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Mifepristone increases gamma-retroviral infection efficiency by enhancing integration of virus into the genome of infected cells

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Abstract

Gamma-retroviruses are commonly used to deliver genes to cells. Previously we demonstrated that the synthetic anti-glucocorticoid and anti-progestin agent, mifepristone, increased gamma-retroviral infection efficiency in different target cells, independent of viral titer. In this paper, we examine how this occurs. We studied the effect of mifepristone on different steps of viral infection (viral entry, viral survival, viral DNA synthesis and retrovirus integration into the host genome) in three distinct retroviral backbones using different virus recognition receptors. We also tested the potential role of glucocorticoid and progesterone receptors in mediating mifepristone's ability to increase gamma-retroviral infectivity. We show that mifepristone increases gamma-retroviral infection efficiency by facilitating viral integration into the host genome and that this effect appears to be due to mifepristone's anti-glucocorticoid, but not its anti-progestin, activity. These results suggest that inhibition of the glucocorticoid receptor enhances retroviral integration into the host genome and indicates that cells may have a natural protection against retroviral infection that may be reduced by glucocorticoid receptor antagonists.

Keywords

mifepristone; gamma-retroviruses; integration; glucocorticoid receptor

Introduction

Retroviral vector-mediated gene transfer is the easiest way for stable gene delivery into cells and can be used to permanently modify the host cell nuclear genome. Previously we showed

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Conflict of interest

The authors declare no conflict of interest.

that the synthetic glucocorticoid and progesterone receptor antagonist, mifepristone, increased retroviral infectivity of uninfected target cells independent of viral titer in a moloney murine leukemia virus (MMLV) based gene delivery system¹. The ability of mifepristone to increase retroviral infectivity was demonstrated in several different types of target cells including rat pulmonary artery smooth muscle and endothelial cells, human epithelial cells, and a glioblastoma cell line. The mechanism, through which this occurred, however, was not clear.

In this paper we demonstrate that mifepristone increases gamma-retroviral infection efficiency by facilitating the integration of the viral genome into the host genome, a process that is mediated by mifepristone's antagonist activity on the glucocorticoid receptor.

Results

Mifepristone increases infection efficiency of different gamma-retroviruses

The classification of retroviruses is arranged by computer analysis of genomes, based primarily on comparisons of the size and morphologic characteristics of the viral genome. All gamma-retroviruses have similarities in genome organization, but each possesses individual characteristics. Moloney murine leukemia virus (MMLV) based vectors are produced by replacing the viral genes required for replication with the desired genes to be transferred and along with other vectors are currently used as gene transfer vehicles². The MSCV (Murine Stem Cell Virus) is an important derivative of MMLV that has been optimized for introducing and expressing target genes into pluripotent cell lines, including murine and human hematopoietic embryonic stem cells, and embryonal carcinoma cells which are usually resistant to retroviral infection³. The friend murine embryonic-stem cell virus (FMEV) which is a hybrid viral backbone [friend mink cell focus-forming (FMCF)/ murine embryonic stem cell virus (MESV)] was also designed to optimize infection and protein expression in primary hematopoietic cells⁴. These last two retroviral backbones can be used to infect the same types of cells as MMLV.

We investigated whether mifepristone could also increase the target cell infectivity of these popular gamma-retroviral systems. Gamma-retroviral backbones containing genes encoding fluorescent proteins as reporters were propagated in Phoenix ecotropic packaging cells. Viral-conditioned supernatant was collected and applied to target cells (rat pulmonary microvascular endothelial cells, PMVEC). Figure 1A shows that mifepristone at 1 $\mu\text{mol/L}$ concentration almost doubled the percentage of infected cells regardless of the viral backbone. This suggests that the ability of mifepristone to increase infectivity of gamma-retroviruses is not restricted to MMLV retroviral vectors, but also occurs in other popular gamma-retroviral gene delivery vectors. In contrast mifepristone did not increase the target cell infectivity of lentiviral vectors (packed into VSV-G based viral particles).

Mifepristone increases target cell infection efficiency independent of viral or target cell recognition receptors

A viral receptor is conventionally defined as a component(s) on the cell surface to which a virus specifically binds and which may result in virus entry. Virus entry occurs following

fusion between the retroviral envelope and the cellular plasma membrane⁵. To determine if the type of viral receptor was critical to mifepristone's ability to increase target cell infectivity, we infected cells with MMLV packaged into viral particles with different envelope proteins – ecotropic, amphotropic, and VSV-G based pantropic. Ecotropic envelope proteins recognize the ecotropic receptor (mCAT1)⁶ and can efficiently infect a broad range of mouse and rat cells. Amphotropic viral envelope proteins recognize the amphotropic receptor (Ram-1) and infect a broad range of mammalian cells⁷. Viral entry with VSV-G pantropic envelope glycoproteins from the vesicular stomatitis virus does not require a cell surface receptor, but mediates viral entry through lipid binding and plasma membrane fusion⁸.

As shown in figure 1B, the infection efficiency of rat PMVEC varied with the different viral particles, with VSV-G envelope glycoprotein demonstrating the greatest baseline infectivity while amphotropic viral particles demonstrated the least. While the baseline infectivity of ecotropic, amphotropic, and VSV-G based viruses was different (amphotropic receptors are less abundant in rodent cells compared to ecotropic ones, while VSV-G base viruses do not use any protein receptors for cell entry), mifepristone doubled the percentage of infected cells in each case. These results suggest that the ability of mifepristone to increase target cell infectivity is not dependent on the type of envelope protein expressed by the viral particles.

Adding mifepristone after exposure to retrovirus still increases viral infection efficiency

Target cell entry is the first step of viral infection. This requires contact between the viral envelope and the cell membrane. Receptors on the viral envelope interact with complementary receptors on the cell membrane and this attachment causes the two membranes to remain in mutual proximity. To enter the cell through the phospholipid bilayer membrane, viral receptors attach to the receptors on the surface of the cell and either puncture the cell membrane or allow fusion of the viral envelope with the host cell. The virus's envelope then releases its contents into the cell⁹.

To determine whether mifepristone increased retroviral infection efficiency by facilitating viral entry into target cells, PMVEC were infected for one hour in the absence of mifepristone. Cells were then washed (to remove the virus) and incubated in fresh medium for an additional hour. This allowed sufficient time for the virus to enter the cell, but not sufficient time to complete its infection cycle. Mifepristone (at 1 μ mol/L concentration) was then added to the medium. Seventy-two hours later, target cells were trypsinized and analyzed for GFP expression by fluorescence activated cell sorting (FACS). Figure 2 demonstrates that adding mifepristone after the initial infection had occurred (i.e. after the virus had been removed from the medium following its one hour incubation) also increased viral infection efficiency approximately two-fold (from 2.1 to 3.7%). The lower infection rate seen in figure 3 compared with figure 1 is due to the shorter retroviral incubation time (one versus 18 hours). This result suggests that the increased infection efficiency seen with mifepristone is not due to its effect on viral entry into target cells.

Mifepristone did not promote retroviral infection in non-dividing cells

Gamma-retroviruses and many other RNA viruses can only infect dividing cells because they must access the host DNA to replicate. The interior of the nucleus is separated from the cytoplasm by a double-layer membrane called the nuclear envelope. Embedded in the nuclear envelope are nuclear pores, which act like selective filters that allow diffusion of ions and molecules, but nothing larger¹⁰. Gamma-retroviruses, therefore, cannot enter the nuclear compartment of non-dividing cells and integrate into the host genome. As cells undergo replication and division, the nuclear envelope becomes more permeable which allows greater cytoplasmic-nuclear trafficking and allows viruses to enter the nucleus. To determine whether mifepristone's ability to increase retroviral infection efficiency was due to its ability to infect non-dividing cells, we compared the infection efficiency in dividing (growing) and non-dividing (confluent) PMVEC with or without mifepristone. We grew cells to either 100% confluency (to induce cell arrest) or 15% confluency and then infected them with MMLV (at the same multiple of infectivity) in the presence of either 1 $\mu\text{mol/L}$ of mifepristone or vehicle (EthOH). Seventy-two hours later, target cells were trypsinized and analyzed for GFP by FACS as previously described. Figure 3, demonstrates that the retrovirus was only able to infect dividing cells and the presence of mifepristone had no effect on this.

Mifepristone does not prolong survival of non-integrated gamma-retroviral intermediates in infected cells

Since transduction of target cells by gamma-retroviruses is dependent on cell replication, it is important to know how long a virus that has successfully entered the target cell retains its ability to integrate. Some authors have demonstrated that if cells have not divided within 6 hours of infection, gene transfer does not occur¹¹. This suggests that retroviruses do not remain viable within the cell cytoplasm for a prolonged period of time. Other studies have shown that growth-arrested cells infected with retroviruses can undergo infection later; however, suggesting that some stable viral intermediates can persist in growth-arrested cells. This intermediate may be viral RNA, viral DNA (after reverse transcriptase) or a mixture of partially reverse-transcribed forms. The intracellular half-life of MMLV has been determined to be in the range of 5.5 to 7.5 hours^{11,12}.

A potential mechanism by which mifepristone could increase the number of infected cells would be to prolong survival of the virus in infected but temporarily arrested cells. To evaluate this hypothesis we tested the ability of mifepristone to increase retroviral infectivity of growth-arrested cultured rat PMVEC as they reentered the cell cycle over 48 hours. Rat PMVEC stop proliferating once they attain confluence and therefore are resistant to retroviral infection. We have previously demonstrated that once these growth-arrested confluent cells are reseeded at lower confluency, they start to enter the S-phase approximately 24 hours and G2-M phase about 30 hours later. Figures 4A and 4B show the percentage of PMVEC entering G2-M phase at various time points after their reseeded from a confluent monolayer. Within this first 30 hours after reseeded, cells are not dividing and therefore while they may be able to take up virus, they are unable to complete an infection cycle by integrating it into the host genome.

PMVEC that were growth-arrested and then re-seeded at lower confluence as described above, were infected at various time points after reseeded in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle. Cells were incubated with virus for only 12 hours, but at different time points after re-seeding. Seventy-two hours later, the cells were analyzed for GFP using FACS.

Figure 4D shows that cells infected immediately after re-seeding (i.e. 0 to 12 hours) demonstrated less infection efficiency compared to cells infected later (24 to 36 and 36 to 48 hours). Since arrested cells can take up the virus, but are unable to integrate it into the host genome, the decreased infection efficiency in cells infected immediately after re-seeding was likely due to the fact that by the time these cells began to divide (approximately 30 hours), most of the virus that had been taken up by the cells was already degraded. By the time the early-infected cells begin to divide (at about 30 hours), the virus has been degraded and is no longer capable of integration. As expected, mifepristone increased the infectivity rate at all time points (about 2.9 fold), but did not demonstrate an enhanced effect at the earlier time points (figure 4E). This suggests that mifepristone did not prolong the survival of non-integrated viral intermediates in these infected but temporarily non-dividing target cells. Had mifepristone prolonged viral survival, the earlier infected cells should have demonstrated a greater infectivity *rate* when compared to those cells infected later. Figure 4E demonstrates that the increase in infectivity rate following incubation with mifepristone was similar at all time points, however. This correlates with our previously published results¹ demonstrating that mifepristone does not prolong viral viability in cell culture. Figure 4C shows representative flow cytometry data of retroviral infectivity for all experimental conditions.

Mifepristone does not enhance viral DNA synthesis in target cells

Since mifepristone did not affect viral entry or survival in target cells but did increase the number of stably infected cells, we examined whether mifepristone stimulated other post-infection events in target cells including viral DNA synthesis (reverse transcriptase) or integration into the host genome. Reverse transcription—the transcribing of genetic information from RNA to DNA—is a hallmark of the retroviral replication cycle. The enzyme reverse transcriptase catalyzes this process and plays a critical role in viral cycling¹³. To determine if viral DNA synthesis was stimulated by mifepristone, we performed quantitative PCR (qPCR) on total DNA isolated from target cells at various time points after infection (figure 5A). To better synchronize infection events, we exposed target cells to MMLV for only 1 hr in the presence of mifepristone or vehicle. After that, virus was removed from the medium. The viral DNA content in infected cells was measured by qPCR using primers to the GFP region of viral DNA. Mifepristone or vehicle was present in the medium from the beginning of infection until analysis (up to 7 days). Viral DNA content peaked six hours after infection and then began to decrease. There was no difference in viral DNA levels between mifepristone- and vehicle- treated cells in the first 6 hours suggesting that mifepristone did not affect viral DNA synthesis catalyzed by reverse transcriptase. Twenty four hours after infection the viral DNA content was decreased in all cells likely due to a combination of viral degradation and target cell proliferation resulting in the dilution of non-integrated viral DNA. The content of viral DNA in mifepristone-treated target cells was

higher than that in vehicle-treated cells at 24 hours, a difference that persisted throughout the seven days of the experiment. This twofold increase in viral DNA level observed in mifepristone-treated target cells at 3 days post infection closely correlated with the two-fold increase in the number of infected cells shown in figure 1 and to our earlier published results. Since the viral DNA content during log phase replication (0 to 6 hours) was not affected by mifepristone, it is unlikely that mifepristone increased target cell infectivity by stimulating viral reverse transcription.

Mifepristone enhances viral integration into host DNA

While newly synthesized viral DNA can persist either as linear forms, one LTR circles, or two LTR circles for a period of time, ultimately this viral DNA must be either integrated (to complete an infection cycle) or degraded. If it is integrated into the host genome, viral DNA should be detectable within the genomic DNA immediately after incorporation. To examine whether mifepristone increased the amount of viral DNA integrated into the host genome, we infected cells with MMLV for 1 hour in the presence or absence of mifepristone, isolated total DNA from target cells at the time points described above and resolved the DNA on an agarose gel. 4Kb DNA fragments (the size of non-integrated viral DNA) and genomic DNA (>50Kb) were cut out and the DNA from each fraction was extracted and analyzed for the presence of viral sequences by qPCR. The level of free viral DNA (i.e. the viral 4 Kb DNA fragment) was markedly increased 6 hours after infection in both vehicle and mifepristone-treated cells and then declined towards baseline by 3 days (figure 5B). At no time did the mifepristone-treated cells demonstrate greater free viral DNA levels than vehicle-treated cells. This is consistent with our previous observation that mifepristone did not increase viral DNA synthesis. In contrast, the amount of viral DNA that was integrated into the genomic fraction was greater in mifepristone-treated cells at all time points (figure 5C). This increase in the level of integrated viral DNA was the only event in the viral infection cycle that was altered by the presence of mifepristone.

Mifepristone increases gamma-retroviral infectivity through its effect on the glucocorticoid receptor

Mifepristone is a synthetic steroid that can act as an antagonist to both glucocorticoid and progesterone receptors¹⁴. At the molecular level, mifepristone binds to the glucocorticoid or progesterone receptors with high affinity interacting with the receptor at the phenylaminodimethyl group in the 11 β -position within a specific region of the receptor binding pocket¹⁵. Mifepristone induces a conformational change within the ligand-binding domain and reduces the receptors affinity for its natural hormone ligands. Mifepristone's ability to increase viral infectivity of target cells could be due to its effects on either the glucocorticoid receptor or the progesterone receptor.

To determine whether the increase in retroviral infectivity was some specific effect of mifepristone or whether it occurred with other glucocorticoid or progesterone receptor antagonists, rat PMVEC were infected with retrovirus in the presence of mifepristone or another glucocorticoid antagonist, Org 34517, kindly gifted by Schering-Plough. The ic_{50} of Org 34517 for the glucocorticoid receptor is 17.9 nmol/L (mifepristone's ic_{50} is 12.2 nmol/L)¹⁶. Org 34517 was used in a range of 0.1–30 μ mol/L and the results were compared

to rates of infectivity in non-treated or mifepristone-treated cells. Org 34517 significantly increased retroviral infectivity above control but was less potent than mifepristone. A progesterone receptor antagonist {11b-[4-(acetylphenyl)-4',5'-dihydro-2'-ethyl-5'-methylenespiro[estra-4,9-dien-17b,4' oxazole]-3-one} which has an ic_{50} of 0.34 nmol/L for the progesterone receptor (graciously provided by RTI International, RTP, NC) (mifepristone's ic_{50} for the progesterone receptor is 0.054 nmol/L), had no effect on retroviral infection efficiency (figure 6A) ¹⁷. Adding both the specific glucocorticoid (Org 34517) and the progesterone (7453-102) receptor antagonists at the same time did not change the rate of target cell infectivity (data not shown). This suggests that the improved infection efficiency seen with mifepristone compared to ORG is not due to a synergistic effect of progesterone blockade.

We next examined the effect of mifepristone on retroviral infectivity in cells that lacked either progesterone or glucocorticoid receptors. U87MG cells (a human glioblastoma cell line) lack functional progesterone receptors, but express functional glucocorticoid receptors ¹⁸ whereas T47D cells (a human breast cell line) express progesterone receptors, but do not have functional glucocorticoid receptors ^{19,20}. Both cell lines were infected with retrovirus in the presence or absence of mifepristone. Seventy-two hours later, cells were analyzed for GFP expression (figure 6B). Mifepristone increased retroviral infectivity in U87MG cells, but not in T47D cells. These results, combined with those using selective progesterone and glucocorticoid receptor inhibitors, suggest that mifepristone's ability to increase retroviral infectivity of target cells is likely due to its glucocorticoid receptor antagonist properties.

Mifepristone did not enhance gamma-retroviral integration in T47D cells

Since mifepristone appeared to enhance target cell infectivity by increasing the rate of retroviral integration into the host genome, (through a glucocorticoid receptor mediated action) we examined whether this effect would be lost in T47D cells. T47D cells were infected with amphotropic MMLV for 1 hr in the presence of 1 μ mol/L of mifepristone or vehicle. Virus was then removed and cells were either analyzed immediately or incubated with mifepristone or vehicle for 6 hours, or 1, 3 or 7 days. Similar to previous experiments, the total cellular DNA was isolated and resolved on a 0.8% agarose gel. 4Kb DNA fragments (the size of non-integrated viral DNA) and genomic DNA (>50Kb) were cut out and the DNA from each fraction was extracted and analyzed for the presence of viral sequences by qPCR. Similar to our results in endothelial cells (figure 5) the level of free (i.e. non-integrated) viral DNA was elevated 6 hours after infection and then gradually declined towards baseline (figure 7A). The amount of integrated viral DNA was elevated above baseline 24 hours after infection in both mifepristone and vehicle-treated cells (figure 7B), but unlike our results in endothelial cells (figure 5) there was no difference in the amount of either free or incorporated viral DNA at any time after infection between mifepristone and vehicle-treated cells. Therefore, it appears that T47D cells, which lack functioning glucocorticoid receptors, are insensitive to mifepristone's ability to increase gamma-retroviral enhanced integration into the host genome. This likely explains the failure of mifepristone to increase viral infection in these cells.

Discussion

Retroviral vectors are popular and widely used gene delivery vehicles because of their efficiency and precision of integration. Retroviruses can be used to correct genetic diseases through the permanent integration of therapeutic genes into chromosomes of affected cells, for generating transgenic animals and plants, and for basic biomedical research. Determining how retroviruses infect target cells is important not only as a guide towards building better vectors, but to further our understanding of the basic biology of retroviral infection in disease.

MMLV-derived vectors are simple and effective gene delivery vehicles in part because they tend to integrate near transcription initiation sites dramatically increasing expression levels of delivered proteins. The biggest disadvantage of using MMLV-derived vectors is low infection efficiency. While infected cells show a high expression level of delivered protein(s), many cells remain resistant to infection even after several infection cycles. One strategy to increase retroviral infection efficiency is to generate higher viral titers in virus-producing (packaging) cells by maximal activation of enhancers within the retroviral promoter. Since MMLV-derived vectors have one or more response elements within its promoter, glucocorticoids stimulate viral propagation in virus-producing cells that leads to an increase in retroviral infectivity due to an increase in viral titer. We have shown that the glucocorticoid receptor antagonist, mifepristone, blocks this increase in viral titer¹. Our previous work demonstrated, however, that while mifepristone blocked the dexamethasone-induced increase in viral titer, it actually increased target cell infectivity. The mechanism underlying that unexpected finding was unclear. In this paper we demonstrate that mifepristone increased the number of infected target cells by facilitating integration of the viral genome into the host genome. It did not appear to affect any other steps involved with viral infection such as recognition of target cells, virus entry, virus stability, or viral DNA synthesis.

Integration of a retroviral genome into a host chromosome completes the viral replication cycle and makes the infection permanent. The integrated viral DNA can be transcribed, resulting in RNA for translation of viral proteins and formation of viral particles, which can be released to infect other cells. Without viral integration, however, cells eventually degrade the virus and remain uninfected. Mifepristone's ability to increase retroviral infection efficiency appears to be due to its ability to facilitate retroviral integration into the host genome and thus complete the infection. Mifepristone enhanced the efficiency of viral infection in three different gamma-retroviral backbones demonstrating that its effect is not limited to a specific vector. Mifepristone had no effect on the target cell infectivity of the two lentiviral vectors we studied, however. The reason for this is not clear, but may be due to the lack of functional glucocorticoid receptors or other differences in how these lentiviruses infect cells.

The exact mechanism through which mifepristone enhances viral integration in gamma-retroviruses is not clear, but it appears to involve its antagonistic effect on the glucocorticoid receptor. Another anti-glucocorticoid, Org 34517, also increased gamma-retroviral infectivity, whereas an anti-progesterone agent had no effect. Consistent with this

observation, mifepristone did not increase gamma-retroviral infection efficiency in cells which lack functional glucocorticoid receptors (T47D cells), but did in those lacking functional progesterone receptors (U87MG cells).

These results suggest that inhibition of the glucocorticoid receptor enhances gamma-retroviral integration into the host genome and indicates that cells have a natural protection against viral infection that may be reduced by glucocorticoid receptor antagonists. As a research tool, mifepristone can be used to enhance gene delivery and provide a way to study different aspects involved in retroviral integration.

Materials and methods

Materials

Dexamethasone (D4902), Mifepristone (M8046), hexadimethrine bromide (H9268), propidium iodide (PI), RNase, and 5'-bromo-2'-deoxyuridine (BrdU) were all purchased from Sigma (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), trypsin-ethylenediaminetetraacetic acid (EDTA) and L-glutamine were all purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). HyBond-P membrane was purchased from Amersham (Buckinghamshire, England). FuGENE 6 Transfection Reagent (11 814 443 001) was purchased from Roche Diagnostics (Indianapolis, IN). Org 34517 was kindly gifted by Schering-Plough. Progesterone receptor antagonist 11b-[4-(acetyl)phenyl]-4',5'-dihydro-2'-ethyl-5'-methylenespiro[estra-4,9-dien-17b,4'-oxazole]-3-one (RTI # 7453-102) was synthesized by Dr. Chunyang Jin at RTI International following the previously published procedure¹⁷.

Cell cultures

Phoenix Retroviral Expression System ecotropic and amphotropic packaging cells were purchased from Orbigen, Inc. (San Diego, CA). The Pantropic Retroviral Expression System (VSV-G based, with pantropic packaging cells) was purchased from Clontech (Palo Alto, CA). Rat pulmonary microvascular endothelial cells (PMVEC), were obtained from our cell culture core. Human glioblastoma cells (U87MG), and human mammary carcinoma cells (T47D) were obtained from ATCC. All cells were cultured in DMEM, 10% FBS, 2mmol L-Glutamine and used for experiments at passages 4–9. All cells were grown in humidified incubators at 37°C in 5% CO₂. Cells were harvested by 0.05% trypsin/0.53 mM EDTA digestion, washed, re-suspended in cultured medium and analyzed directly by FACScan in the University of South Alabama Flow Cytometry Core. For cell cycle analysis cellular DNA was stained with 10 mmol/l of propidium iodide for 20 minutes as described elsewhere²¹ and then analyzed by FACScan.

Vectors and delivery systems

pBMN-GFP (Orbigen, Inc., San Diego, CA), a retroviral vector that expresses GFP driven by the MMLV's promoter in Phoenix and other cells was used. In some experiments Murine Stem Cell Virus (MSCV) expressing mCherry and the hybrid retroviral vector Friend Murine Embryonic-stem cell Virus (FMEV) [for Friend Mink Cell Focus-forming (FMCF)/

Murine Embryonic Stem cell Virus (MESV)]⁴ expressing DsRed2 were used in addition to MMLV. Two lentiviral vectors pLemiR (Open Biosystems, Huntsville, AL) and pLVX-DsRed-Monomer-C1 (Clontech Laboratories, Inc., Mountain View, CA) harboring turboRFP and DsRed as reporters were also used for comparison to gamma-retroviruses. Packaging cells were transfected with retroviral vectors using FuGENE 6 reagent following the manufacturer's instructions. For virus propagation, packaging cells were cultured in growth medium for 12–18 hours. Virus was collected with cultured medium, filtered, supplemented with hexadimethrine bromide (polybrene) to a final concentration of 4µg/ml and applied directly to target cells.

Viral DNA measurement

The total DNA from infected cells was extracted by DNeasy Blood & tissue kit (Qiagen). The level of viral DNA in total DNA extract isolated from infected cells was determined by quantitative PCR using iScript One-Step RT-PCR kit (with SYBR Green, Bio-Rad, 170–8893) with primers for the GFP or the internal ribosome entry site regions of the viral DNA following the manufacturer's instructions. No reverse transcriptase was used in order to exclude contamination from a viral RNA signal. In integration studies, the total DNA isolated from infected cells was first resolved in a 0.8% agarose gel. 4Kb DNA (the size of non-integrated viral DNA) and genomic DNA pools (>50Kb) were excised. DNA from each fraction was extracted and analyzed for the presence of viral sequences by qPCR as previously described.

Statistical analysis

Data are expressed as means ± SE. Changes in cell cycle profile, infectivity and changes in fluorescent protein expression were compared using ANOVA combined with Fisher post hoc analysis, with a *P* value < 0.05 considered significant.

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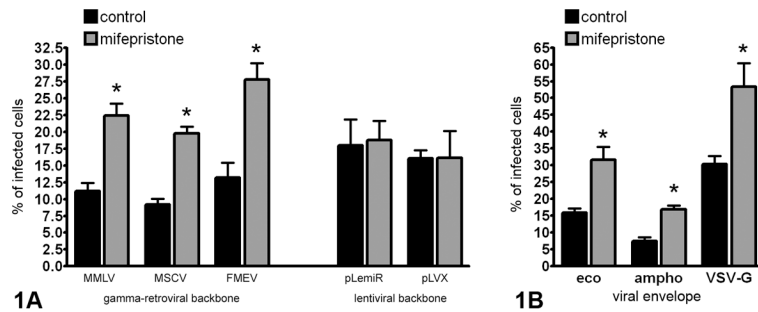


Figure 1.

Mifepristone increased the percentage of infected target cells (PMVEC) regardless of the type of gamma-retroviral backbone or viral envelope. **1A**: Ecotropic gamma-retroviruses MMLV, MSCV, and FMEV; and VSV-G pantropic lentiviruses pLemiR and pLVX-DsRed-Monomer-C1 were used to infect PMVEC in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control. **1B**: MMLV packaged in viral particles with different envelope proteins were used to infect PMVEC in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control. The percentage of infected (fluorescent) cells was determined 72 hours after infection. (n = 4 experiments, * indicates $p < .05$).

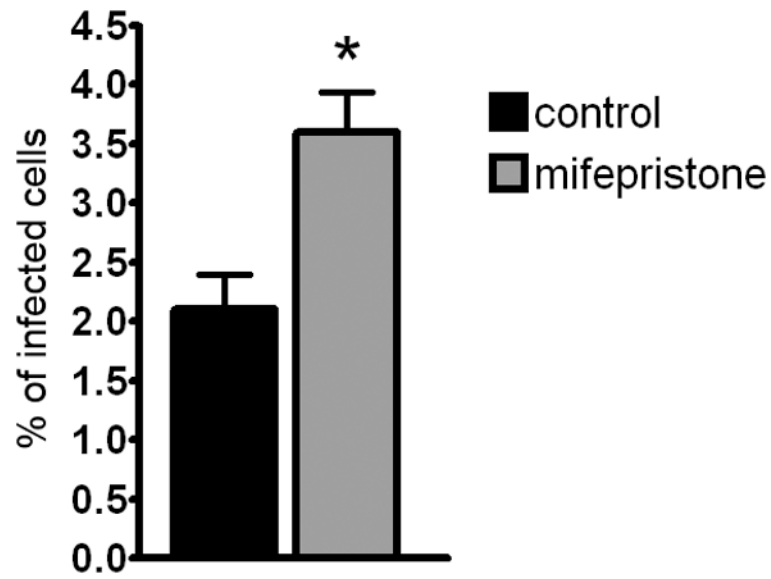


Figure 2.

Adding mifepristone after the initial retroviral infection also increased target cell infection efficiency. PMVEC were infected with ecotropic MMLV for 1 hour. After virus removal, cells were incubated in fresh medium for an additional 1 hour and then 1 $\mu\text{mol/L}$ mifepristone or vehicle control was added. The percentage of GFP positive (infected) cells was measured 72 hours after infection. (n = 4 experiments, * indicates $p < .05$).

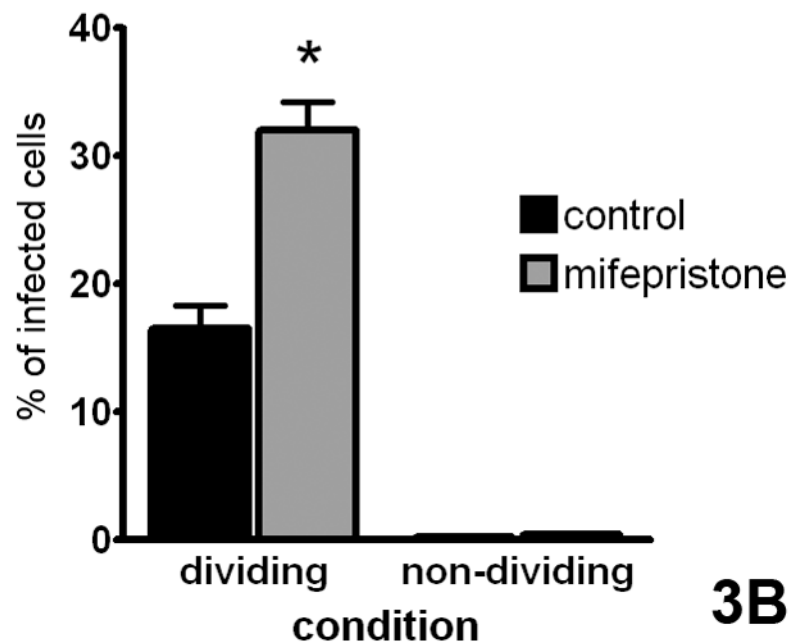
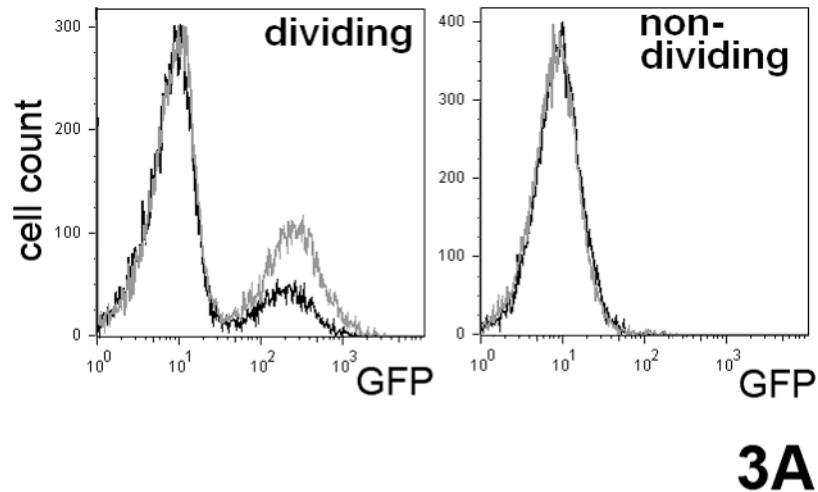


Figure 3. Mifepristone does not increase infection efficiency by promoting infection of non-dividing cells. Growing (15% confluent) or growth-arrested (100% confluent) PMVEC were infected with ecotropic MMLV at the same MOI for 18 hours in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control. The percentage of GFP positive (infected) cells was determined 72 hours after infection. (n = 4 experiments, * indicates $p < .05$).

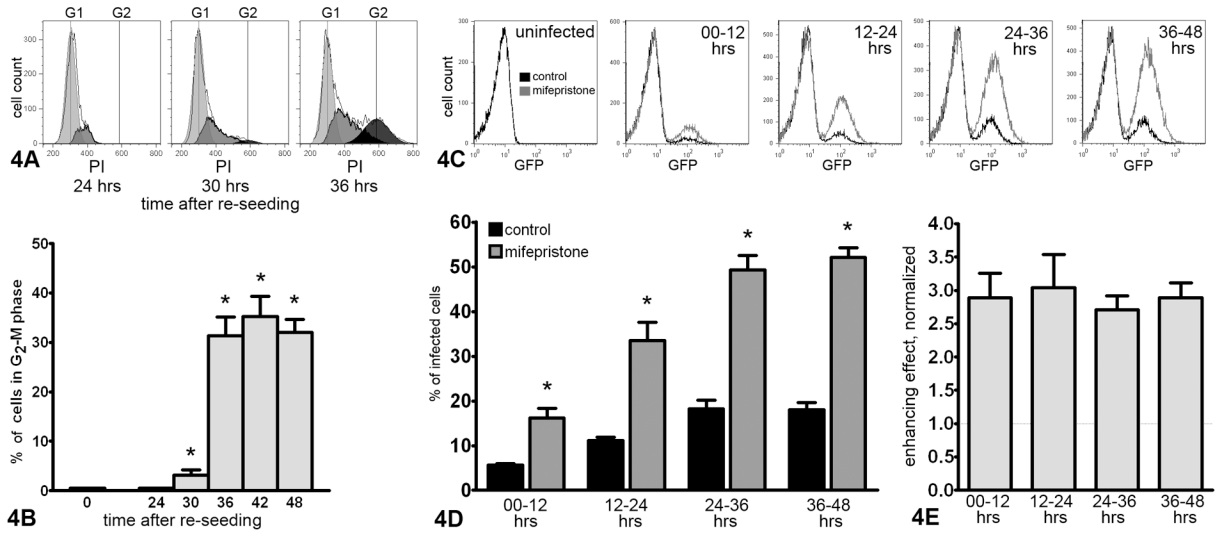
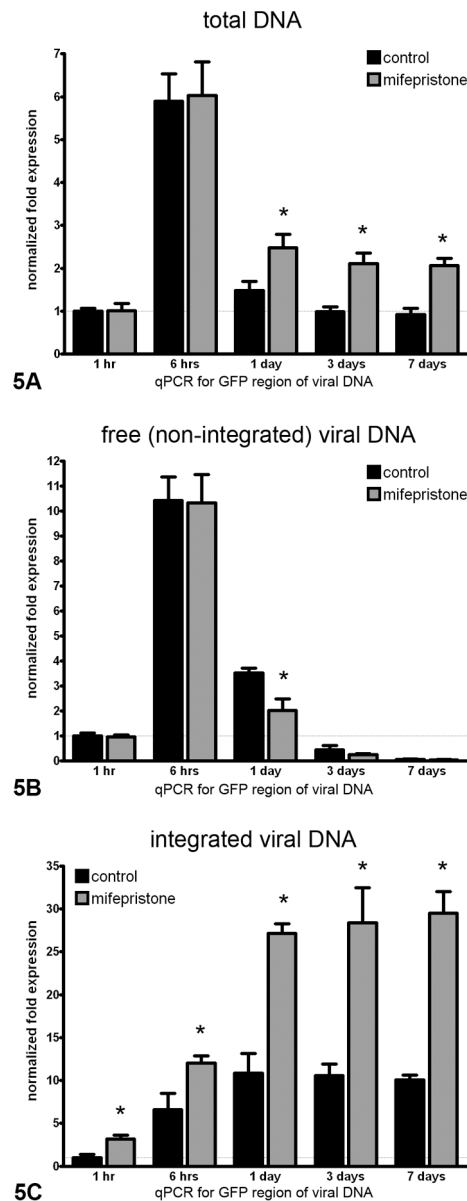


Figure 4.

Mifepristone had no effect on the survival or stability of the non-integrated gamma-retrovirus in infected cells. Growth-arrested PMVEC from a confluent monolayer were trypsinized and re-seeded at 15% confluency. Cell cycle progression was monitored by PI staining using FACS. **4A:** Representative data of cell cycle progression 24, 30 and 36 hours after cell re-seeding. **4B:** Statistical data for the percentage of G₂-M phase endothelial cells at different time points after re-seeding. PMVEC enter G₂-M phase 30 hours after release from confluency-induced growth arrest. Only cells that enter G₂-M phase can be infected by gamma-retroviruses. After re-seeding from confluent monolayer, cells were also incubated with an ecotropic MMLV retrovirus encoding GFP for 12 hours in the presence or absence of 1 μ mol/L mifepristone immediately, 12, 24 or 36 hours after re-seeding to lower confluency. The percentage of GFP-positive (infected) cells was measured 72 hours after infection. **4C:** Representative data showing the infection efficiency of PMVEC infected with MMLV at different time periods after re-seeding in the presence or absence of mifepristone. **4D:** Statistical data indicating the percentage of infected endothelial cells at different periods after re-seeding. **4E:** The relative enhancing effect of mifepristone on retroviral infection at different periods after re-seeding. (n = 4 experiments, * indicates p < .05).

**Figure 5.**

Mifepristone enhances viral integration into host DNA. PMVEC were infected with ecotropic MMLV for 1 hour in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control. The virus was removed by washing and cells were then cultured in fresh medium supplemented with 1 $\mu\text{mol/L}$ mifepristone or vehicle until analysis. **(5A)** Total DNA (host and viral) was extracted from target cells and the presence of viral DNA (using primers to the GFP coding sequence that is present only in the viral DNA) was determined. Total target cell DNA was extracted and then separated in a 0.8% agarose gel. Non-integrated viral DNA migrates to about 4Kb whereas genomic DNA migrates > 50Kb. Each fraction was extracted from the gel and analyzed for the presence of viral DNA separately. For each time point, equal amounts of DNA were examined. **(5B)** qPCR from the 4Kb fragment representing the relative amounts of non-integrated viral DNA in mifepristone or

vehicle treated cells. (5C) qPCR from the > 50Kb genomic DNA representing the relative amounts of integrated viral DNA in mifepristone or vehicle treated cells. qPCR results in each experimental design were normalized to the signal obtained from vehicle-treated cells 1 hour after infection. DNA was extracted and analyzed 1, 6 and 24 hours and also 3 and 7 days after initial infection. These qPCR results were confirmed using another set of primers encoding for the internal ribosome entry site of viral DNA (present in the pBMN-GFP vector). (n = 4 experiments, * indicates $p < .05$).

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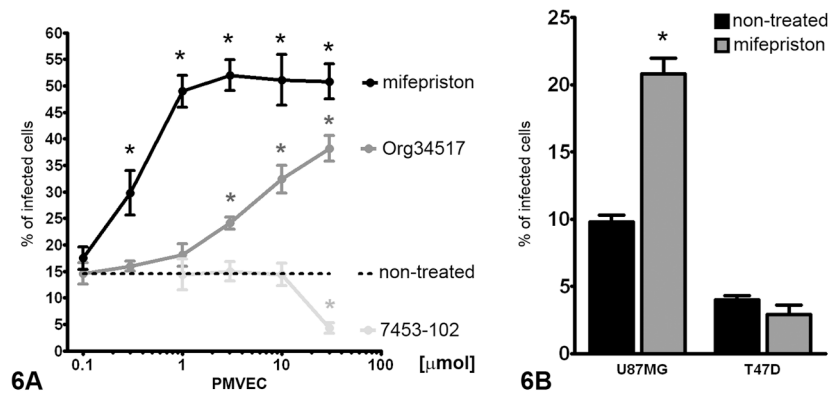


Figure 6. Mifepristone increases gamma-retroviral infection efficiency through its effect on the glucocorticoid receptor. **6A:** PMVEC were infected with ecotropic MMLV in the presence of increasing concentrations of the glucocorticoid receptor antagonists - mifepristone or Org 34517, or the progesterone receptor antagonist, 7453-102. The percentage of GFP positive (infected) cells was determined 72 hours after infection. **6B:** Two different human cell lines (U87MG which contain no functional progesterone receptors, and T47D which contain no functional glucocorticoid receptors) were infected with amphotropic MMLV in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control. The percentage of GFP-positive (infected) cells was determined 72 hours after initial infection. Mifepristone did not increase infection efficiency in the T47D cells. (n = 4 experiments, * indicates $p < .05$).

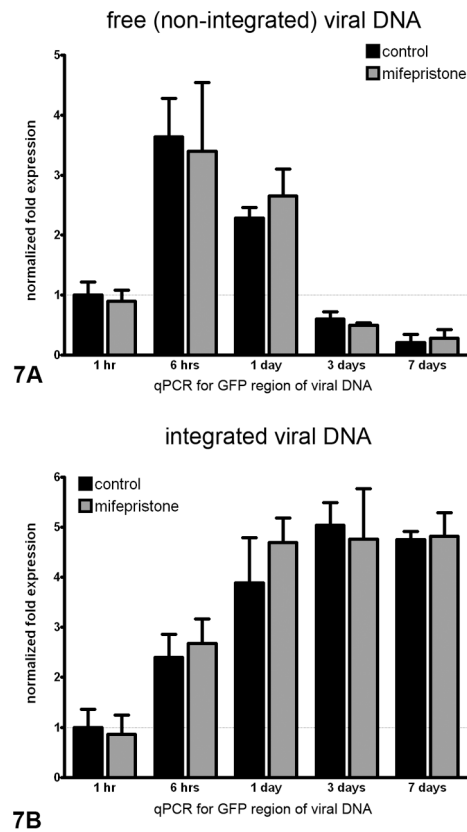


Figure 7.

Mifepristone does not increase viral integration into host DNA of T47D cells. T47D cells were infected with amphotropic MMLV for 1 hour in the presence of 1 $\mu\text{mol/L}$ mifepristone or vehicle control (using a similar protocol as described in figure 5). The virus was removed by washing and cells were then cultured in fresh medium supplemented with 1 $\mu\text{mol/L}$ mifepristone or vehicle until analysis. Non-integrated viral and genomic DNA were separated in and extracted from a 0.8% agarose gel at same way as was described for PMVEC and analyzed for the presence of viral DNA separately. For each time point, equal amounts of DNA were examined. **7A:** qPCR from the 4Kb fragment representing the relative amounts of non-integrated viral DNA in mifepristone or vehicle treated T47D cells. **7B:** qPCR from the > 50Kb genomic DNA representing the relative amounts of integrated viral DNA in mifepristone or vehicle treated cells. qPCR results in each experimental design were normalized to the signal obtained from vehicle-treated cells 1 hour after infection. DNA was extracted and analyzed 1, 6 and 24 hours and also 3 and 7 days after initial infection. These qPCR results were confirmed using another set of primers encoding for the internal ribosome entry site of viral DNA (present in the pBMN-GFP vector). (n = 4 experiments, * indicates $p < .05$).