Evaluation of the innate immune modulator acitretin as a strategy to clear the HIV reservoir

Edurne Garcia-Vidal, Marc Castellví, Maria Pujantell, Roger Badia, Antoni Jou, Lucia Gomez, Teresa Puig, Bonaventura Clotet, Ester Ballana, Eva Riveira-Muñoz[1], and José A. Esté[1]

AIDS Research Institute – IrsiCaixa, AIDS Unit and Health Research, Institute Germans Trias i Pujol (IGTP), Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, Spain

[1] Corresponding author: eriveira@irsicaixa.es, jaeste@irsicaixa.es, Tel. +34934656374, fax +34934653968

Running title: Acitretin as HIV reactivating agent

Abstract

The persistence of HIV despite suppressive antiretroviral therapy is a major roadblock to HIV eradication. Current strategies focused on inducing the expression of latent HIV fail to clear the persistent reservoir, prompting the development of new approaches for killing HIV+ cells. Recently, acitretin has been proposed as a pharmacological innate cellular-defense network enhancer that led to virus reactivation and preferential death of infected cells. We have evaluated the capacity of acitretin to reactivate and/or facilitate immune-mediated clearance of HIV+ cells. Acitretin did not induce HIV reactivation in latently-infected cell lines (J-Lat or ACH-2). We could only observe a modest induction of HIV reactivation by acitretin in latent GFP-HIV Jurkat cells, comparable to suboptimal concentrations of vorinostat a known latency-reversing agent (LRA). However, acitretin induction was insignificant when compared to LRAs optimal concentrations. Acitretin failed to reactivate HIV in a model of latently infected primary CD4+ T-cells but induced RIG-I and MAVS expression in infected and uninfected cells, confirming the role of acitretin as an innate immune modulator. However, this effect was not associated with selective killing of HIV+ cells. In conclusion, acitretin-mediated stimulation of the RIG-I pathway over HIV reactivation is modest and thus may not meaningfully impact the HIV reservoir. Stimulation of the RIG-I-dependent IFN cascade by acitretin may not significantly affect the selective destruction of HIV+ latently infected cells.

Keywords: HIV, latency, reactivation, RIG-I, interferon

Introduction

The use of antiretroviral therapy (ART) has significantly transformed the face of HIV-1 infection from a terminal illness to a chronic manageable disease (1). Despite intensive investigation, no strategy so far has resulted in sustained control of HIV in the absence of antiretroviral therapy and HIV persists through multiple mechanisms (2). Thus, the eradication of HIV-1 will require novel approaches to purge the reservoir of latently infected cells from a patient (3, 4). The quest for long-term control of HIV-1 in the absence of ART has led to numerous therapeutic approaches aimed at increasing host-mediated control of HIV and clearance of latent virus reservoirs (5-7) while maintaining the beneficial effects of immune reconstitution.

HIV infection and recognition by infected cells triggers a signaling cascade leading to increased activity of interferon regulatory factors (IRF) and production of interferons (IFN) and inflammatory cytokines (8). The innate immune response may also be responsible, in part, for a virus-induced cell death response, either through caspase-1-mediated program cell death triggered by abortive viral infection (9) or IFN-induced apoptosis (10, 11). As others, we have shown that established HIV-1 infection of monocyte-derived cells induces upregulation of the pattern recognition receptors melanoma differentiation-associated protein 5 (MDA5) and retinoic acid-inducible gene I (RIG-I), production of IFN-β, and transcription of interferon-stimulated genes (ISG) (12, 13). Additionally, HIV-1 infection may limit the deoxynucleotide pool by downregulation of the ribonucleotide reductase subunit R2 (RNR2) and reactivation of the virus restriction factor SAMHD1 (14, 15) together with increased cell death (12), mediated in part by the HIV-1 Vpr (16, 17).

Pharmacological stimulation of the RIG-I pathway has been proposed as an alternative mechanism to kill cells in the latent HIV reservoir, following viral reactivation. Enhancement of RIG-I signaling *ex vivo*, was shown to increase HIV transcription, and induce preferential apoptosis of HIV-infected cells (18), recapitulating the effect observed by chronic infection of macrophages (12). Li et al. (18) showed that the retinoic acid derivative acitretin enhanced

63 RIG-I signaling *ex vivo*, increased HIV transcription, and induced preferential apoptosis of HIV-
64 infected cells. Acitretin is an oral retinoid that may be used in the treatment of severe resistant
65 psoriasis in HIV+ individuals (19), suggesting that treatments to revert HIV latency and
66 potentially eliminate the virus reservoir are already available and thus, require further and
67 careful examination.

68 Here, we have explored the effect of acitretin in HIV infection hoping to confirm and expand
69 the observations made by Li et al.(18) We found that acitretin-mediated stimulation of the
70 RIG-I pathway over HIV reactivation is modest, and lacked selective destruction of HIV positive
71 cells and thus, may not meaningfully impact the HIV reservoir.

Materials and Methods

Viruses and cells

The HIV-1 viral strain NL4–3 was obtained from the MRC Centre for AIDS Reagents (London, UK). NL4-3 strain was grown in lymphoid MT-4 cell line. The envelope-deficient HIV-1 NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP) was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described.(20, 21) Viral stocks were titrated for its use in MT-4 cells.

Peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes, naïve CD4+ T lymphocytes or monocytes by negative selection (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. Buffy coats were purchased anonymously from the *Catalan Banc de Sang i Teixits* http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease. CD4+ T lymphocytes were kept in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Life Technologies). Monocytes were cultured in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (Gibco) and differentiated to monocyte derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech). Macrophages were infected with a VSV pseudotyped NL4-3-GFP virus and viral replication was measured 48h later by quantification of GFP+ expression by flow cytometry.

CD4+ T cells were activated with anti-CD3 and anti-CD28 (at 1 µg/ml each, StemCell technologies) for 3 days or left untreated with IL-2 (16 U/ml, Roche). Cells were acutely

97 infected with a VSV pseudotyped NL4-3-GFP virus by spinoculation before adding the
98 corresponding drugs and incubated for 48 h.
99 The human cell lines ACH-2,(22) Jurkat (J-Lat) clone 9.2 and 8.4 (23) and CD4- TZM-bl (24) cells
100 were obtained from AIDS Reagent Program, National Institutes of Health (Bethesda, MD). All
101 cell lines were grown in RPMI 1640 medium, supplemented with 10% of heat-inactivated fetal
102 calf serum (FCS, Gibco, Life Technologies, Madrid, Spain) and antibiotics 100 U/ml penicillin,
103 100 µg/ml streptomycin (Life Technologies) and maintained at 37ºC in a 5% CO_2 incubator.
104 TZMbl were infected with the NL4-3 virus and drugs were added at the time of infectioin. Viral
105 replication was measured 48h later by quantification of luciferase production in a
106 luminometer.
107 **Generation of latently infected cells**
108 Latently infected Jurkat cells (J-Hig) were generated following a modified protocol described by
109 Li et al (18). Briefly, cells were generated after acute infection of CD4+ Jurkat cells with HIV-1
110 HIG and maintained in culture for 10 days to allow for the attrition of productively infected
111 cells.
112
113 Latently infected primary $CD4^+$ T cells were generated according to the cytokine-polarized
114 primary T cells model of latency (25, 26) with few modifications. Briefly, naïve $CD4^+$ T cells
115 were activated with αCD3/αCD28 antibodies (1 µg/ml each; BD, Madrid, Spain) and
116 supplemented with TGFβ1 (10 µg/mL, Peprotech), αIL-12 (2 µg/mL) and αIL-4 (1 µg/mL,
117 Peprotech). Medium supplemented with rIL-2 (30 IU/mL, Roche) was replaced every 3 days.
118 After 7 days of activation, CD4+ T cells were infected with VSV-NL43-GFP by spinoculation
119 (1200xg, 1h 30 min at 37 ºC) and latently infected/GFP negative cells sorted three days later
120 using a FACSAria II flow cytometer (BD Biosciences).
121 **Compounds**

122 Acitretin was purchased from Selleckchem, vorinostat (VOR) was purchased from Prochifar srl
123 (Italy) and panobinostat (PNB) was purchased from LC Laboratories. Antiretroviral agent 30-
124 azido-30-deoxythymidine (zidovudine; AZT) was obtained from the NIH AIDS Research and
125 Reference Reagent Program. P300 inhibitor curcumin (CURC) was purchased from Sigma-
126 Aldrich. All compounds were reconstituted in DMSO and stored at -20 ºC until use. Control
127 (untreated) cell cultures contained an equivalent DMSO concentration to drug treated
128 cultures.
129
130 **HIV reactivation in vitro in latently infected cells.**
131 HIV reactivation was measured as described before (25). Briefly, J-Lat, which harbor an HIV
132 provirus containing the Green Fluorescent Protein (GFP) ORF instead of *nef* and a frame shift
133 mutation in *env* (23), or J-Hig cells were incubated for 24h with different concentrations of
134 acitretin, LRAs panobinostat and vorinostat were used as controls for HIV-1 reactivation.
135 Reactivation of HIV was monitored as the percentage of living GFP+ positive cells according to
136 forward and side laser light scatter flow cytometry analysis in a FACS LSRII flow cytometer (BD
137 Biosciences). The data were analyzed using the FlowJo software.
138 Similarly, ACH-2 cells, a T cell latent model with one integrated proviral copy, were cultured for
139 48h in the presence or absence of LRA and reactivation was measured by the production of
140 HIV CAp24 antigen using Genscreen HIV-1 Ag ELISA (BioRad) according to manufacturer's
141 instructions, or by detection of viral mRNA by quantitative PCR as described below. 48h
142 incubations were used to minimize cytotoxic effect commonly observed with known latency
143 reversing agents (data not shown)(25, 27)
144 Sorted latently infected/GFP negative naïve CD4+ T cells were incubated for 12h with PNB,
145 VOR or acitretin. Treatment with anti-CD3 and anti-CD28 was used as reactivation control.
146 Subsequently, cells were washed with PBS and kept in fresh media containing rIL-2 for 3 days
147 at 37 ºC and 5 % CO2, before measuring reactivation by flow cytometry (GFP positive cells).

Quantitative PCR

To assess HIV-1 reactivation in ACH-2 cells, total RNA was isolated using the QIAamp® Viral RNA Mini kit (Qiagen, Hilden, Germany), as recommended by the manufacturer, and retro-transcribed to cDNA by PrimeScript RT Master Mix (Takara Bio USA, Inc.). Quantification of HIV-1 reactivation was determined using a two-step quantitative polymerase chain reaction (qPCR) as described before (25) with few modifications. Briefly, samples were run in triplicate on cDNA using TaqMan Universal Master Mix II (Applied Biosystems) on a 7500 Real Time PCR System (Applied Biosystems) Real-Time PCR instrument. We used the following primer and probe set for conserved regions of the 5'LTR of HIV-1 mRNA: Forward primer (5'-3') GACGCAGGACTCGGCTTG; reverse primer (5'–3') ACTGACGCTCTCGCACCC and probe (5'–3') FAM-TTTGGCGTACTCACCAGTCGCCG-TAMRA. Cycling conditions were as follows: 50 ºC for 2 min followed by 95 ºC for 10 min for polymerase activation, followed by 50 cycles of 95 ºC for 15 s and 60 ºC for 1 min. A standard curve from 10^6 to 10^2 copies of HIV-1 5'LTR was performed using ACH-2 DNA and run in parallel with samples in order to quantify absolute viral RNA copy numbers in cell supernatant.

Immunoblot

Treated cells were rinsed in ice-cold PBS and extracts prepared in lysis buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM Na Pyrophosphate, 270 mM sucrose and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride. Lysates were subjected to SDS-PAGE and transferred to a PVDF membrane (ImmunolonP, Thermo). The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce); anti-human Hsp90 (1:1000; 610418, BD Biosciences); anti-PARP cleaved (1:1000 or 1:10000 depending on the cell type used; ab32046, abcam) and anti-GAPDH (1:1000; ab9485, abcam); anti-MDA-5 (1:500; 5321), anti-phospho-

STAT1 (9177), anti-RIG-I (3743), anti-phospho-IRF3 (4947), anti-IRF3 (11904) and anti-MAVS (3993) (all 1:1000; Cell Signalling Technologies).

Flow cytometry

For evaluation of cell death, cells were stained for 30 minutes with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) in PBS according to manufacturer's instructions. Cells were washed and fixed in 1% formaldehyde before the analysis. Acitretin selective clearance of HIV+ cells was measured by annexin V expression which stains for apoptotic cells. Cells were suspended in the Annexin-V APC antibody (BD Pharmigen) 30 minutes before the cytometry analysis. Antibodies were diluted 1/20 in Annexin V Binding Buffer 1X (BD Pharmigen). Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using the FlowJo software (BD Biosciences). Viability determinations were performed in triplicates and data calculated from three independent experiments as done before (15).

Statistical analyses

Data are presented as Mean ± standard deviation (SD). All p-values were calculated using a t-student test with the GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). A p-value of 0.05 was considered to be statistically significant.

Results

Acitretin efficacy as an HIV-1 latency reversing agent

To evaluate the efficacy of acitretin as a LRA, we compared its effect to that of known LRA panobinostat and vorinostat in two commonly used models of HIV reactivation. We were unable to detect HIV reactivation in acitretin-treated cells (up to 25 μM) in two different clones of J-Lat cells (clones 8.4 and 9.2) at conditions in which a clear and dose-dependent effect was observed for panobinostat or vorinostat (Figure 1A and 1B). Combination of acitretin with LRA panobinostat or vorinostat did not show any significant difference in HIV reactivation to the LRA alone (Fig. S1a). Similarly, curcumin, a p300 inhibitor used to counteract the effect of acitretin (18), did not have any relevant effect in the presence of acitretin (Fig. S1B and S1C). Moreover, acitretin did not induce significant HIV reactivation in ACH-2 cells, as seen by both p24 and viral mRNA copies in the cell supernatant (Fig. 1C and 1D).

J-Lat clones and ACH-2 cells may differ in the number of HIV integrated copies and their integration site in the cell genome and thus may have differential susceptibility to LRA. To exclude a cell-dependent lack of potency, HIV latently infected cells were generated in house (J-Hig) and HIV reactivation in the presence of LRA and acitretin was tested. In this model, acitretin was able to induce HIV reactivation. However, the effect of acitretin on J-Hig cells was modest as compared to optimal panobinostat or vorinostat concentrations (Figure 2A and Fig. S2). In addition, acitretin was not able to induce HIV reactivation in latently infected primary $CD4^+$ T lymphocytes generated *in vitro* (Fig. 2b). Conversely, acitretin induced the expression of integrin α4 and β7 in activated CD4+ T cells, a common marker of retinoid induced T cell activation (Fig. 2C), indicating that acitretin was indeed active at the concentrations used. Taken together, these results indicate that acitretin is, at most, a modest or weak inducer of HIV reactivation.

Acitretin did not selectively clear HIV-infected cells

Pharmacological HIV reactivation is thought as a mean to trigger the death of HIV+ cells that otherwise would remain latent and unrecognized by the immune system (28, 29). We explored the capacity of acitretin to selectively induce apoptosis in HIV-reactivated cells as suggested by Li et al (18). Induction of HIV reactivation in J-Hig cells was followed in parallel to the evaluation of cell death. Panobinostat and vorinostat induced a cell-dependent cytotoxic effect on all cell lines tested, but acitretin did not (Table 1, and Fig. S3). In order to evaluate the selected killing of HIV+ cells, we measured annexin-V staining in J-Hig and in HIV latently infected CD4+ T cells after 12h and 24h of treatment. Following Li et al (18), the double positive (annexin+/HIV+) fraction was compared to the annexin+/HIV- fraction (Fig. 3A and 3C). The majority of dead cells induced by panobinostat, vorinostat or acitretin belong to the annexin+/HIV- fraction (Fig. 3B). That is, neither panobinostat, vorinostat nor acitretin selectively killed HIV+ cells (Fig. 3D). This result was confirmed in primary CD4+ T cells, since none of the conditions presented more apoptotic GFP+ (HIV+) cells than the untreated control (Fig. 3E and Fig. S5). Acitretin concentrations were up to >5-fold and >10-fold higher than those used by Li et al (18).

Acitretin is a weak inducer of the RIG-I-signaling-pathway

To evaluate acitretin capacity to enhance RIG-I signaling pathway we observed and quantified RIG-I protein expression and its downstream effectors such as MAVS and IRF3 in several cell lines. Similarly to Li et al (18), TZMbl cells were treated with acitretin, vorinostat and panobinostat for 48h in the presence or not of HIV-1 . Acitretin enhanced RIG-I, MAVS in both uninfected and infected cells (Fig. 4A) Moreover, a slight but not significant increase of the apoptosis marker PARP cleaved, was also observed. Similar results were obtained in both ACH-2 cells (Fig. 4B), and uninfected and infected primary monocyte derived macrophages (Fig. 5A) and primary resting and activated CD4+ T cells (Fig. 5B); all of them showing modest increases in MDA-5, RIG-I, MAVS or IRF-3 expression after acitretin treatment. The modest effects on RIG-I signaling pathway after culture with acitretin, concur with the lack of antiviral effect of

242 acitretin observed in acute infection (Fig. S4). However, LRA such vorinostat also are able to

243 induce some of the observed changes (Fig. 4 and Fig. 5).

Discussion

The identification of acitretin as a LRA and selective inducer of HIV+ cell death(18) offered a fast-lane opportunity to treat and cure HIV infection: it is an approved drug used to treat autoimmune diseases such as psoriasis, triggers what appears to be a favorable immune response and was shown to culminate in the preferential apoptotic death of the reactivated HIV reservoir cells(18). We strongly challenge these conclusions, as we could not recapitulate the promising results previously shown with acitretin.

Li et al. compared the effect of acitretin at 5 μM to that of vorinostat at 0.350 μM. That is, 11.3-fold lower vorinostat concentration than its reported fifty percent effective concentration (EC_{50})(27). Indeed, we found that the latency reversal activity of acitretin, at 25 μM, was only commensurate to suboptimal concentrations of an already relatively weak LRA, vorinostat(30, 31), and negligible if compared to second generation HDAC inhibitors such as panobinostat (27) (Fig. 2A). Acitretin LRA activity was undetectable in a HIV latency model in primary CD4+ T cells(25), and was only weakly observed in one of three different cell line models used, suggesting that acitretin's effect on HIV reactivation would be insufficient to trigger a desirable effect.

Despite early indications that RNA sensing of HIV-1 infection may be counteracted by a protease-mediated sequestration of RIG-I(32), we, as others, have shown that HIV-1 infection may induce the expression of genes involved in antiviral signaling, including MDA-5 and RIG-I in primary cell cultures (12, 13, 33). RIG-I expression may also be associated to disease progression in HIV+ individuals(34). These effects have been associated with IFN-mediated cell death. However, the effect of acitretin in the stimulation of the RIG-I pathway leading to IFN production was again mild in the laboratory adapted HeLa-derived TZMbl cells, in ACH-2 cells or primary macrophages and activated and resting CD4+ T cells. Importantly, the effect of acitretin on RIG-I stimulation was neither specific nor selective for HIV+ cells (Figure 4) as stimulation of RIG-I, MDA-5 and IRF3 were not differentially observed in infected acitretin-

treated cells. These results are in line with a mild cytotoxic effect of acitretin in both infected and uninfected cells.

The proposed selective killing of HIV+ latently infected cells deserves particular attention when considering the method employed to evaluate its significance by Li et al (18). Comparing the percentage of HIV+ dead cells to that of HIV- dead cells in the presence or absence of an apoptosis inducer may indeed provide clues to purging and eliminating unwanted HIV+ cells. Selective destruction of HIV+ cells is the goal of a "shock and kill"(28, 35) therapeutic strategy. However, we failed to detect a significant number of HIV+ dead cells by acitretin as compared to dead of uninfected cells at acitretin concentrations that effectively affect α4 and β7 integrin expression. Of note, the absolute number of uninfected dead cells was significantly higher than that of infected dead cells, indicating lack of selectivity for HIV+ cells. We do not discard the possibility of differences in virus strains or cell culture conditions used that could explain our discrepancies with the results shown by Li et al. The virus genome between the two studies is different and we used a Nef-deleted virus in our cultures. Nevertheless, loss of the accessory protein Nef is not necessary for HIV replication in tissue culture.

Innate immune protection from HIV-1 infection may be associated with the inability of the virus to surpass cell restriction without negatively affecting the cells' proliferative capacity (36) and be detected by pattern recognition receptors (12, 13), indicating that stimulation of such recognition is justified as a potential therapeutic strategy. However, our results suggest that acitretin-mediated stimulation of the RIG-I pathway over HIV reactivation is modest and thus may not meaningfully impact the HIV reservoir. Effective LRAs may only be defined when they can by themselves induce measureable clearance of persistent HIV infection or, when they can be appropriately paired with viral clearance strategies that result in depletion of latency (37). Stimulation of the RIG-I-dependent IFN cascade by acitretin alone may not significantly affect the selective destruction of HIV+, latently infected cells.

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431 **Table 1. Citotoxicity concentration (CC_{50}*) of the compounds in the cell lines tested**

	J-Lat (clone 8.4)	J-Lat (clone 9.2)	ACH-2	J-Hig
Panobinostat (µM)	>4	<0.16	<0.16	>4
Vorinostat (µM)	>4	1,16	1,16	>4
Acitretin (µM)	>125	>125	>125	>25
Curcumin (µM)	39.18	34.38	20.64	-

432 * 50% effective concentration or the concentration needed to induced 50% cell death induced

433 by the indicated drug.

434 **FIGURE LEGENDS**

435 **Fig. 1.** Acitretin does not induce HIV reactivation in J-lat and ACH-2 cells. (a, b) HIV reactivation
436 induced by acid retinoic (AR) derivative acitretin (25 – 1 µM) in J-Lat cells clone 8.4 (a) and 9.2
437 (b). HDAC inhibitors (HDCAi) panobinostat (PNB, 0.16µM) and vorinostat (VOR, 4 – 0.16 µM)
438 were used as controls. Reactivation was determined by the quantification of GFP+ cells (%)
439 after culturing J-Lat with HDACi and acitretin for 24h. (c, d) HIV reactivation induced by
440 acitretin and HDACi in ACH-2 cells. Reactivation was determined after 48h of incubation by
441 quantification of CAp24 (c) and HIV RNA copy number (d) in the supernatant. Values represent
442 mean±SD of at least three independent experiments performed in triplicate. UN, untreated. *P
443 < 0.05; **P < 0.01; ***P < 0.001.

444 **Fig. 2.** Acitretin effect as a HIV reactivator in latently infected Jurkat cells and primary naïve
445 CD4+ T lymphocytes. (a) HIV reactivation induced by acitretin (25 – 1 µM) in HIV latently
446 infected Jurkat (J-Hig) cells. Panobinostat (PNB, 0.16 µM), vorinostat (VOR, 4 – 0.16 µM) were
447 used as controls. Reactivation was quantified after 24h as GFP+ (%) cells in the non-apoptotic
448 population, measured with a αAnnexin V antibody. (b) HIV reactivation induced by acitretin
449 and the HDACi in primary latently infected CD4+ T cells. Naïve CD4+ T cells were activated for 7
450 days followed by VSV-NL43-GFP infection. After three days, GFP- cells were sorted and
451 incubated for 12h with acitretin. Panobinostat (PNB, 0.16µM), vorinostat (VOR, 4 – 0.16µM)
452 were used as controls. (c) Acitretin activity as a retinoic acid derivative in activated peripheral
453 blood mononuclear cells (PBMCs). Integrin α4 and β7 expression induced by acitretin was
454 assessed in activated PBMCs with IL-2 and αCD3-αCD28 for 7 days followed by 72h incubation
455 with IL-2 in presence or absence of acitretin (25 µM). Integrin overexpression was measured by
456 flow cytometry. Values represent Mean ± SD of at least three independent experiments
457 performed in triplicate. UN, untreated. *P < 0.05; **P < 0.01; ***P < 0.001.

458 **Fig. 3.** Acitretin does not selectively kill HIV-reactivated cells. (a) Apoptosis percentage in HIV
459 reactivated (GFP+) and not reactivated (GFP-) latently infected Jurkat cells (J-Hig). Cells were
460 incubated for 24h with acitretin (25 – 1 µM), panobinostat (PNB, 0.16 µM) and vorinostat
461 (VOR, 4 – 0.16µM). Apoptosis percentage shown in the graphic represents a fraction of the
462 total GFP+/- subpopulation. Data was evaluated by flow cytometry with a double staining for
463 HIV reactivation (GFP+) and cell apoptosis (Annexin V+). (b) Cell distribution of apoptotic
464 and/or HIV reactivated J-Hig cells of the results shown in (a), without taking into account the
465 fraction of GFP+ or GFP- cells. Assays were evaluated by flow cytometry. (c) Representative
466 cytometry plots from (a) showing the four subpopulations: GFP+/AnnexinV+, GFP+/AnnexinV-,
467 GFP-/AnnexinV+ and GFP-/AnnexinV-. (d) Apoptosis ratio between the HIV reactivated (GFP+)
468 population and the no-reactivated (GFP-) population in J-Hig cells. A selective drug against HIV
469 reactivated cells is expected to have >1 ratio. (e) Apoptosis percentage in HIV reactivated
470 (GFP+) and not-reactivated (GFP-) latently infected primary CD4+ T cells. Cells were incubated
471 for 12h with acitretin, panobinostat and vorinostat. Anti-CD3 anti-CD28 condition was used as
472 a reactivation control. Apoptosis percentage shown in the graphic represents a fraction of the
473 total GFP+/- subpopulation. Values represent mean ± SD of at least three independent
474 experiments performed in triplicate. UN, untreated.

475 **Fig. 4.** Effect of acitretin compared to panobinostat and vorinostat in different cell lines. (a)
476 Representative immunoblot (left panel) and graph representing density band peaks
477 quantification (right panel) of TZMbl cells with expression of RIG-I, MAVS, pIRF3, total IRF3,
478 PARP cleaved and Hsp90 in uninfected or infected conditions with NL4-3 at 48h post-
479 treatment with AZT 1 µg/mL, acitretin 1 µM, panobinostat 0.5 µM and vorinostat 0.35 µM.
480 Data was relativized to untreated control of at least three independent experiments. A
481 representative donor is shown. (b) Representative immunoblot (left panel) and graph
482 representing density band peaks quantification (right panel) of ACH-2 cells with protein
483 expression of MDA-5, RIG-I, MAVS, total IRF3 and GAPDH after 48h treatment with acitretin 25

µM or 5 µM and vorinostat 0.35 µM. Data was relativized to untreated control of at least three independent experiments. A representative experiment is shown. ND; No drug, AZT; zidovudine, ACI; acitretin, PNB; panobinostat, VOR; vorinostat, UN; uninfected, INF; infected. * p<0.05; ** p<0.005; *** p<0.0005.

Fig. 5. Effect of acitretin compared to panobinostat and vorinostat in primary cells. (a) Representative immunoblot (left panel) and graph representing density band peaks quantification (right panel) of treated monocyte derived macrophages (MDM) with acitretin at 10 µM and 2 µM. Protein expression of RIG-I, MAVS, total IRF3, PARP cleaved and GAPDH 24h post-treatment is shown. Data was relativized to untreated control of at least two independent experiments. A representative donor is shown. (b, c) Representative immunoblot (left panel) and graph representing density band peaks (right panel) of resting (b) or activated (c) CD4+ T cells with protein expression of MDA-5, RIG-I, MAVS, total IRF3, PARP cleaved and GAPDH in uninfected or infected conditions after 48h treatment with acitretin 5 µM and vorinostat 0.35 µM. Data was relativized to untreated control of at least three independent experiments. A representative donor is shown. ND; No drug, AZT; zidovudine, ACI; acitretin, PBNB; panobinostat, VOR; vorinostat, UN; uninfected, INF; infected. * p<0.05; ** p<0.005; *** p<0.0005.

Figure 1

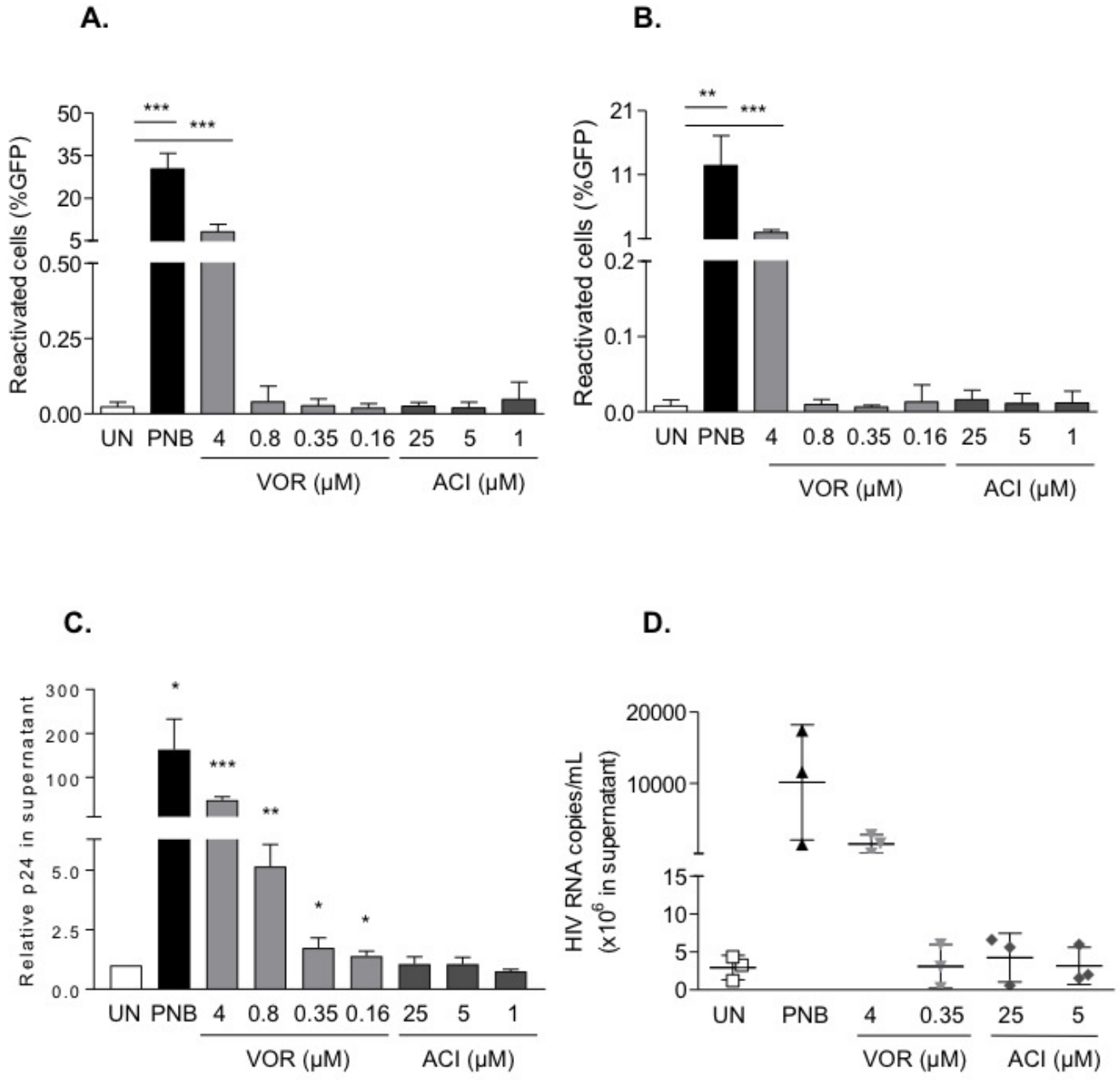

Figure 2

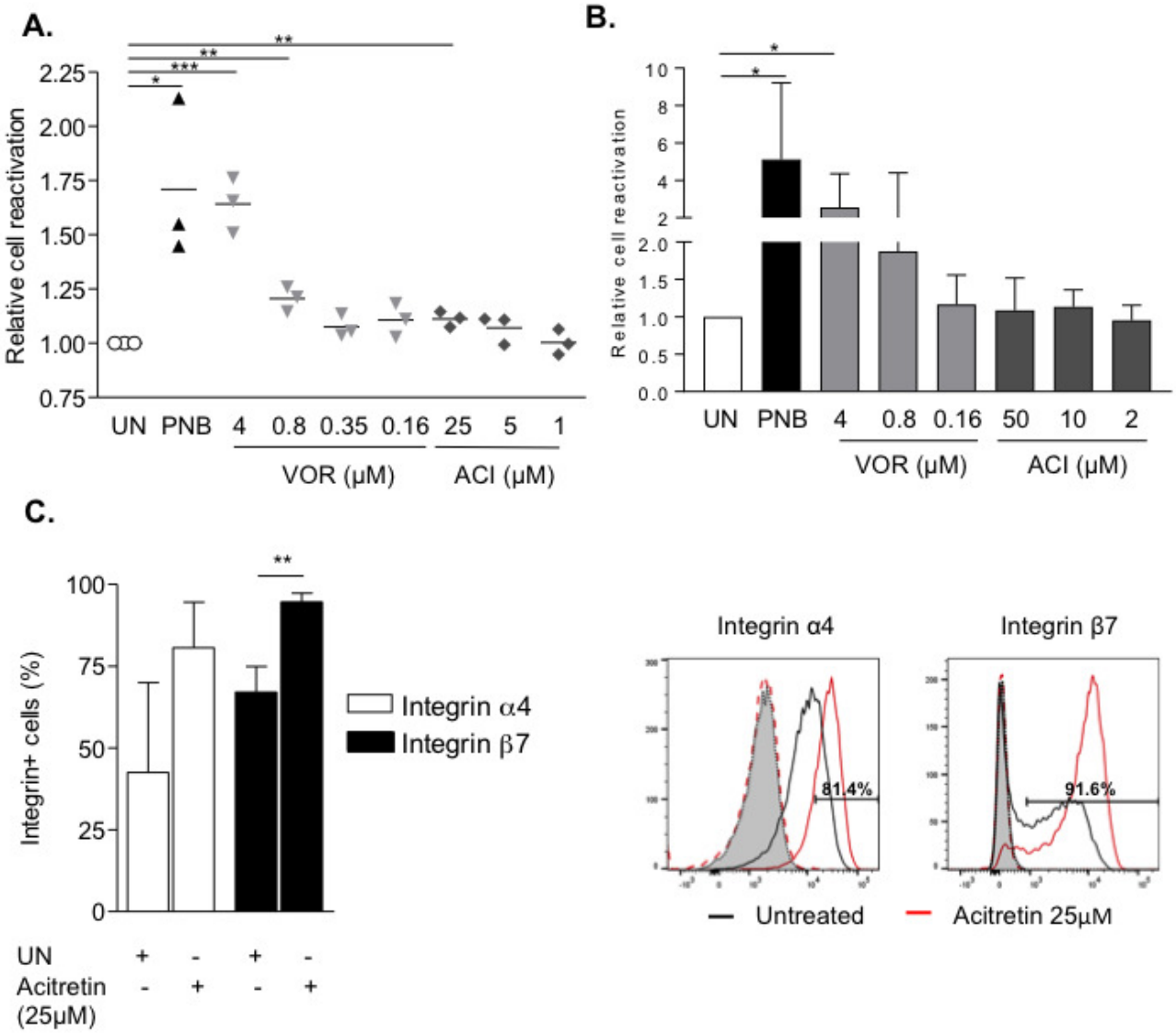

Figure 3

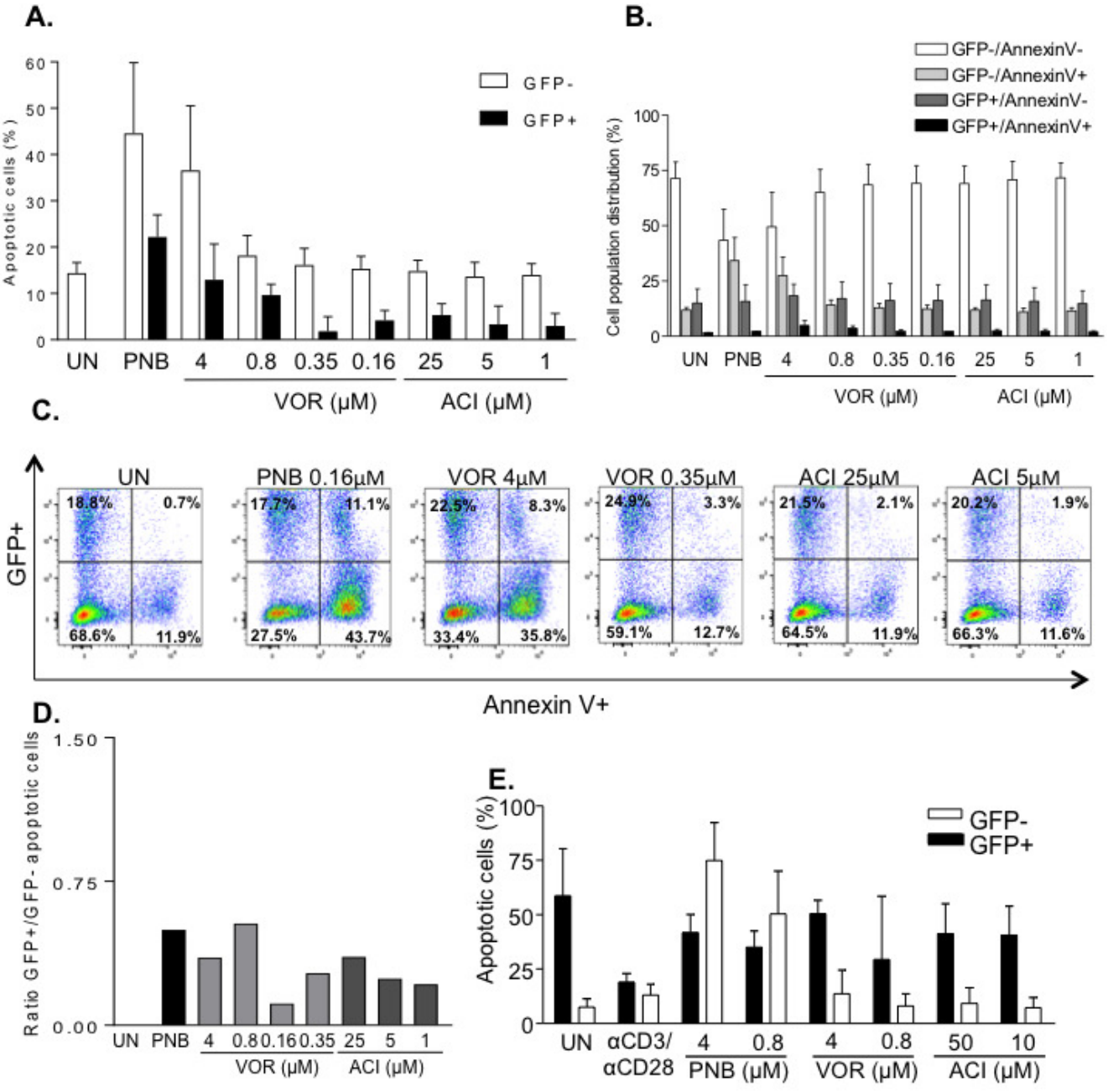

Figure 4

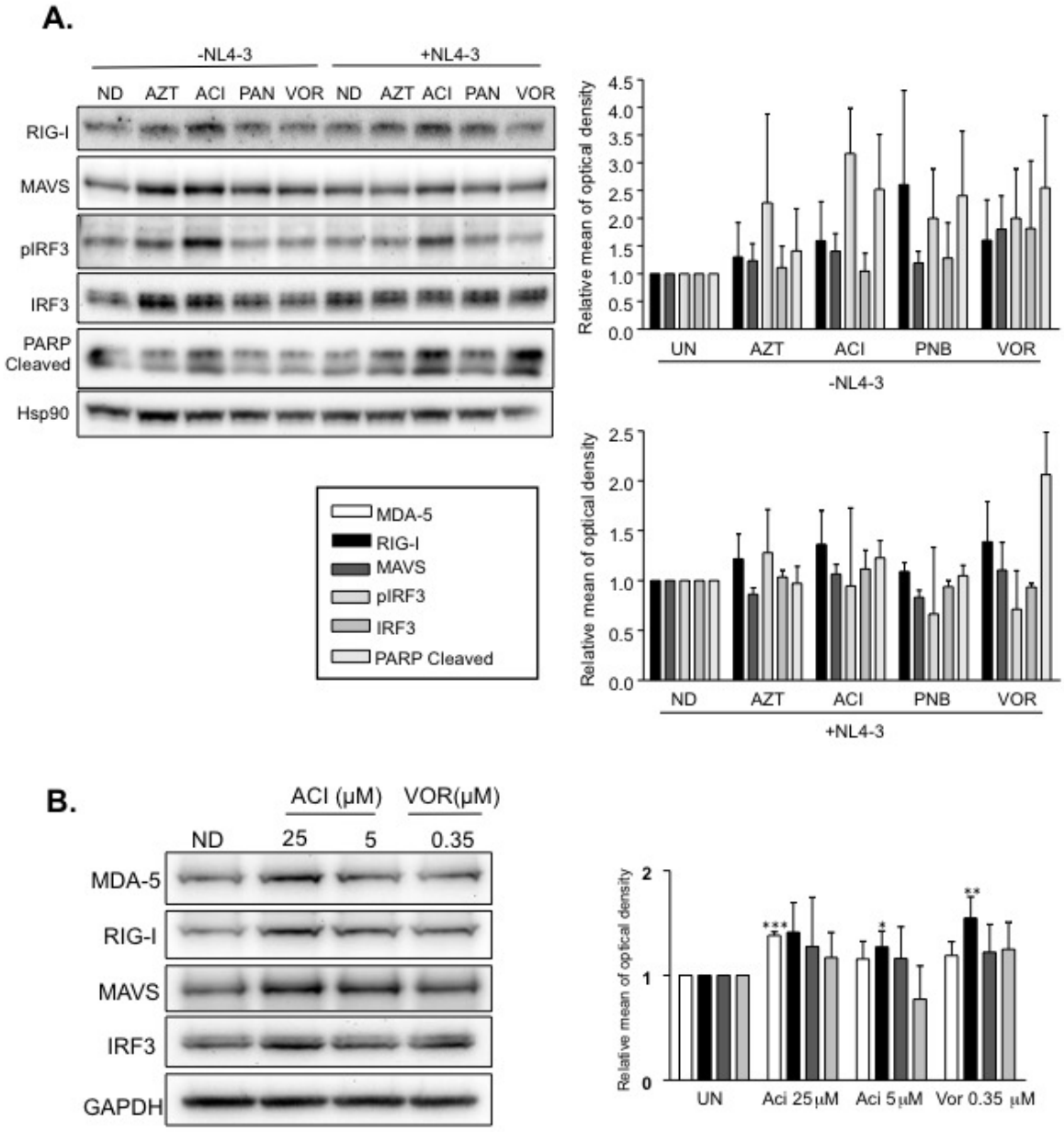

Figure 5