear shifts in fluorescence intensity show that GRFT (Fig. 1 I) and CV-N (Fig. 1K) efficiently bind the surface of human PBMC relative to GRFT\(^{Lec-}\) (Fig. J), for which we observed only minimal binding. Binding of GRFT to the surface of PBMC was significantly reduced when occluding the glycan binding pockets by pre-incubation with yeast mannan (Fig. I). Interestingly, the binding of CV-N to PBMC was reduced, but not eliminated, by mannan binding, which implies a second mode of binding between CV-N and the cell surface (Fig. 1K). We assume that distinct populations of differently labeled cells seen in the flow histograms reflect differences in the amount of labeled protein that binds different subpopulations of leukocytes in the unfraccionated PBMC samples.

**Griffithsin Safety and Efficacy Profile**

When freshly-isolated PBMC were pre-incubated for 24 hrs with GRFT at various concentrations, washed and then infected with HIV-1 R5 strain BaL (without adding new compound), GRFT inhibited viral replication for 9 days of cell culture (Fig. 2). As a control maraviroc (MVR) at 2 \(\mu\)M was included and this also showed anti-HIV activity after 9 days in culture, as this compound is known to bind specifically to the CCR5 receptor. However,
lower concentrations of maraviroc (at 0.4 μM) did not retain its antiviral activity in this assay protocol (data not shown).

**High concentrations of GRFT are not cytotoxic to cervico-vaginal cell lines**

An MTT assay was used to assess the effects of GRFT on End1/E6E7, Ec1/E6E7 and VK2/E6E7 cell viability by measuring the metabolic activity of treated cells. In these experiments (Fig. 3) we observed no loss in cell viability after a 3 day exposure of the endocervical and ectocervical cell lines to concentrations of up to 1 mg/ml (84 μM) GRFT, at least 10-times more concentrated than a likely microbicide formulation [23]. High doses of GRFT did, however, slightly reduce viability of the vaginal keratinocyte (VK2) cells. In marked contrast to GRFT, the mannose-specific mitogenic

![Figure 1](image-url)
lectin ConA showed clear concentration-dependent cytotoxicity towards all three cell lines. As shown in Fig. 3, treatment with 1 μM ConA killed approximately 94% of the ectocervical cells and vaginal keratinocytes, and 80% of the endocervical cells. PHA, another lectin known to be mitogenic in vivo and in vitro also caused dose-responsive cell death, but to a lesser extent than ConA.

**GRFT does not stimulate cell proliferation**

We previously showed that GRFT exhibits no mitogenic stimulatory effect in human PBMC, using incorporation of tritiated thymidine as a marker for cell proliferation [23]. In the present study we investigated the mitogenic activity of GRFT on PBMC by flow cytometry, evaluating changes in size and morphology of cells treated with GRFT in comparison with cells treated with the vehicle (PBS). Cells treated with 1 or 4 μM GRFT had flow cytometry profiles similar to the control cells (Fig. 4A, B and C). In contrast treatment with lectins ConA and PHA at doses (0.37 μM ConA and 10 μg/ml PHA) that do not negatively affect cell viability, resulted in completely different flow cytometric plots, with emergence of a subpopulation composed of larger cells (higher forward scatter FSC) with perceptibly higher side scatter (SSC) values gated in Region R2, as shown in Fig. 4D and E. In addition, a clear decrease in cell number was observed in region R1 after treatment with ConA and PHA, as quantified in Fig. 4F. In Fig. 5, we show that GRFT also does not induce cell proliferation in any of the three cultured human cervical and vaginal epithelial cells. Cell division was assessed by monitoring BrdU incorporation in newly synthesized DNA of actively dividing cervicovaginal cell lines. Treatment with 1 or 8 μM GRFT did not induce proliferation of any of the cell lines since BrdU counts were not elevated in comparison with control cells treated with vehicle alone (PBS). In contrast Pokeweed agglutinin (PKM), a well characterized mitogen, caused concentration-dependent increase in cell division, especially in the cervical cell lines End1/E6E7 and Ect1/E6E7.

**Effects of GRFT treatment on PBMC activation markers**

To evaluate the effect of GRFT on cell surface markers of immune activation, we measured expression of the following membrane proteins: (i) CD69, a marker of activated T-lymphocytes, considered an “early” marker of T-cell activation; (ii) CD25, the alpha chain of the IL-2 receptor, upregulated on
activated T-cells, B-cells, and some thymocytes and myeloid precursors, considered a “middle” marker of T-cell activation; and (iii) HLA-DR, a component of the type II major histocompatibility (MHC) complex, and a “late” marker of T-cell activation. Activated T-cells are highly susceptible to HIV-1 infection, and hence induction of these markers indicates an undesirable side effect of lectin treatment of PBMC. PBMC were incubated in the presence of the test compounds for 72 hours. The vehicle control was PBS, and positive controls were 10 μg/ml PHA [20], as well as 0.37 mM ConA, chosen as this concentration was not cytotoxic.

In PBS treated cells, 1.2% PBMC in average were double positive (CD4+ /CD25+) and a non significant increase of this population was observed after incubation in presence of 1 or 4 μM GRFT (Fig. 6, left panel and Fig. S2A). Treatment with PHA and ConA resulted in an impressive increase in the number of CD4+/CD25+ cells (Fig. 6, left panel and Fig. S2A). In addition, the numbers of CD4+ /CD25+ were elevated when PBMC were cultured in presence of PHA or ConA compared to their PBS and GRFT counterpart (Fig. 6, left panel and Fig. S2A). A detailed overview of the cytokine profiles of GRFT-treated PBMC from multiple blood donors is given in Fig. 7. For reference, we provide previously published data where PBMC with the same donor origins were treated with CV-N at 2 μg/ml (182 nM), lower than the GRFT concentration tested, since higher concentrations of CV-N proved too toxic to PBMC [19]. The concentration of the separate cytokines was compared with that of the untreated PBMC and calculated as a fold increase value. In the previous studies on PBMC treatment with CV-N and MVN, considerable variability in the lectin-induced cytokine profile was observed between the different PBMC donors. Therefore, the fold increase values obtained from the different donors were divided over different ranking groups (i.e. 1–3-, 3–10-, 10–100-, 100–500-, and >500-fold increase), and the number in each rank is given as a percentage of the total and indicated by a specific color (Fig. 7).

Confirming our data in Fig. 6 that GRFT has very little effect on lymphocyte activation markers, we also see minimal alterations in the cytokine and chemokine release for the majority of markers, in most donor PBMC. This profile indicates that GRFT induces significantly less response from PBMC than has been previously reported for CV-N, MVN and ConA [19]. The only chemokine
induced weakly by GRFT in the majority of donors (75%) was MCP-1 [19]. Although these studies were performed at a later date than the previously published CV-N and MVN studies, we used exactly the same donor panel, and the assays were performed in the same laboratory (D. Schols), justifying comparison between the experiments.

Validation of the utility of cultured cervico-vaginal cell lines for detection of off-target effects of GRFT treatment

In our previously published studies, we showed that treatment of human cervical explants with a range of GRFT concentrations had no significant effects on expression of a panel of chemokines and cytokines [23]. In the present studies, we used cultured human endocervical, ectocervical and vaginal cell lines for more detailed analysis of potential “off-target” effects of GRFT treatment, since the cell lines are easier to procure than fresh human cervical tissues, and experiments with the cell lines are more easily reproduced without question of variability in genetic background and physiological conditions of the donor. To validate the utility of these cell lines (which are immortalized by induction of papillomavirus E6 and E7 oncogenes) for analysis of any “off-target” effects of GRFT treatment, since the cell lines are donor panel, and the assays were performed in the same laboratory (D. Schols), justifying comparison between the experiments.

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Effect of GRFT Treatment on Gene Expression in Ect1/E6E7 cells

Gene expression in Ect1/E6E7 cells in response to treatment with GRFT was compared with vehicle (PBS), GRFT, CV-N...
Figure 6. Effect of GRFT on PBMC activation. Cells were incubated for three days in culture medium alone, with 1 μM GRFT, with 4 μM GRFT and with 10 μg/ml PHA and dual stained using FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25 (left panel), anti-CD69 (middle panel) and anti-HLA-DR (right panel). The percentages of receptor positive cell populations are indicated in the individual dot plots. doi:10.1371/journal.pone.0022635.g006
and ConA treatments (all qualified with endotoxin levels less than 0.05 EU per milligram). This was studied using a microarray experiment in which the whole human genome was represented by the 41,000 known genes and transcripts [27]. The microarray data were deposited in the Gene Expression Omnibus database, under accession number GSE28584. The heat map shown in Fig. 9A indicates that cells exposed for 24 hours to GRFTLec- (1 and 8 \(\mu M\), and low concentrations of GRFT (0.1 \(\mu M\)) and CV-N (0.05 \(\mu M\)) showed comparable gene expression profiles to those that were incubated in presence of PBS alone. Treatment of Ect1/E6E7 with 1 \(\mu M\) GRFT resulted in minor changes in the expression profile while 4 \(\mu M\) GRFT appeared to alter the expression of many genes but to a much lesser extent compared to CV-N (0.5 and 4 \(\mu M\)) and ConA at 1 \(\mu M\) (Fig. 9). Thus, the microarray studies confirm the data showing that GRFT has substantially lower “off-target” effect in comparison with CV-N and ConA, although there are clearly several genes that are regulated by the carbohydrate binding activity of GRFT (compare with GRFTLec-). As predicted from the heat map (Fig. 9A), no gene was identified as regulated by 0.1 \(\mu M\) GRFT, even when the cutoff was brought to 1.0. However, a first analysis using Benjamini-Hochberg low stringency correction with a cutoff of 2.0 yielded 107 and 35 entries as differentially expressed in samples treated with 1 and 4 \(\mu M\) GRFT, respectively (Fig. 9B). Treatment with CV-N (0.5 and 4 \(\mu M\)) or ConA (1 \(\mu M\)) resulted in regulation of impressive numbers of human genes (Fig. 9B). We then employed stricter criteria by keeping only the positive entries that showed GRFT concentration dependent gene expression. This yielded 2 and 32 genes for 1 and 4 \(\mu M\) GRFT, respectively. The entries which showed an increased gene expression after treatment with 1 \(\mu M\) GRFT included a gene annotated as immunoglobulin-like and fibronectin type III domain containing 1 (IGFN1). The entries that were found to be affected by 4 \(\mu M\) GRFT are summarized in Table S2. Among the 26 genes mapped by the Ingenuity database, there was overrepresentation of genes in the following canonical pathways: NRF2-mediated oxidative stress response (MAF, HMOX1, SOD2), phospholipid degradation, glycerophospholipid metabolism and Endothelin-1 Signaling (HMOX1, WISP2), cAMP mediated signaling (CALML5, PKIB) and acute phase response signaling (HMOX1, SOD2). Furthermore using the Ingenuity software we identified five toxicological functions with an overrepresentation of genes including liver hyperbilirubinemia and steatohepatitis, cardiac arteriopathy, renal and liver necrosis (data not shown). None of these is relevant to mucosal treatment with GRFT. We used quantitative RT-PCR (Q-RT-PCR) to validate the microarray results. Expression of 14
genes was studied using 18S RNA and β-actin mRNA as controls. With the exception of MYCN, all genes studied showed comparable expression levels in both experimental systems including microarrays and Q-PCR (Table S3).

Discussion

Natural product lectins have received considerable attention as potential antiviral drugs, particularly in the context of prevention of HIV-1 transmission via mucosal surfaces (reviewed recently in [8]). Although the volume of literature supporting their use as antivirals in vitro is dwarfed by a comprehensive set of data showing potent in vitro antiviral activity, there are reports of impressive in vivo efficacy of CV-N in animal models of HIV-1 prevention [17,18], influenza prevention and treatment [28], and Ebola virus prophylaxis and treatment [29], and of GRFT in prevention of SARS-CoV infection [30]. Despite the myriad of potential prophylactic and therapeutic applications of antiviral lectins, enthusiasm for their development as pharmaceuticals is tempered by a long history of research into natural product lectins, which characterizes many members of this broad class as erythrocyte agglutinins, lymphocyte mitogens, and potentially lethal toxins [10,11]. The pharmacological basis of natural product lectin toxicity is generally poorly understood, but at a basic mechanistic level is thought to reside in the lectins’ capacity for multivalency of binding to cell surface glycans, resulting in cell agglutination and/or cross-linking of cell surface receptor molecules with consequent activation of signaling pathways. Why different lectins that bind identical glycan moieties have quite distinct biological effects in vitro remains a paradox that is well illustrated by our data which, show very different in vitro activity profiles of four different oligomannose-binding lectins: GRFT, CV-N, MVN [19] and ConA.

Characterization of GRFT is the primary focus of our studies, but understanding the molecular pharmacology and toxicology of this potent antiviral lectin is informed by comparison to CV-N, for which a rich set of in vitro and in vivo data is available. Both molecules have comparable HIV-1 neutralization activities, with mean IC50 values against Clade C viruses of 42.7 ± 4.4 nM for GRFT and 77.0 ± 18.2 for CV-N [15]. Both proteins bind oligomannose glycans, with GRFT targeting terminal mannose residues found on Man5–9-GlcNAc2 [13] and CV-N specific for the Manα1R2Man linkages found on Man6–9-GlcNAc2 [16,31,32]. They thus share overlapping binding specificities, and should target identical cell surface and viral glycans. If anything, GRFT would be predicted to bind to a larger number of glycan targets than CV-N since it can bind pentamannose structures that lack the α1R2 mannose linkages that CV-N targets [13]. In this context, it is surprising that CV-N appears to bind more promiscuously than GRFT throughout the cervical epithelium and sub-epithelial stroma (Fig. 1A–D, and Fig. S1). Given that GRFTLec- (the carbohydrate-binding deficient form of GRFT) hardly bound the epithelial sections or cultured cervical and vaginal epithelial cells (Fig. 1), and GRFT binding to cultured epithelial cells was blocked by mannan, we conclude that GRFT’s binding activity to the cell surface is exclusively via its

![Figure 8. Effect of GRFT on the secretion of selected key cytokines in cervico-vaginal cell lines.](image-url)

Individual ELISA experiments for detection of IL-1β (A), IL-2 (B), IL-6 (C) and IL-8 (D) after 24 hours treatment of cervico-vaginal cells with 2 μM ConA (black), 8 μM GRFT (grey) and PBS (white).

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carbohydrate binding activity. It is very interesting to note that CV-N binding to the surface of PBMC is not entirely eliminated by blocking its carbohydrate binding sites with mannan (Fig. 1K). This implies that its capacity to bind and induce signaling in cells may not reside entirely in its lectin activities, and supports prior studies with CV-N [21]. Another important result showed that GRFT seems to bind somewhat selectively to terminally differentiated keratinocytes on the epithelial surface, presumably reflecting presence of optimal glycoprotein binding targets on the surface of those cells only in the intact epithelium (Fig. 1C and D; and Fig. S1).

The data presented in Fig. 2 strongly support GRFT’s candidacy as a microbicide and antiviral, since they show that GRFT retains antiviral activity even when complexed to the surface of PBMC. This contrasts with many other lectins or compounds we have tested in this assay. The most plausible mechanism that might explain GRFT’s retention of HIV-1 entry inhibition activity is that fewer than all six carbohydrate binding sites are occupied when the lectin is docked on cell surface glycoprotein/s, leaving sites available for binding to the viral envelope glycoprotein. The crystal structure of GRFT suggests that the three glycan-binding pockets of each GRFT monomer are on opposite ends of the double prism homodimer [13], suggesting that only one monomer of the homodimer is engaged in binding the cell surface, leaving the “free” monomer competent to bind and cross-link two oligomannose structures on the surface of HIV-1. Since the PBMC were washed extensively 24 hours after treatment and prior to addition of the HIV-1 inoculum, this suggests that cell surface-bound GRFT irreversibly inactivated the inoculum, with no evidence of breakthrough infection at 9 days post infection. This duration of antiviral activity is unprecedented. Conventionally, antiviral potencies of virus-targeted entry inhibitor drugs are measured without washing the cells prior to addition of viruses. In traditional antiviral assays the IC_{50} of GRFT against HIV-1 Bal is 0.2 nM in PBMC, and 0.1 nM against HIV-1 BaL in monocytes/macrophages (data not shown). Remarkably, the IC_{50} for GRFT in the washed PBMC assay (Fig. 2), when the test agent is applied 24 hours prior to cell washing and infection, is 0.78 nM, showing quite exceptional antiviral activity of GRFT. In the same assay we show that activity of CCR5 antagonist maraviroc persists for 24 hrs, although a high concentrations, but GRFT’s activity persists substantially longer, a property that may facilitate non-coital linked administration of microbicides containing GRFT as an active ingredient.

The cardinal rule in development of an anti-HIV-1 microbicide must be “first, do no harm”. Randomized controlled preclinical and clinical studies with detergent-based microbicides such as nonoxynol-9 showed a trend towards evidence of harm, with increased incidence of not only HIV-1 infection, but also HSV-2 and HPV seen in the experimental arms [33,34,35,36,37]. The microbicidal field therefore requires stringent and extensive in vitro and in vivo safety studies before human trials initiate. Initial studies showed that GRFT was not cytotoxic; had no mitogenic activity; did not induce secretion of chemokine and cytokine-mediators of inflammation in treated cervical explants; and showed a good safety profile in the rabbit vaginal irritation test [23,24,25].
Griffithsin Safety and Efficacy Profile

Materials and Methods

Lectin reagents

Recombinant Griffithsin (GRFT) was produced in Nicotiana benthamiana plants. Recombinant CV-N was produced in Escherichia coli. Methods for expression and purification of both products have been described previously [23,42]. A synthetic cDNA encoding a lectin activity-deficient mutant of GRFT, termed GRFTLec−, was designed with a conservative amino acid substitution of aspartic acid to asparagine in each of the 3 carbohydrate binding pockets identified in the primary amino acid sequence and crystal structures of GRFT [24,26]. GRFTLec− was expressed in N. benthamiana and purified exactly as described for GRFT [23]. Proteins were purified to >99% purity, and formulated in phosphate buffered saline (PBS), pH 7.4 at 10 mg/ml protein concentration. Endotoxin was removed from GRFT, GRFTLec−, and CV-N protein samples using Detoxi-Gel endotoxin-removing gel gravity flow columns (Thermo Scientific). Endotoxin levels were measured using the ToxinSensor™ chromogenic LAL endotoxin assay kit from GenScript (Piscataway, NJ). Only products with final Endotoxin readings less than 0.05 Endotoxin Units (EU) per milligram were used in the in vitro studies, and all dilutions were performed in endotoxin-free buffers. GRFT, GRFTLec−, and CV-N were fluorescently-labeled with amine-reactive Alexa Fluor 488 carboxylic acid, succinimidyl ester using a kit from Molecular Probes/Invitrogen, according to the manufacturer’s specifications. Control lectins Concanavalin A (ConA), phytohemagglutinin A (PHA) and Pokeweed agglutinin (PKM) were purchased from Sigma.

Lectin activity measurements using HIV-1 gp120-binding ELISA

Immobilized HIV-1 gp120 (Protein Sciences Corporation) was used to measure the lectin activity of purified GRFT, CV-N and fluorescently labeled conjugates thereof, and to confirm that GRFTLec− lacked gp120 binding activity. Nunc Maxisorp ELLSA plates were coated overnight with 1 µg/ml gp120 (strain H1B, Protein Sciences) diluted in PBS. The wells were blocked with 5% (w/v) non-fat dry milk in PBS+0.05% Tween (PBS-T; Immunowash, Bio-Rad) and washed before addition of serial dilutions of lectin analyte (GRFT, GRFTLec−, CV-N or Alexa-Fluor 488-labeled conjugates thereof) diluted in 1× PBS for 1 h. After three washes with PBS-T, a primary polyclonal antiseraum (rabbit anti-GRFT or CV-N or Alexa-Fluor 488 (Invitrogen), as appropriate) diluted 1:10,000 in PBS was added for 1 h at room temperature. The wells were again washed before goat anti-rabbit IgG-HRP (Southern Biotech) was added at a 1:10,000 dilution. Colorimetric values reflecting HRP activity were derived using KPL SureBlue TMB Microwell Peroxidase Substrate, with the reaction stopped by addition of 1 N H2SO4. The plates were read at 450 nm on a BioTek Synergy HT reader with data collected using Gemini Software. We confirmed that the labeled products retained lectin activities comparable to the unlabeled product by gp120-binding ELISA. We used ELISA with anti-AlexaFluor 488 detection to measure the total amount of label conjugated to GRFT, GRFTLec− and CV-N, which displayed quantitatively similar labeling efficiency.

Cervico-vaginal cells lines and human PBMC (PBMC)

End1/E6E7, Ect1/E6E7 and VK2/E6E7 are well characterized immortalized cell lines derived from normal human endocervical, ectocervical and vaginal epithelia, respectively [30]. All 3 cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cervico-vaginal cell lines were grown as previously described [30] in keratinocyte serum-free medium (KSF) supplemented with recombinant human epidermal growth factor (0.1 ng/ml), bovine pituitary extract (50 µg/ml), calcium chloride (0.4 mM) and an antibiotic cocktail composed of penicillin and streptomycin at final concentrations of 100 U/ml and 100 µg/ml, respectively. Reagents were obtained from Invitrogen (SanDiego CA, or from Sigma Chemical Company). Cryopreserved human PBMC used in assays of inflammatory cytokines and chemokines were purchased from SeraCare Life Sciences Inc. (Milford, MA) and were immediately cultured for the experiments in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and the penicillin-streptomycin antibiotic cocktail (to 100 U/ml and 100 µg/ml final concentrations, respectively).

Microscopic analysis of lectin interaction with human cervical tissues and cultured cervicovaginal cells

Slides with paraffin embedded human cervical tissue sections from a healthy 21 year old female (US Biomax, Inc.) were deparaffinized and rehydrated. Alexa-Fluor 488-labeled proteins of interest were added to the slides and incubated overnight at 4°C in a humidity chamber. The slides were rinsed with PBS twice for 10 minutes and coverslipped using VectaShield Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). For light microscopy, tissue sections were stained with hematoxylin and eosin by standard methods. Ect1/E6E7, End1/E6E7 and VK2/E6E7 cultured cells were seeded onto eight-well Lab-Tek chamber slides (Nalgene Nunc) in duplicate at 10,000 cells per well and allowed to incubate at 37°C with 5% CO₂. After eight hours the fluorescently labeled proteins of interest were added to the wells and incubated overnight at 37°C with 5% CO₂. The slides were washed twice with PBS and coverslipped using VectaShield Mounting Media with DAPI (Vector Laboratories, Burlingame, CA). Slides were visualized using the Axio Observer Z1 microscope with ApoTome assembly (Carl Zeiss, Thornwood, NY).

Analysis of lectin interaction with PBMC surface molecules by flow cytometry

Human PBMC (SeraCare Life Sciences, MD) were thawed and seeded onto 48-well culture plates (CellTreat, MA) at 2.5×10⁶ cells per well. Three dilutions of each Alexa-Fluor 488-labeled protein were used to determine the expression of selected genes validated the microarray data presented here. It is unclear what is the biological relevance and significance of this level of regulation. Using the same cell line (Ect1/E6E7) Sharkey et al. classified a gene as differentially expressed when the fold change was more than 2.0 and found a total of 444 probe sets that fell in this category after treating the cells 12 h with 10% human seminal plasma [41]. This is in sharp contrast to treatment with 4 µM GRFT, a level at least 1,000 fold greater than the average antiviral EC₅₀, which regulated expression of only 32 genes.

In summary, our data provide further evidence that GRFT, an exceptionally potent antiviral lectin, has very minor effects on the molecular physiology of human cells. At this point, the molecular basis for the distinct biological activities of different antiviral lectins is uncharacterized, we propose that the specific spatial arrangement of the carbohydrate binding sites may determine the nature and extent of cross-linking of cell surface glycoproteins. GRFT clearly has superior binding and cross-linking activity with the HIV-1 envelope glycoprotein, which displays dense clusters of oligomannose type NGL, but does not induce off-target cellular signaling to the extent that other lectins do. We believe this provides further data in strong support of focused clinical development of HIV-1 microbicides containing GRFT as an active ingredient.
added to the cells for overnight incubation at 37°C with 5% CO₂. Samples were also prepared with 5 and 10 mg of mannan (Sigma, St. Louis MO) at each protein dilution. All samples were analyzed in duplicate. Following incubation, cells were briefly trypsinized (TrypLE Express, Gibco) and placed in 5 mL polystyrene tubes (BD Falcon, MA). Cells were washed twice with PBS and analyzed using the BD FACSAria (BD Biosciences, NJ) flow cytometer.

**Antiviral activity assay in PBMC**

Freshly isolated PBMC were cultured in the presence of GRFT, CV-N and maravirac for 24 hrs. Then the cells were collected, washed in culture medium, suspended in RPMI medium with 2 ng/ml IL-2 and seeded in a 48-well flat bottom plate (5×10⁵ cells in 450 μl) and 30 μl of the CCR5-tropic clade B HIV-1 BaL stock was added at 100 TCID₅₀. The supernatant of each sample was collected after 9 days and viral replication measured by a specific p24 Ag ELISA (Perkin Elmer, Zaventem, Belgium).

**Cell viability assays**

Viability of cervico-vaginal cell lines was measured using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay kit from BIOITUM Inc. (Hayward, CA) following the manufacturer’s instructions. Briefly, 10⁴ cells per well were seeded (96 well plates), the test reagents added and the cultures incubated for three days in a humid environment with 5% CO₂ at 37°C. Afterwards, 10 μl of MTT solution were added to each well followed by 4 hours incubation at 37°C. Then the medium was gently removed and the insoluble purple formazan product dissolved in DMSO to yield a colored solution which absorbance was read at 570 nm with a background at 630 nm.

**Mitogenicity assays**

Proliferation of cervicovaginal cell lines was measured by bromodeoxyuridine (BrdU, a thymidine analog) incorporation in newly synthesized DNA using a Cell Proliferation ELISA kit from Roche, according to the manufacturer’s instructions. For human PBMC, cells were treated with GRFT for three days and analyzed by flow cytometry for any changes in size and/or morphology using forward scatter (FSC) and side scatter (SSC) with a FACSCalibur (BD, San Jose, CA) counting 10,000 events per sample. Data were acquired and analyzed using CellQuest Pro from BD. ConA (0.37 μM), PHA (10 μg/ml) and PBS were used as controls.

**Evaluation of cellular activation markers**

Three day old PBMC were analyzed flow-cytometrically after dual fluorescent staining with anti-mouse antibodies purchased from BD Pharmingen (San Diego, CA). Briefly, cell cultures were transferred from plates to a 5 ml round bottom tubes and washed with PBS containing 5% inactivated FBS (washing solution). After 10 min blocking with purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block), cells were incubated in dark with FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25, anti-CD69 or anti HLA-DR mAb for 30 min on ice. Finally PBMC were washed and analyzed with a FACSCalibur (BD, San Jose, CA), counting 10 000 events per sample. Data were acquired and analyzed using CellQuest Pro from BD. ConA (0.37 μM) and PHA (10 μg/ml), and PBS were used as positive and negative controls, respectively.

**Immunoaassays for cytokine detection in cultured cervicovaginal cell supernatants**

Multiplex immunoassays were carried out on a Bio-Plex instrument (Biorad) using a Milliplex Human Cytokine/Chemo-
gene expression analysis methods above.

**Supporting Information**

**Figure S1** Analysis of binding specificity of GRFT in comparison with GRFTlec- and CV-N. In the first column we depict the identical fluorescence micrographs to Fig. 1, these show binding of AlexaFluor 488-labeled GRFT (B), GRFTlec- (C) and CV-N (D) to paraffin-embedded cervical tissue sections from a 21-year old female. In (A) we show a hematoxylin and eosin-stained light micrograph of cervical epithelial tissue, showing the general microanatomy of the cervical epithelium. We have provided additional fluorescence micrographs of tissues stained with labeled GRFT (B2 and B3), GRFTlec- (C2 and C3) and CV-N (D2 and D3). (TIF)

**Figure S2** Quantitation of the effect of test compounds on the expression of activation markers in CD4+ positive PBMC. Percentages of double positive PBMC are reported for cells after 3 days treatment with PBS, 1 μM GRFT, 4 μM GRFT, 10 μg/ml PHA and 0.37 μM ConA and dual staining using FITC-conjugated anti-CD4 mAb in combination with PE-conjugated anti-CD25 (A), anti-CD69 (B) and anti-HLA-DR (C). (TIF)

**Table S1** Genes used in Q-PCR experiments with corresponding TaqMan assay identities. (DOC)

**Table S2** List and fold changes (FC) of mapped genes differentially expressed after treatment with 4 μM GRFT. (DOC)

**Table S3** Relative expression of selected genes after treatment with GRFT [1 and 4 μM] and 1 μM ConA as assessed by Q-PCR and microarrays (μArrays). (DOCX)

**Author Contributions**

Conceived and designed the experiments: JCK DH DS AJ SKR NM BRO KEP. Performed the experiments: JCK DH DS AJ SKR NM BRO KEP. Analyzed the data: JCK DH DS AJ SKR NM BRO KEP. Contributed reagents/materials/analysis tools: KEP. Wrote the paper: KEP JCK.

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