Published in final edited form as:

Virus Res. 2007 February ; 123(2): 178–189.

The Tyrosine Kinase Inhibitor Genistein Blocks HIV-1 Infection in Primary Human Macrophages

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Abstract

Binding of HIV-1 envelope glycoprotein (Env) to its cellular receptors elicits a variety of signaling events, including the activation of select tyrosine kinases. To evaluate the potential role of such signaling, we examined the effects of the tyrosine kinase inhibitor, genistein, on HIV-1 entry and infection of human macrophages using a variety of assays. Without altering cell viability, cell surface expression of CD4 and CCR5 or their abilities to interact with Env, genistein inhibited infection of macrophages by reporter gene-encoding, β -lactamase containing, or wild type virions, as well as Env-mediated cell-fusion. The observation that genistein blocked virus infection if applied before, during or immediately after the infection period, but not 24h later; coupled with a more pronounced inhibition of infection in the reporter gene assays as compared to both β -lactamase and p24 particle entry assays, imply that genistein exerts its inhibitory effects on both entry and early post-entry steps. These findings suggest that other exploitable targets, or steps, of the HIV-1 infection process may exist and could serve as additional opportunities for the development of new therapeutics.

Keywords

HIV; macrophage; tyrosine kinase; genistein; entry; fusion; receptor; CD4; CCR5; CXCR4; envelope glycoprotein; gp120; infection

INTRODUCTION

HIV-1 envelope glycoprotein (Env) interacts with CD4 and subsequently with a member of the chemokine receptor family, predominantly CCR5 and/or CXCR4, to gain access to the intracellular environment (reviewed in (Berger et al., 1999)). It is now well established that during the interactions with its receptors, the HIV-1 Env gp120 elicits various intracellular signaling events both in primary cells and cell lines (reviewed in (Popik and Pitha, 2000; Stantchev and Broder, 2001)), which are similar, but not identical to that caused by chemokines (Freedman et al., 2003; Liu et al., 2000).

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Often given as an example of G-protein coupled, 7 transmembrane domain receptors (GPCR), that associates exclusively with pertussis toxin (PT) sensitive $G_{\alpha I}$ proteins, the chemokine receptors may also couple with other subclasses of G_{α} molecules, such as $G_{\alpha q}$ or $G_{\alpha s}$. The mode of these interactions is complex and may depend on the chemokine receptor molecule itself, the cell type or even the activation state of the cell. In addition to modulation of intracellular cAMP levels, chemokine receptors are also able to activate various non-receptor tyrosine kinases in both PT-sensitive and/or insensitive modes (reviewed in (Rodriguez-Frade et al., 2005; Stantchev and Broder, 2001)). The signaling pattern of CD4 is less complex and has been primarily associated with activation of the CD4 cytoplasmic tail-associated Src related tyrosine kinase, p56^{lck}. Interestingly however, a recent study has shown that CD4 is able to induce Ca^{2+} influx, tyrosine phosphorylation and activation of phospholipase C gamma (PLC- γ), phosphatidylinositol 3-kinase (PI-3K) and up-regulation of the stress activated protein kinases (SAPK) branch of the mitogen activated protein kinases (MAPKs) pathway in promonocytic cell lines, which like primary monocytes/macrophages do not express p56^{lck} (Graziani-Bowering et al., 2002).

In macrophages gp120 binding caused an elevation of intracellular Ca^{2+} and interestingly the response to HIV-1 Envs from R5 strains, which efficiently replicate in these cells, was markedly higher and more sustained in comparison to Envs derived from X4 strains (Arthos et al., 2000; Freedman et al., 2003). R5 Envs were also more potent than X4 ones in the activation of an array of genes in resting CD4⁺ lymphocytes, that would subsequently favor the productive infection of these cells (Cicala et al., 2006). Furthermore, the gp120 induced Ca^{2+} elevation and activation of proline rich tyrosine kinase Pyk2 in macrophages occurred in a PT-independent manner (Freedman et al., 2003). Recently, CCR5 dependent activation of the Src kinase Lyn, in response to gp120 from the R5 strain JR-FL, has been recently described in macrophages as well (Tomkowicz et al., 2006). In addition, some of these processes such as the up-regulation of PI-3K, c-Jun and certain MAPKs in primary macrophages, following gp120 stimulation, have been described as events that are likely downstream effects of Ca^{2+} and/or tyrosine kinase signaling (Del Corno et al., 2001; Freedman et al., 2003; Lee et al., 2003; Tomkowicz et al., 2006). At present, the gp120 elicited signaling is usually associated with augmentation of HIV-1 replication in already infected cells (Cicala et al., 2002; Kinter et al., 2003) or the production of several pro-inflammatory cytokines, which in auto or paracrine fashion may alter the activation status of the cell, and hence HIV-1 infection (reviewed in (Freedman et al., 2003)). However, any direct role of gp120 induced signaling in the establishment of infection, such as with CCR5 dependent strains in macrophages, has yet to be shown.

Because of the multiple domains of GPCRs involved in their G protein dependent and independent signaling processes (Hall et al., 1999; Pierce et al., 2002), the generation of chemokine receptors completely devoid of any tyrosine kinase signaling properties, while at the same time maintaining normal cell surface expression-levels and gp120 binding ability have been difficult if not an impossible task. Also, gp120 has the potential to induce tyrosine kinase activation via both CD4 and CCR5 or CXCR4, suggesting the possibility that an impaired signaling through one of the receptors may be compensated for by the other. Finally, during progeny virion budding, HIV-1 itself may incorporate into its membrane a variety of different molecules including proteins which may subsequently interact with their counterparts on the host cell membrane (reviewed in (Tremblay et al., 1998)), resulting in tyrosine kinase activation and facilitation of virus fusion (Liao et al., 2000; Tardif and Tremblay, 2003). Here, we sought to explore the significance of tyrosine kinase signaling in the establishment of HIV-1 infection in a primary cell target by examining the effects of the broad spectrum tyrosine kinase inhibitor genistein using a battery of virus entry and infection assays.

MATERIALS AND METHODS

Reagents

The tyrosine kinase inhibitor, genistein, was obtained from Biomol (PA) and initially resuspended in hybridoma grade, endotoxin negative DMSO (Sigma-Aldrich(MO)) to initial concentration of 20 mg/ml. Potential endotoxin contamination of the genistein was excluded by the Kinetic Turbidimetric Method (test performed by Associates of Cape Cod, Inc., Falmouth, MA). The pNL4-3 HIV-1 backbone plasmids encoding the luciferase (Luc) or green fluorescence protein (GFP) reporter gene and pSV7d-Ba-Lgp160 were provided by Dr. R. Doms (University of Pennsylvania). The plasmids pSV7d-JR-FLgp160, pcDNA3.1(Zeo)-Ba-Lgp160, pDK38 (pLNCX2-CD4-Stag), pCG-VSV-G and pMM310 (pcDNA3.1(Zeo)-Vpr-BlaM) were supplied by Dr. G. Quinnan (Uniformed Services University), Dr. T.R.Fouts (University of Maryland), Dr. D. Khetawat (Uniformed Services University), Dr. P. Cannon (Childrens Hospital, LA) and Dr. M. Miller (Merck & Co., Inc., West Point, PA), respectively. The wild-type CD4-encoding recombinant vaccinia virus vCB3 was previously described (Broder et al., 1993). Recombinant human JR-FL gp120 (rgp120) was produced in BS-C-1 cells by recombinant vaccinia virus and purifying the protein by affinity chromatography using lentil lectin Sepharose 4B (Amersham Pharmacia Biotech, NJ) as previously described (Earl et al., 1994). pYK-JRCSF (Cann et al., 1990; Koyanagi et al., 1987), HIV-1_{Ba-L} (Gartner et al., 1986) and HIV-1_{92/UG/024} (the UNAIDS Network for HIV Isolation and Characterization and DAIDS, NIAID) were received through the AIDS Research and Reference Reagent Program, NIAID, NIH. The pAdVantage vector and the Luciferase Assay System were purchased from Promega Corp. (Madison, WI). The CCF2/AM Beta lactamase Loading Kit (GeneBLazer Reporter Assay) and Calcein AM were ordered from Invitrogen Corp. (CA). Eugene 6 and Complete Mini protease inhibitor tablets were obtained from Roche Diagnostics. HIV-1 p24 and TNF α ELISA Kits were purchased from Beckman Coulter GmbH (Germany).

Cells and culture conditions

The 293T cells were obtained from Dr. G Quinnan (USUHS) and maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD), 10% bovine calf serum (BCS), 2 mM L-glutamine, and antibiotics (DMEM-10) at 37°C in a humidified 5% CO₂ atmosphere. Peripheral blood mononuclear cells (PBMC) were isolated from human blood following leukapheresis of HIV-1 seronegative donors and subsequent density gradient centrifugation; monocytes were purified by countercurrent centrifugal cell elutriation as previously described (Gerrard et al., 1983). Macrophages were prepared from elutriated monocytes by differentiation in 100 mm square Petri dishes (Bibbi Sterilin Ltd., Stone Staffs, UK) in DMEM supplemented with 10% human AB serum from several different sources, 2 mM L-glutamine and antibiotics (Broder et al., 1994; Lazdins et al., 1990). Macrophages were obtained after 7 to 14 days differentiation without exogenous growth factors and were either used immediately after the differentiation period or kept frozen in liquid nitrogen. The day before the experiment, the frozen cells were thawed, washed, centrifuged, resuspended in DM-10, put in the desired plate format and incubated at 37°C overnight.

Cell-fusion assay

The effect of genistein on HIV-1 Env induced fusion was assessed by the fluorescent dye Calcein AM transfer from labeled, HIV-1_{Ba-L} Env expressing 293T cells to unlabeled primary macrophages. HIV-1_{Ba-L} Env was expressed on 293T cells by transfection with pcDNA3.1 (Zeo)-BaLgp160 and pAdVantage vectors. After transfection, the cells were further cultured for an additional 24h to allow protein expression and subsequently loaded with 0.02 μ M Calcein AM (in 100% DMSO) for 40 min at 37°C. The excess dye was removed by two washes in phosphate buffered saline (PBS) and the labeled cells mixed with the primary macrophages at

a 3:1 ratio. The extent of fusion was quantified as the ratio of bound, dye-redistributed 293T cells to the total number of bound 293T.

HIV-1 infection studies

For the reporter gene HIV-1 Env pseudotyping system (Connor et al., 1995), viral stocks were prepared as previously described by transfecting 293T cells with plasmids encoding the luciferase virus backbone pNL4-3-Luc-E_R⁺ or pNL4-3-GFP and pSV7d-JR-FLgp160 or pSV7d-Ba-L. After transfection the cells were washed extensively with DMEM and further incubated for 24-48 h. The resulting supernatants were clarified by centrifugation for 10 min at 1500 rpm, filtered through low protein binding 45 µM syringe filter (Millipore, Bedford, MA) and used immediately or kept at 4°C for up to 48 h. Macrophages were prepared in 96 (75x10³ cells/well) or 48 well (150x10³ cells/well) plates and infected with 25 or 50 µl virus suspension per well, respectively. The experiments were performed in triplicate wells for each genistein concentration. No DEAE-Dextran or polybrene were used to facilitate fusion. Genistein was applied as indicated in the figure legends. After the 2.5-3 h period of infection, the cells were briefly treated with trypsin (0.25% EDTA/0.02% trypsin) to remove cell surface bound virus particles, washed and incubated for 48 h before lysis with 0.5% Triton-100 in PBS (Luc reporter gene studies). A 50 µl aliquot of the resulting lysate was assayed for luciferase activity using luciferase substrate (Promega, Madison, WI). The efficiency of macrophage infection by GFP reporter gene encoding virus particles in the presence of different concentrations of genistein was evaluated by counting the number of green cells 48h post-infection using Olympus IX81 fluorescent microscope.

HIV-1 entry measured by cytoplasmic p24 antigen was performed in general as described by V Marechal, et al. (Marechal et al., 1998). Briefly, macrophages (5 x 10⁶/sample) were infected for 1.5 h with JR-FL Env pseudotyped virus particles, treated with trypsin, washed and detached by 5 mM EDTA (30 min at 4°C) and gentle scraping. After resuspension in 100 mM sodium phosphate buffer plus protease inhibitors (Complete Mini, Roche Diagnostics), cell were disrupted by dounce homogenation using a Teflon micro-douncing tool. The resulting lysates were centrifuged for 5 min (3000 rpm at 4°C, Sorvall RT7) to pellet nuclei and cell debris and further subjected to ultracentrifugation (10 min, 60 000 rpm at 4°C, Beckman TL100 centrifuge) to remove the intracellular membrane vesicles. The resulting cytosolic fractions were adjusted to 0.5% Triton-100 and the p24 concentrations were determined by ELISA (p24 Antigen Immunoassay Kit, Beckman Coulter) following manufacturer's instructions.

For the BlaM assay, the cells were plated in sterile Lab-Tek II 2 well chamber slides (Nalge Nunc, IL) at 300x10³ cells/well. β-lactamase (BlaM) containing virus particles were produced by cotransfection of 293T cells with the cloned HIV-1 proviral DNA pYK-JRCSF and pMM310. The pMM310 construct encodes BlaM fused to the amino-terminus of the viral protein Vpr, which directs BlaM into the forming virions (Tobiume et al., 2003; Wyma et al., 2004). After infection for 2 h, macrophages were briefly treated with trypsin, washed and loaded with the fluorescent dye CCF2/AM (2 µM final concentration) for 1 h, washed to remove the extracellular dye and incubated in phenol red free DM-10 in the presence of the nonspecific anion transport inhibitor probenecid (2.5 mM) for 12-14 h before fixing with 1.5% paraformaldehyde (Cavrois et al., 2002). The chambers were removed and the slides were prepared as for fluorescent microscopy by mounting the cells in Fluoromount-G (SouthernBiotech, AL). The extent of CCF2/AM cleavage by the virus-introduced intracellular BlaM, detected by the change in the dye emission from the green to the blue spectrum, was evaluated by measuring the fluorescence using Laser Scanning Cytometer (CompuCyte, Cambridge, MA) generating light at 407 nm and equipped with HQ460/10 and HQ530/20 filters for detection of the blue and green emission, respectively. Individual negative controls (virus containing no BlaM added) were prepared for both DMSO and genistein treated

macrophages to define more precisely the region of BlaM positive cells in the Laser Scanning Cytometer created histograms.

For wt HIV-1 infection studies, macrophages were plated in 24 well plates (750×10^3 cells/well), pre-incubated with genistein, and infected in the presence of the reagent with HIV-1_{Ba-L} or HIV-1_{92/UG/024} for 3 h (~ 2000 virus particles/well), prior to extensive washing. Every third day, two-thirds of the medium from each well was collected for analysis and replaced with fresh DM-10. The p24 concentration in the cell culture supernatants was determined by ELISA (p24 Antigen Immunoassay Kit, Beckman Coulter).

Flow Cytometry

For detection of CD4 and CCR5, the two well characterized monoclonal antibodies Leu 3a (Beckton-Dickinson, Franklin Lakes, NJ) and 2D7 (Pharmingen, San Diego, CA) were used ($2.5 \mu\text{g}$ for 10^6 cells), respectively. Cells were detached by incubation in 5 mM EDTA (in PBS) for 30 min at 4°C and scraping. The incubation time for the primary and subsequently for the secondary (goat anti-mouse FITC conjugated IgG F_{(ab)2}, Chemicon, CA) antibodies was 1 hour (on ice). At the end of the procedure, cells were fixed with 1.5 % paraformaldehyde and the readings were made using Coulter EPICS Elite ESP flow cytometer.

The possibility that genistein may induce apoptosis in primary macrophages during our experiments was excluded by using Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen). Since scraping resulted in extensive Annexin V-FITC staining of both DMSO and genistein treated macrophages, for this experiment the cells were cultured in Teflon coated screw cap containers (Tuf-Tainer, Pierce, IL) to prevent firm adherence and were detached only by gentle pipetting. Macrophages were stained with Annexin V-FITC and Propidium Iodide (PI) without fixation and analyzed immediately by flow cytometry.

Western Blot Analysis and CD4-gp120 complex formation

VSV-G Env pseudotyped, CD4-Stag encoding virus particles were produced by co-transfection of 293T cells with pDK38 (pLNCX2-CD4-Stag) and pCG-VSV-G. After overnight transduction, macrophages (5×10^6 /sample) were cultured for an additional 24 h to allow CD4-Stag expression. Subsequently, cells were pre-incubated with genistein ($10 \mu\text{g}/\text{ml}$) or DMSO (at 37°C), treated with JR-FL rgp120 ($1 \mu\text{g}$ per 1×10^6 cells, 2h at 4°C) in the presence of the reagents and lysed (1% Brij97, 150 mM NaCl, 20 mM Tris pH 8, 20 mM EDTA and protease inhibitors). After cell lysis and removal of the nuclear fraction ($17000 \times \text{g}/20 \text{ min}$, Sorvall Biofuge), the CD4_{Stag}/gp120 complexes were precipitated by S-beads and their amount was quantified by SDS-PAGE electrophoresis followed by Western blot analysis using anti-gp120 polyclonal rabbit serum.

For formation and detection of CD4-CCR5-gp120 complexes, the level of CD4 cell-surface expression was boosted in primary macrophages by infecting them with the relevant recombinant vaccinia virus vCB3. Preincubation of macrophages with DMSO or genistein, treatment with JR-FL rgp120 and cell lysis were performed as described above. After removal of the nuclei and cell debris by centrifugation ($17000 \times \text{g}/20 \text{ min}$), gp120-CD4-CCR5 complexes were precipitated by the anti-CCR5 MAb 5C7 (provided by Dr. L. Wu (Wu et al., 1997)) and further quantitatively analyzed by Western blot using anti-gp120 polyclonal rabbit serum.

TNF secretion in response to gp120

Macrophages in 48 well plates (150×10^3 cells/well) were pre-incubated with different concentrations of genistein for 2.5 h and subsequently challenged with HIV-1_{JR-FL} recombinant gp120 ($0.25 \mu\text{g}/\text{ml}$) for 5 h in the presence of the reagent. The TNF- α concentration

in the different cell culture supernatants was determined by ELISA (Beckman Coulter GmbH, Germany) following manufacturer's instructions.

RESULTS

Genistein inhibits R5 Env pseudotyped virus infection in primary macrophages

To examine the effects of genistein on HIV-1 entry, primary human macrophages were chosen as a relevant HIV-1 target cell because they are non-dividing and terminally-differentiated, since tyrosine kinase inhibitors are known to suppress mitosis (Gozlan et al., 1998). As a first step to evaluate the significance of tyrosine signaling in HIV-1 infection, we examined the effect of genistein using luciferase and green fluorescent protein (GFP) reporter gene encoding, HIV-1_{JR-FL} or HIV-1_{Ba-L} Env pseudotyped virus particles. We speculated that if present, an inhibitory effect by genistein would be more easily detected using these single cycle pseudotyped reporter virus assays than in a spreading *in vitro* infection experiment. After 3-3.5 h of pre-incubation, primary macrophages were infected with virus in the presence of genistein, followed by a brief trypsin treatment and extensive washout of the reagent without replacement. After 48 h, the cells were lysed to measure luciferase activity or examined by fluorescent microscopy for GFP expression. Genistein could potentially block pseudotyped virus infection up to ~ 80-85% in a dose dependent, non-linear fashion when used at a concentration range of 1 to 10 µg/ml (Fig. 1). Although the ability of macrophages from different donors to support R5 Env pseudotyped virus infection varied (from ~ 1×10^4 to ~ 2×10^5 light units for the DMSO treated controls), the dose dependent inhibitory curves were almost identical (Fig. 1A) and similar inhibition profiles by genistein were seen when macrophages from the same donor were infected by virions pseudotyped with Envs from two different R5 strains (Fig. 1B). There was also no significant difference in the ability of genistein to suppress virus infection with GFP reporter gene encoding JR-FL Env pseudotyped virus particles when 1 versus 2 week differentiated macrophages from the same donor were examined (Fig. 1C). Reduced activity of the multidrug resistant protein 1 (MRP-1) by genistein may also contribute to the inhibition of HIV-1 infection. However, this effect is unlikely to influence the virus entry process, since MRP-1 has not been localized in cellular membrane fractions (Speck et al., 2002).

Genistein inhibits cell-fusion between macrophages and HIV-1_{Ba-L} Env expressing cells

Fusion between HIV-1 Env expressing and CD4/coreceptor positive cells is a well known phenomenon allowing direct cell to cell spread of the infection (Phillips, 1994). The recombinant HIV-1 Env cell-fusion system was originally devised to study solely the HIV-1 Env-mediated membrane fusion step of virus infection, since the typical virus infection assay depends on both entry and post-entry events (Broder and Berger, 1995). To evaluate the effect of genistein on HIV-1 Env-mediated fusion, we measured the efficiency of Calcein AM fluorescent dye transfer between pre-labeled HIV-1_{Ba-L} Env expressing 293T cells and primary macrophages. The pre-incubation of macrophages with genistein, and its presence during the cell-fusion assay, strongly inhibited the transfer of fluorescent dye between the HIV-1 Env expressing cells and macrophage targets in a dose dependent manner (Fig. 2).

Genistein treatment does not alter cell viability

The ability of genistein to suppress proliferation and induce apoptosis in tumor cells or tumor derived cell lines is well established. At the same time primary cells, particularly the non-dividing ones, were considerably more resistant to the pro-apoptotic influence of genistein (Kyle et al., 1997; Linford et al., 2001). Consistent with these latter studies, use of genistein at the concentrations and timeframe indicated for our experiments did not cause changes in trypan blue exclusion by macrophages before the efficiency of viral infection was evaluated. However, since the trypan blue test does not detect early stages of apoptosis, we also performed double staining of macrophages with Annexin V-FITC and Propidium Iodide (PI) (Vermes et

al., 1995) and observed no significant difference between genistein and DMSO treated cells (Table 1).

Genistein treatment does not alter CD4 or CCR5 surface expression and has no effect on gp120-CD4-CCR5 trimolecular complex formation

To discern the mechanism of the genistein mediated inhibitory effect on HIV-1 infection of primary macrophage we explored its effects on CD4/CCR5 receptor expression and the formation of gp120-CD4-CCR5 trimolecular complexes which appear to be an important prerequisite for the productive Env-mediated membrane fusion (Dimitrov et al., 1999; Xiao et al., 2000). Using immunostaining and Flow Cytometric analysis, we determined that pre-incubation of primary macrophages with 10 µg/ml genistein for up to 8 h did not alter cell surface expression levels of CD4 or CCR5 (Fig. 3A). We next examined whether genistein interfered with gp120 binding to CD4 and the subsequent assembly of individual gp120/CD4/CCR5 trimolecular complexes. In these experiments, to optimize complex detection, we elevated the low level cell surface expressed CD4 on macrophages by infecting with either VSV-G pseudotyped, CD4-Stag encoding virus particles or wild-type CD4-encoding recombinant vaccinia virus. In the pLNCX2 retroviral vector, the S-tag sequence was cloned immediately before the stop codon at the COOH terminus of CD4 molecule. After preincubation with genistein at 37°C, the cells were treated with recombinant gp120 at 4°C in the presence of the reagent and lysed. CD4-gp120 binding was evaluated by adding S-beads to the cell lysate from CD4-S tag expressing macrophages (Fig. 3B). gp120/CD4/coreceptor trimolecular complexes, formed on recombinant vaccinia virus infected cells, were immunoprecipitated by the anti-CCR5 MAb 5C7 and G beads (Xiao et al., 1999). In both cases, the precipitated complexes were quantified by SDS-PAGE followed by Western blot analysis using polyclonal rabbit anti-gp120 serum and no differences were observed between control (DMSO) and genistein treated samples (Fig. 3B and 3C).

Time dependence of genistein inhibition

The establishment of HIV-1 infection is a complex process involving entry, uncoating, reverse transcription, preintegration complex formation, nuclear translocation/integration and replication. Therefore, we next sought to determine the stage(s) at which genistein exerted its inhibitory effect by applying the reagent at different time points. (The effect of genistein on HIV-1 infection was examined as a function of time) (Fig. 4). The reduction in reporter gene expression was strongest when genistein was present before and during infection. The suppression was weaker, especially at lower concentrations, when macrophages were only pre-incubated with genistein or if the reagent was added immediately after the infection period. No significant inhibition was observed when genistein was applied 24 h post-infection. These results suggest that genistein's inhibitory effect on HIV-1 infection was at the level of entry and possibly early post-entry stages that at least preceded nuclear integration.

Genistein inhibits HIV-1 particle fusion with primary macrophages

The inhibitory effect of genistein on HIV-1 Env-induced cell-fusion implied an inhibition of the virus entry process. However, there are concerns that cell-fusion assays may not reflect all the variables governing the fusion of actual virions with their target cells, due to the significant differences in the composition of virus and plasma membranes (Cavrois et al., 2002). Therefore, we employed the cytoplasmic p24 (Marechal et al., 1998) (Fig. 5A) and the recently developed β-lactamase (BlaM) (Cavrois et al., 2002; Tobiume et al., 2003) (Fig. 5C) assays to examine to what extent and stage of inhibition by genistein could be attributed to reduced virus particle entry. In both assays, we observed that genistein caused consistent and significant inhibition (~ 45%) of virus penetration into target cells. The infection efficiency of primary macrophages by the BlaM containing virions in our experiment (~ 4.25% for DMSO samples)

was comparable to previous reported results (5.2%) concerning infection of primary lymphocytes by NL4-3 BlaM virus particles (Cavrois et al., 2002). The stronger inhibitory effect of genistein at 10 $\mu\text{g/ml}$ seen in the reporter gene virus infection assay (**Fig. 5B**), compared to these types of entry assays, support the concept that genistein can also suppress certain post-fusion steps in the virus life cycle.

Genistein suppresses gp120-induced TNF- α secretion by primary macrophages

It was previously demonstrated that macrophages respond to gp120 exposure by secretion of several pro-inflammatory cytokines, including TNF- α . Its production was found to be sensitive to PI-3K and MAPK inhibitors (Del Corno et al., 2001; Lee et al., 2005) and more recently to the inhibition of the Src kinase Lyn (Tomkowicz et al., 2006). TNF- α production by macrophages in response to several other stimuli was also shown to be dependent on Src or Src related tyrosine kinases (Morris et al., 1999; Orlicek et al., 1999). In light of these reports, we analyzed the effect of genistein on TNF- α secretion by gp120 challenged macrophages and observed a consistent, dose dependent inhibition (**Fig. 6**). These results further support the involvement and significance of tyrosine kinase signaling in the gp120-induced responses in primary macrophages upstream of PI-3K and MAPK. Our findings also expand upon previous observations that the gp120 induced secretion of other pro-inflammatory mediators by primary macrophages, such as certain β -chemokines, was sensitive to a different tyrosine kinase inhibitor, herbimycin A (Del Corno et al., 2001).

Infection of primary macrophages by HIV-1_{Ba-L} is suppressed by genistein

To confirm genistein's inhibitory effects on HIV-1 infection of primary macrophages, acute infection assays were carried out with a replication-competent HIV-1 (**Fig. 7**). In comparison to the HIV-1 Env pseudotyped virus infection assays, there was no effect on virus replication at a genistein concentration of 1 $\mu\text{g/ml}$ and the percent inhibition induced at 2.5 $\mu\text{g/ml}$ was also significantly lower. However, concentrations of genistein between 5 and 10 $\mu\text{g/ml}$ resulted in almost complete inhibition of virus replication and spread in the macrophage cultures (**Fig. 7**). In a parallel experiment, genistein was also capable of inhibiting the infection of primary macrophages by the X4 HIV-1 strain UG024 (Yi et al., 1999) and also had no effect on cell surface expression levels of CXCR4 (Feng et al., 1996) (data not shown), indicating that its effects were not coreceptor specific.

DISCUSSION

It was reported over a decade ago, before the discovery of the HIV-1 coreceptors, that the tyrosine kinase inhibitors herbimycin A (Cohen et al., 1992) or genistein (Yoshida et al., 1992) could inhibit HIV-1_{LAV} or HIV-1_{IIIB} Env-mediated syncytia formation. Later studies using mutated CD4 (Bedinger et al., 1988) or chemokine receptors (Amara et al., 2003; Atchison et al., 1996; Farzan et al., 1997; Gosling et al., 1997) have supported the view that signaling is dispensable for HIV-1 Env-mediated fusion. Nevertheless, it is now evident that signaling via both CD4 and, more importantly, the chemokine coreceptor molecules is far more complex than was appreciated just a few years ago (Laudanna and Alon, 2006; Rodriguez-Frade et al., 2005). In the light of these more recent findings it is now not certain whether the mutant receptors employed previously were completely devoid of signaling properties, nor has an examination on HIV-1 entry and infection in which both CD4 and the coreceptor molecules were altered in such a manner been conducted.

It has been reported that pertussis toxin (PT) did not interfere with the capacity of HIV-1 to infect cell lines (Alkhatib et al., 1997; Cocchi et al., 1996), however in primary lymphocytes and macrophages PT and especially its B oligomer were able to suppress HIV-1 infection at both entry and post-entry levels.(Alfano et al., 2005; Alfano et al., 1999; Alfano et al., 2001).

Since the B oligomer does not inhibit G_i, but is able to bind to certain cell surface glycoproteins and induce intracellular signaling, its effect on HIV-1 entry was attributed to heterologous desensitization of CCR5 (reviewed in (Wang and Oppenheim, 1999)). Two recent reports indicated that the Rho family GTPases Rac1 (Pontow et al., 2004) or RhoA (del Real et al., 2004) played a substantial role in HIV-1 Env-induced fusion. Indeed, certain steps in the activation pathways of both these molecules require tyrosine kinase activity that was shown to be sensitive to genistein (Murasawa et al., 2000; Sakurada et al., 2001). In agreement with the previously demonstrated inhibition of syncytia formation by tyrosine kinase inhibitors and the already established role of Rac1 and RhoA in HIV-1 infection, we also observed that genistein was able to suppress both virion entry in primary macrophages, as well as Env-mediated membrane fusion. Importantly, the genistein treatment period employed in our experiments, did not alter the plasma membrane expressed levels of CD4 and CCR5 and also did not alter the formation of individual CD4/CCR5/gp160 trimolecular complexes.

We speculate that the interference with the function of lipid rafts and actin cytoskeleton are the most likely mechanisms, responsible for the observed effects of genistein. It has been suggested that HIV-1 entry requires rearrangement and coalescence of lipid rafts (reviewed in (Manes et al., 2003)). Also, rafts do differentially associate a variety of signaling molecules, including tyrosine kinases (Simons and Toomre, 2000) and certain stimuli, including tyrosine kinase signaling, may be able to induce patching of lipid rafts and changes in their composition and size (Simons and Toomre, 2000; Viola et al., 1999).

A number of studies suggest that actin cytoskeleton rearrangements may be necessary for series of events required for productive HIV-1 infection: 1) remodeling of cellular microvilli (Popik and Alce, 2004; Viard et al., 2002) where CD4, CCR5 and CXCR4 are preferentially localized (Singer et al., 2001); 2) clustering of a sufficient number of CD4 and co-receptor molecules in a timely manner for fusion pore formation (Iyengar et al., 1998; Kuhmann et al., 2000; Stantchev and Broder, 2001; Viard et al., 2002); 3) assembly of the newly described “viral synapse”, which enhances HIV-1 spread and infection (Jolly et al., 2004); 4) passage of the viral nucleocapsid through the cortical actin (Komano et al., 2004; Pelkmans and Helenius, 2003) and 5) the formation and transport of the viral reverse transcription complex (Bukrinskaya et al., 1998; Stevenson, 1996). It has been shown that HIV-1 Env-mediated cell-fusion is inhibited by reagents which interfere with actin function (Pontow et al., 2004; Viard et al., 2002), and the sensitivity to this inhibition was inversely correlated to the levels of CD4 and coreceptors on the cell surface (Viard et al., 2002). Inhibition of CD4/CXCR4 co-capping and HIV-1 infection by cytochalasin D was observed in primary peripheral blood mononuclear cells (PBMC) (Iyengar et al., 1998), but not in HOS CD4/CXCR4 positive cell line (Campbell et al., 2004). The apparent inconsistency between these studies may reflect variations in CD4/CXCR4 expression and/or differences in actin cytoskeleton regulation between primary cells and cell lines. In agreement with the preponderance of evidence from the studies cited above, we have also found significant inhibition of R5 HIV-1 Env-mediated cell-fusion and virus entry in primary macrophages by reagents that interfere with the actin cytoskeleton ((Stantchev and Broder, 2001) and our unpublished results).

There is evidence from studies on the immunological synapse formation that tyrosine kinase signaling, lipid raft coalescence and actin cytoskeleton reorganization are closely interconnected events (Delon et al., 1998; Trautmann and Randriamampita, 2003). Further, the activity of many key actin regulatory proteins as WASP (Cory et al., 2002), cortactin (Daly, 2004), ezrin (Bretscher, 1999; Ivetic and Ridley, 2004), gelsolin, villin, Cap G and profilin (De Corte et al., 1997; Zhai et al., 2002; Zhai et al., 2001)), have been shown to be directly modulated by tyrosine phosphorylation. The Rho family GTPases Rac1 (Pontow et al., 2004) or RhoA (del Real et al., 2004), are also closely involved in actin function (Burridge and Wennerberg, 2004).

It has also been reported that in macrophages HIV-1 may be internalized and fuse with the membrane of macropinosomes (Marechal et al., 2001), but this is considered an inefficient pathway whereby most of the endocytosed virus is degraded (Pelkmans and Helenius, 2003; Schaeffer et al., 2004). Macrophages do readily support membrane fusion with HIV-1 R5 Env expressing cells (Broder and Berger, 1995; Dimitrov et al., 1999), suggesting that the macropinosome environment is not obligatory for virus infection and this pathway would also be inhibited by genistein, since macropinocytosis is tyrosine kinase dependent process (Racoosin and Swanson, 1992; Veithen et al., 1996).

Genistein, at concentrations comparable or higher than ones used in our study, has been also shown capable of suppressing the entry of several types of viruses that exploit different endocytic ways to gain access to the cytoplasm: SV40 (Pelkmans et al., 2002), adenoviruses (Li et al., 2000) and human herpesvirus 8 (HHV-8) (Sharma-Walia et al., 2004). Unfortunately, the general need of tyrosine kinases mediated signaling for entry/infection of a diverse group of viruses makes it impossible to reveal the specific requirements of HIV-1 by just comparing the effects of a broad spectrum inhibitor in different viral systems.

The considerably more potent inhibition by genistein that we measured in pseudotyped virus reporter gene expression experiments (>80%), as compared to the inhibition observed in the BlaM and cytoplasmic p24 assays, implies that tyrosine kinase signaling may also play a role in some early post-fusion stages of HIV-1 infection. Since virus entry is often defined as delivery of the virus nucleocapsid into the cytoplasm with the presumption that successful virus replication will follow (Young, 2001), it is a matter of debate as to whether the virus movement through the cortical actin network should be considered a part of its entry process. This is an important consideration since both the cytoplasmic p24 and BlaM assays are most likely unable to detect the potential inhibition of HIV-1 nucleocapsid passage through cortical actin by genistein (Cavrois et al., 2004) following successful virion - host cell membrane fusion. There are several post-fusion stages at which genistein might be exerting its inhibitory effects. For example, the assembly, function and transport inside the cell of the reverse transcription complex are known to be dependent on the actin cytoskeleton (Bukrinskaya et al., 1998; Stevenson, 1996) whose regulation may be influenced by certain tyrosine kinases. Further, genistein sensitive tyrosine phosphorylation of the HIV-1 matrix protein (gag MA) was shown to be required for the nuclear import of the preintegration complex in non-dividing cells (Bukrinskaya et al., 1996), and the gag MA phosphorylation was shown to occur prior or during virus entry (Bukrinskaya et al., 1996; Gallay et al., 1995a; Gallay et al., 1995b). Nevertheless, even if gag MA is phosphorylated during virus budding, it is likely that tyrosine kinase activity may be necessary to keep the matrix protein phosphorylated after virus entry due to the activity of intracellular phosphatases. In agreement with these studies, our Env-pseudotyped virus entry experiments demonstrate that genistein inhibits the luciferase reporter gene expression if applied before, during or immediately after the infection period, but has no effect 24 hours post-infection. Alternatively, the interference with some of the tyrosine kinase-mediated effects of Nef (Collette and Olive, 1997; Renkema and Saksela, 2000) could also be a potential mechanism(s) by which genistein is inhibiting HIV-1 infection at post-entry level.

Finally, we also observed nearly complete inhibition of HIV-1 replication in primary macrophages using genistein concentrations of 5 -10 µg/ml. In a previous study, Gozlan et al. (Gozlan et al., 1998) reported an increase in p24 production in the culture supernatants of OM10 and U1 cell lines after treatment with genistein at concentrations higher than 25 µM (7.35µg/ml). However, in that study genistein was applied to chronically infected cells, i.e., after proviral DNA integration into the cell genome, while here we have focused on the role of tyrosine kinase signaling and the effects of genistein in the very early events of the virus life cycle. That enhancing effect of genistein was also linked to cell cycle arrest in G2, whereas

and in contrast to our model here which employs well differentiated, non-dividing primary cells.

The suppressive effect of genistein on TNF- α production by primary macrophages after gp120 challenge (see result section) and/or TNF- α induced signaling (Avdi et al., 2002) would also be expected to contribute to genistein's overall inhibitory effect on a spreading wild type HIV-1 infection in cell culture. The reduced TNF- α production by genistein indicates an effective interference with the gp120 induced signaling as well. Further, we also exclude the possibility that the inhibition of HIV-1 infection was due to increased apoptosis in the macrophage target cells. Interestingly, previous studies revealed that gp120 cytotoxic effects were tyrosine kinase mediated and were substantially attenuated by tyrosine kinase inhibitors, including genistein (Corasaniti et al., 2003; Russo et al., 2003).

In summary, the potential of HIV-1 to mutate and become resistant to different antiviral agents is well known, even to the newly FDA approved entry/fusion inhibitor enfuvirtide (T-20) (Miller and Hazuda, 2004). A better understanding of the conserved cellular factors required for productive HIV-1 entry and infection of important cellular targets like macrophages, such as those tyrosine kinase targets that appear to be critical for HIV-1 infection, may facilitate the discovery of new and exploitable therapeutic avenues.

Acknowledgements

This study was supported by NIH grant AI43885 to C.C.B. We thank Drs. Robert Doms, Gerald Quinnan, Michael Miller, Timothy Fouts, Dimple Khetawat and Paula Cannon for providing plasmid constructs used in the present study and Ms. Karen Walcott for her expert assistance with the Flow Cytometry.

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Figure 1.

Effect of genistein on infection of primary macrophages with HIV-1 Env pseudotyped reporter gene encoding virus particles. Cells were pre-incubated with different concentrations of genistein for 3 h and genistein was also present during the 3 h infection period. HIV-1 Env pseudotyped reporter gene virus particles were produced and infection was carried out as performed in Materials and Methods. The graphs, including the best fitted curves, were created using Prism 4 software (GraphPad Software, Inc., San Diego, California). **Panel A:** two different donors infected with JR-FL Env pseudotyped virus particles. **Panel B:** macrophages from one donor infected in parallel with two different HIV-1 Env pseudotyped

particles: JR-FL (;) or Ba-L (;). **Panel C:** JR-FL Env, GFP-encoding virus particles infecting one (;) or two weeks (X;) differentiated macrophages from the same donor.

Figure 2.
Effect of genistein on membrane fusion between primary macrophages and HIV-1_{Ba-L} Env-expressing cells. After preincubation for 3 h with different concentrations of genistein, macrophages were mixed with HIV-1_{Ba-L} gp160 expressing 293T cells, pre-loaded with the fluorescent dye Calcein AM. Cells were incubated for 1 h at 37°C in the presence of DMSO or genistein and the HIV-1 Env induced fusion was measured by the fluorescent dye redistribution from labeled 293T cells to unlabeled macrophages. Percent fusion represents the ratio of bound, dye redistributing 293T cells to the total number of 293T cells bound to primary macrophages.

Figure 3.

Effect of genistein on CD4 and CCR5 cell-surface expression and gp120-CD4-CCR5 complex formation. **Panel A:** Primary macrophages were pre-incubated with genistein (10 μ g/ml) (○) or DMSO (●) for 8 h and detached by treatment with 5 mM EDTA and gentle scraping. Cells were labeled for surface expression of CD4 and CCR5 and analyzed by Flow Cytometry. The isotype control fluorescence was subtracted and the graphs were created using WinList software (Verity Software House). **Panel B:** gp120-CD4 complex formation in CD4-S tag expressing macrophages. After transduction with VSV-G Env pseudotyped CD4-S tag encoding virus particles and pre-incubation with genistein (10 μ g/ml) or DMSO for 3.5 h, macrophages were treated with JR-FL rgp120 in the presence of the reagent and the efficiency of gp120-CD-S tag complexes formation were evaluated by precipitation with S-beads and Western blot analysis. The first two lanes represent negative controls: in lane 1; macrophages were not transduced for CD4-S tag expression and in lane 2 no rgp120 was added. Lanes 3 and 4 show gp120-CD4 complex formation in DMSO and genistein treated cells, respectively. **Panel C:** Formation of gp120-CD4-CCR5 complexes. The infection of macrophages with recombinant vaccinia virus, encoding wild type CD4, the preparation of the samples from DMSO (lane 1) or genistein (lane 2) treated cells and the subsequent Western blot analysis were performed as described in Materials and Methods. Lane 3 is a Western blot of just rgp120 and lane 4 is a negative control, where no anti CCR5 MAb 5C7 was added to the cell lysate to precipitate the trimolecular complexes.

Figure 4.
Effect of genistein on Pseudotyped HIV-1 infection of macrophages depending on the time of its application. Human macrophages were infected with JR-FL pseudotyped virus particles for 3 h., briefly treated with trypsin to remove the remaining virus particles on the cell surface and washed. Macrophages were pre-incubated with genistein for 3.5 h before and genistein was also present during infection (•), only pre-incubated with genistein as the reagent was removed before virus application (○), treated with genistein for 3.5 h immediately after the infection and virus removal (△) or incubated with genistein for the same time period 24 h post infection (◼). After infection, macrophages were incubated for 48h before lysis and measurement of luciferase activity.

Figure 5.

Genistein inhibits HIV-1 particle entry into primary macrophages. Parallel virus entry/infection assays were conducted with macrophage targets. **Panel A:** Cytoplasmic p24 assay; **Panel B:** Luciferase reporter gene assay. The same preparation of pseudotyped virus particles was used and the ratio of number of cells/applied virus was kept constant in both experiments. After pre-incubation with genistein (10 μ g/ml) for 3 h, macrophages were infected with JR-FL pseudotyped virus particles for 1.5 h at 37°C in the presence of the reagent and subsequently briefly treated with trypsin to remove the cell surface bound virus. Cells for the Luciferase reporter gene assay were washed and incubated for 48 h at 37°C before lysis. For the virus entry assay, macrophage cytosolic fractions and p24 measurements were performed as described in Materials and Methods. Negative controls of no added virus and the anti-CCR5 MAb 2D7 (5 μ g/ml) were performed in both experiments. **Panel C:** β -lactamase assay. Macrophages were pre-incubated for 3 h with genistein (10 μ g/ml) or for 1 h with 2D7 MAb

(5 µg/ml) at 37°C and infected for 2 h at 37°C with BlaM containing JR-CSF virus particles in the presence of genistein or 2D7. Virions without BlaM were used as a negative control. Macrophages were briefly treated with trypsin, washed and loaded for 1 h at RT with the fluorescent dye CCF2/AM. Subsequently, the cell free dye was removed; macrophages were washed and further incubated for 15 h at RT to allow cleavage of CCF2/AM by the virus introduced intracellular BlaM molecules. Finally, the cells were fixed, imbedded in mounting medium and the percent blue (infected) cells was determined by Laser Scanning Cytometry as described in Materials and Methods. (* P<0.05, ** P<0.01)

Figure 6.

Genistein inhibits gp120-induced TNF- α production by primary macrophages.

Macrophages were pre-incubated with different concentrations of genistein for 2.5 h and subsequently challenged with JR-FL rgp120 (0.25 μ g/ml) for 5 h in the presence of the reagent and the TNF- α concentration in the different cell culture supernatants was determined by ELISA. No detectable levels of TNF- α were found in the supernatants of macrophages treated with DMSO or genistein alone.

Figure 7.

Effect of genistein on HIV-1_{Ba-L} infection of primary macrophages. After pre-incubation for 3 h with different concentrations of genistein, macrophages were infected for additional 3 h in the presence of the reagent with wild type HIV-1_{Ba-L} virus particles. Subsequently, cells were extensively washed and incubated in fresh DM-10 without genistein. After 3 (;) and 6 (;) days, the concentration of p24 in the cell culture supernatants were measured by ELISA. The amount of p24 in the supernatant of DMSO treated cells served as a positive control. Genistein was also effective in inhibiting macrophage infection by HIV-1_{92/UG/024} (data not shown).

Table 1
Apoptosis in primary macrophages treated with genistein.

	Double negative (%)	Annexin V Positive (%)	Annexin V and PI positive (%)
Medium	60.3	25.4	14.1
DMSO (1µl/ml)	60.7	26	12.9
Genistein (10µg/ml)	68	15.4	16.3

Macrophages were treated with genistein (10 µg/ml) for 7.5-8 h in Teflon coated screw cap containers (37°C in a humidified 5% CO₂ atmosphere), washed and resuspended in fresh DM-10. After incubation for 48 h without genistein, macrophages were aspirated, centrifuged, washed, stained with Annexin V-FITC and propidium iodide and then analyzed by Flow cytometry.