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Oxaliplatin antagonizes HIV-1 latency by activating NF-κB without causing global T cell activation

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ABSTRACT

Reactivation of latent HIV-1 is a promising strategy for the clearance of the viral reservoirs. Because of the limitations of current agents, identification of new latency activators is urgently required. Using an established model of HIV-1 latency, we examined the effect of Oxaliplatin on latent HIV-1 reactivation. We showed that Oxaliplatin, alone or in combination with valproic acid (VPA), was able to reactivate HIV-1 without inducing global T cell activation. We also provided evidence that Oxaliplatin reactivated HIV-1 expression by inducing nuclear factor kappa B (NF-κB) nuclear translocation. Our results indicated that Oxaliplatin could be a potential drug candidate for anti-latency therapies.

1. Introduction

Highly active antiretroviral therapy (HAART) has successfully controlled viremia down to undetectable levels. Nevertheless, antiretroviral therapy for HIV-1 infection needs lifelong access and strict adherence to regimens that are both expensive and associated with toxic effects [1] and a sudden rebound of the viral load after interruption of HAART is generally observed. The existence of a stable population of latently infected CD4+ T cells, and perhaps other cell types, which cannot be eliminated by HAART on its own is now regarded as the main barrier to viral eradication. For this reason, the eradication of viral reservoirs is at present an urgent requirement for HIV-1 therapeutics [2].

Notwithstanding that mechanisms allowing HIV-1 to establish and maintain latency are not well defined, a number of factors, and others possibly acting simultaneously may contribute to the molecular mechanisms underlying HIV-1 proviral latency [3,4], mostly through the suppression of transcription involving several activated factors such as nuclear factor kappa B (NF-kB), nuclear factor of activated T cells (NFAT), activating protein-1 (AP-1) and specificity protein 1 (SP1) [5]. Another positive transcriptional elongation factor b (P-TEFb) levels, which impede efficient transcription elongation from the HIV promoter is also involved in transcriptional block observed in latently infected cells [6,7]. Moreover, HIV-1 transcription is determined by the epigenetic silencing such as histone deacetylation, histone methylation and DNA methylation [8].

So far a lot of small molecules targeting the different mechanisms involved in maintaining latency have been explored and can be divided into the following groups: (1) histone deacetylase inhibitors; (2) cytokines and chemokines; (3) DNA methyltransferase inhibitors; (4) histone methyltransferase inhibitors; (5) protein kinase C activators; (6) positive transcription elongation factor b activators; (7) some unclassified agents, such as disulfiram [9]. These have been suggested as agents to reactivate HIV-1 and eradicate the viral reservoirs. However, most of these drugs have either limited efficacy in clinical trials or various side effects. New agents or combinations of agents need to be explored.

Oxaliplatin, an FDA-approved anti-cancer drug, is a third-generation platinum compound that confers a different spectrum of activity compared with cisplatin [10]. Although the therapeutic potential of Oxaliplatin on cancers has been explored, such as ovarian, non-small-cell lung and breast cancer and non-Hodgkin lymphoma [11], research on its potential use in the treatment of HIV-1 remains little known. In our study, we investigated the ability and the molecular mechanisms of the Oxaliplatin to induce expression of HIV-1 in latently infected cells, and the effect of Oxaliplatin in combinations with other activators on HIV-1 production. We found that oxaliplatin reactivates latent HIV-1 in this cell line model of HIV-1 latency through activation of NF-kB and does not induce T cell activation and the reactivation is synergized when combination with valproic acid. Our results suggested that Oxaliplatin regulates HIV-1 LTR gene expression through NF-κB signal pathway and it can be a potential drug for anti-latency therapies.

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2. Materials and methods

2.1. Cell culture and chemical treatment

C11 cells, a type of latently infected Jurkat cell encoding the green fluorescent protein (GFP) as a marker for Tat-driven HIV LTR expression, were constructed in our lab and used elsewhere [12–14]. The C11 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml of streptomycin (Invitrogen) at 37 °C under 5% CO2. Human embryonic kidney 293 cells (HEK 293) were purchased from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL of streptomycin at 37 °C under 5% CO_2.

Oxaliplatin was purchased from ZhongShan Hospital (Shanghai, China). 5-azacytidine (5-Aza) and Aspirin were purchased from Sigma–Aldrich (Shanghai, China) and valproic acid (VPA) was purchased from InvivoGen (San Diego, CA, USA). Prostratin was purchased from LC laboratories (Woburn, MA, USA). Recombinant human TNF-α was purchased from Chemicon International (Temecula, CA, USA). Oxaliplatin (100 mM), TNF-α (1 mg/mL), VPA (100 mM), 5-Aza (10 mM) and Aspirin (1 M) were dissolved in anhydrous dimethyl sulfoxide (DMSO) and stored at −20 °C.

2.2. Flow cytometry

C11 cells were washed with phosphate-buffered saline (PBS) and incubated with the indicated concentrations of Oxaliplatin at different points in time, or pretreated with various concentrations of (0, 2.5, and 5 mM) Aspirin for 3 h and subsequently treated with Oxaliplatin (10 μM) or VPA (2 mM) or 5-Aza (100 nM) or control medium for indicated hours. Oxaliplatin-mediated T-cell activation in PBMCs was monitored after staining for activation markers with FITC conjugated anti-CD25 and PE conjugated anti-CD69 antibodies. The Flow cytometry was performed as previously described [13]. All experiments were performed independently at least three times in triplicate per experimental point.

2.3. Isolation of human peripheral blood mononuclear cells (PBMCs)

Whole peripheral blood from healthy donors was purchased from the Blood Center of Shanghai (Shanghai, China). The PBMCs isolation was made by the difference of gradient density Ficoll-Hypaque (density = 1.077 g/mL, Haoyang Biological manufacture, Tianjin, China). After centrifugation (400 × g; 30 min at room temperature), the PBMCs were found at the plasma/Ficoll-Hypaque interphase and collected carefully with a Pasteur pipette. After that, the cells were washed in PBS twice (240 × g for 10 min) and resuspended in RPMI 1640 medium containing 4.5 g/L glucose supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin and 100 μg/mL of streptomycin.

2.4. Cytotoxicity assay

Proliferation and viability were measured via A Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Gaithersburg, MD, USA) assays [13,15]. HEK 293 and PBMCs, approximately 4×10^4 cells per well (96-well plates were used), were treated with or without Oxaliplatin for 48 h, 10 μL of CCK-8 solution was added to each well of the plate. After 4 h of incubation under 37 °C, measures of the absorbance at 450 nm were taken using a microplate reader. In this assay, the 50% cytotoxic concentration (CC50) was at least three times in triplicate per experimental point.

2.5. Cell nuclear protein extraction and electrophoretic mobility shift assay

Nuclear extracts from C11 cells with different treatments were obtained as previously described [13,16]. C11 cells were pretreated with Aspirin (10 mM) for 1 h and subsequently treated with Oxaliplatin or TNF-α (10 ng/mL) at indicated concentrations for 3 h. Cell nuclear protein extraction and electrophoretic mobility shift assay were performed as previously described [13].

3. Results

3.1. Oxaliplatin potently activates latent HIV-1 replication

To investigate if Oxaliplatin has the ability to induce HIV-1 expression in latently infected cells, we used C11 cells which are latently infected Jurkat T cells with a single provirus integrated into intron of RNPS1 and a GFP gene under control of the HIV-1 LTR as a marker of HIV-1 LTR expression [14]. The structure of Oxaliplatin and VPA are shown in Fig. 1A. C11 cells were treated with Oxaliplatin for 48 h or VPA for 96 h at different concentrations, and then the percentage of GFP-expressing cells was measured by flow cytometry. We found that the percentage of GFP-positive cells increased to 16.7% for Oxaliplatin, 13.5% for VPA (Fig. 1B). As shown in Fig. 1C, addition of micromole concentrations of Oxaliplatin to the culture medium for 48 h increased the GFP-expressing cells by 2–16-fold over background levels. The maximum efficacy of VPA was 13.5% compared with mock controls at the concentration of 2 mM for 96 h, but VPA showed to be toxic to C11 cells at the concentration of 4 mM. These results demonstrated that Oxaliplatin induced HIV-1 LTR reactivation, indicating Oxaliplatin's effect on HIV-1 production in a dose-dependent manner.

To analyze the kinetics of HIV-1 LTR expression induced by Oxaliplatin, we performed a kinetics experiment in which C11 cells were grown for 1–4 days with or without Oxaliplatin (10 μM) or VPA (2 mM). At each time point, GFP-expressing cells were assayed by flow cytometry. As shown in Fig. 1D, after the C11 cells were treated with Oxaliplatin, the percentage of GFP-expressing cells increased for the first 2 days then decreased from the 3rd day. The HIV-1 LTR expression induced by VPA (2 mM) increased over time during the 4 days. These results indicated Oxaliplatin's effect on HIV-1 production in a time-dependent manner.

3.2. Oxaliplatin synergistically reactivates the latent HIV-1 production

To assess whether Oxaliplatin synergistically reactivates the HIV-1 promoter in C11 cells when combined with VPA or 5-Aza, C11 cells were treated with or without Oxaliplatin alone (10 μM), VPA alone (2 mM), 5-Aza alone (100 nM) or Oxaliplatin (10 μM)/VPA (2 mM), Oxaliplatin (10 μM)/5-Aza (100 nM) for 48 h, respectively. Two activators synergize as the combination produces a greater activation level than the sum of the effects produced by individual activators [17]. As shown in Fig. 2, the percentage of GFP-positive cells was 13.63% in Oxaliplatin alone (10 μM), 4.3% in VPA alone (2 mM), 5.69% in 5-Aza alone (100 nM); 42.01% in Oxaliplatin/VPA, 23.74% in Oxaliplatin/5-Aza. These results indicated that Oxaliplatin in combination with VPA resulted in the synergistic reactivation of the latent HIV-1 production in the C11 cells.

3.3. Oxaliplatin exhibits unobvious toxicity in vitro and without inducing global T cell activation

To measure potential toxicity of Oxaliplatin, PBMCs were treated with Oxaliplatin or Prostratin at the designated concentrations for 72 h, and then the cells were subjected to a CCK-8 assay. We did

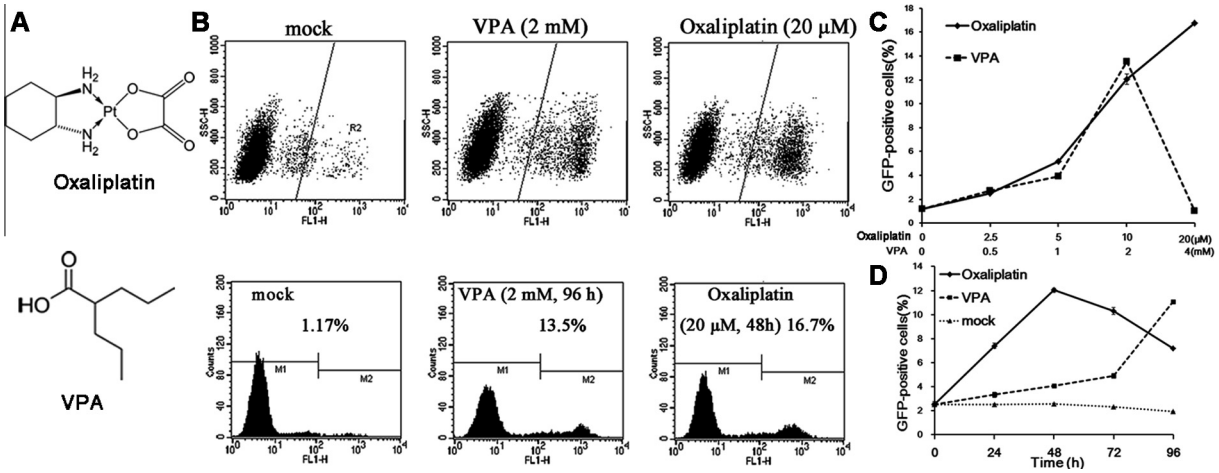

Fig. 1. Reactivation of latent HIV-1 in latently infected cells by Oxaliplatin and VPA. (A) The structure of Oxaliplatin and VPA. (B) Florescence histograms of C11 cells treated with Oxaliplatin (20 μM) for 48 h or VPA (2 mm) for 96 h. (C) C11 cells treated with Oxaliplatin for 48 h or VPA for 96 h at the indicated concentrations. (D) Time-dependent effects of Oxaliplatin and VPA on HIV-1 production. C11 cells were mock treated or treated with Oxaliplatin (10 μM) or VPA (2 mM) at the indicated times. Data show dose-dependent effects of Oxaliplatin and VPA on HIV-1 production in C11 cells and represent the means ± standard deviations of three independent experiments.

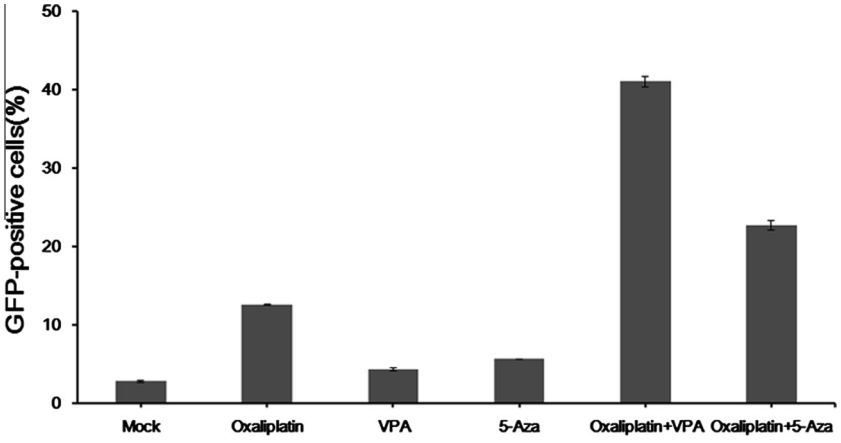

Fig. 2. Synergistic activation of HIV-1 promoter by Oxaliplatin and VPA, 5-Aza in latently infected cells. C11 cells were mock treated or treated with Oxaliplatin (10 μM), VPA (2 mM), 5-Aza (500 nM), Oxaliplatin/5-Aza or Oxaliplatin/VPA. The effects of synergistic activation of HIV-1 promoter were determined by quantifying the GFP-positive cells using flow cytometry 48 h after treatment. Data represent the means ± standard deviations of three independent experiments.

correlation analysis between the concentrations of the drugs and the viability of cells. Oxaliplatin exhibited no significant toxicity in PBMCs at its active concentrations but Prostratin showed toxicity at concentration above 100 μm (Fig. 3A).

Another major disadvantage of some current therapeutic agents is their propensity to non-specifically activate bystander T cells. We therefore investigated the induction of global T cell activation markers by Oxaliplatin. Human PBMCs were treated with Oxaliplatin (10 μm) or Prostratin (1 μm) for 48 h, and the expression of CD25 and CD69 was detected by flow cytometry using antibodies against CD25 and CD69. Prostratin stimulated the expression of these activation markers at a relatively high level, similar to previously published results [18]. In contrast to Prostratin, no significant upregulation of CD25 or CD69 expression was observed in the Oxaliplatin-treated cells at its active concentration (10 μM) compared with untreated cells (Fig. 3B).

3.4. Oxaliplatin activates the HIV-1 LTR through induction of NF-κB

Next we explored the signaling pathway that mediates activation of the HIV-1 LTR by Oxaliplatin. The HIV-1 LTR consists of binding sites for several inducible transcription factors including NF-κB, AP-1, and Sp1 [19]. To testify the role of transcription factors in Oxaliplatin activation of the HIV LTR, HEK-293 cells were transfected with luciferase reporter plasmids containing the wild-type HIV-1 LTR, LTR lacking two κB enhancers, LTR lacking AP-1 enhancers, or LTR lacking Sp1 enhancers. We chose TNF-a as a positive control. Compared with mock controls, TNF-a induced about 5-fold upregulation of the HIV-LTR-Luc reporter, about 2-fold upregulation of the HIV-LTR (ΔAP-1)-Luc and 3-fold upregulation of the HIV-LTR (ΔSp1)-Luc reporters (Fig. 4A). Similarly, Oxaliplatin induced about 3-fold upregulation of the HIV-LTR-Luc reporter, about 1.7-fold upregulation of the HIV-LTR (ΔAP-1)-Luc and 1.3-fold upregulation of the HIV-LTR (ΔSp1)-Luc reporters but failed to activate the LTR (ΔκB)-luciferase reporter, indicating that NF-κB transcription factor plays an important role in Oxaliplatin-mediated activation of the latent HIV-1 LTR, which corresponds to previous findings that Oxaliplatin is a potent activator of NF-κB transcription factor [20].

To further confirm the role of NF-κB factors in Oxaliplatin activation of the HIV LTR, C11 cells were pretreated with Aspirin, the inhibitor of TNF-α-induced activation of NF-κB [21,22], and subsequently treated with Oxaliplatin (10 μM) or TNF-α (10 ng/mL) or control medium. The result showed that Aspirin pretreatment not only inhibit TNF-α-induced GFP expression in a dose-dependent manner but also strongly inhibit GFP expression

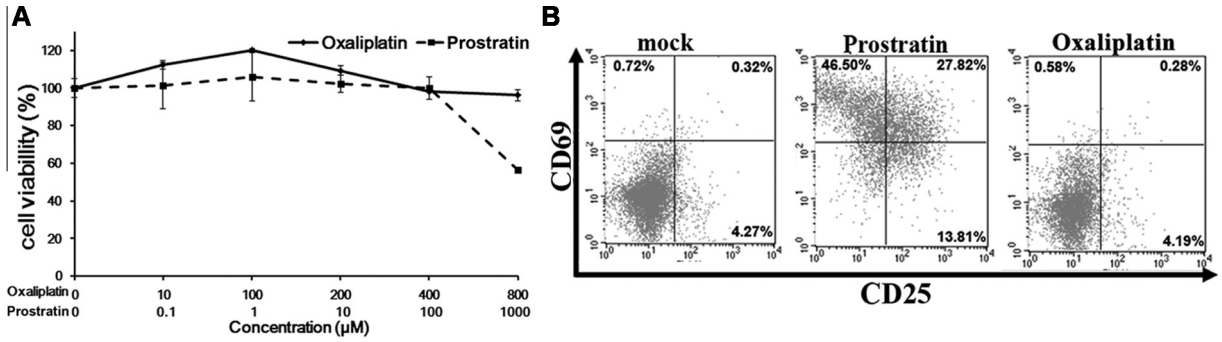

Fig. 3. Oxaliplatin exhibits unobvious toxicity and does not cause global T cell activation. (A) Human PBMCs were treated with Oxaliplatin or Prostratin at the indicated concentrations for 72 h and measured by the CCK-8 method. (B) The effect of Oxaliplatin and Prostratin on the expression of CD25 and CD69. Human PBMCs were treated with either Oxaliplatin (10 μm) or Prostratin (1 μm) for 24 h. Activation marker expression was detected by flow cytometry using antibodies against CD25 and CD69. Data represent the means ± standard deviations of three independent experiments.

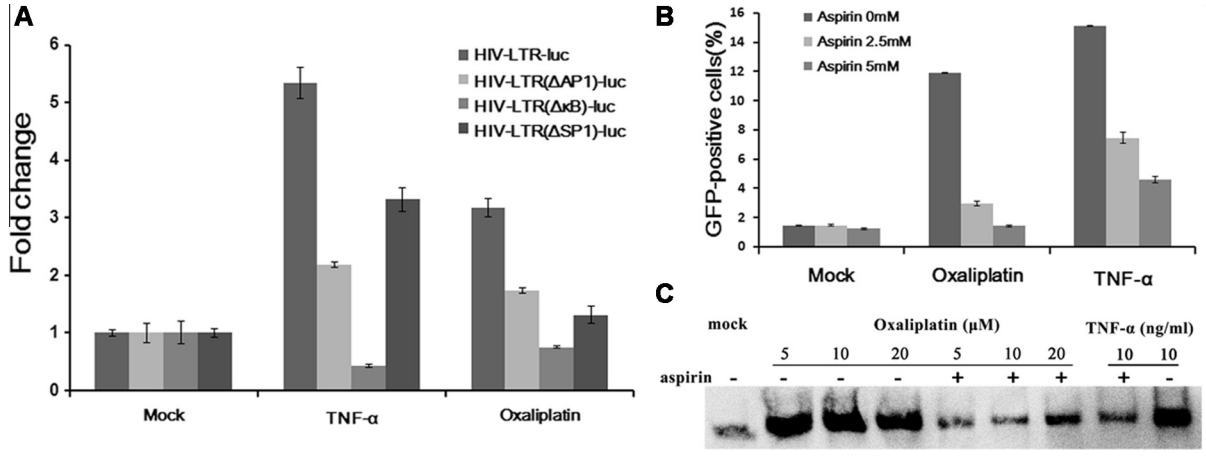

Fig. 4. Oxaliplatin activates the HIV-1 LTR through induction of NF-κB. (A) C11 cells were transfected with HIV1-LTR luc, HIV1-LTR (ΔκB) luc, HIV1-LTR (ΔAP-1) luc, and HIV1-LTR (ΔSp1) luc. At 24 h post-transfection, the cells were mock treated or treated with Oxaliplatin (10 μM) or TNF-α (10 ng/ml). Luciferase activity was measured after 24 h of treatment. The error bars indicate standard deviation. (B) C11 cells were pretreated with various concentrations of Aspirin for 3 h and subsequently treated with Oxaliplatin (10 μM) or TNF-α (10 ng/mL) or control medium for 48 h. The percentage of GFP + cells in Oxaliplatin- or TNF-α- treated cells either in the absence or in the presence of the inhibitor Aspirin was measured by flow cytometry. Data represent the means ± standard deviations of three independent experiments. (C) Oxaliplatin stimulates nuclear NF-κB DNA binding. C11 cells were pretreated with or without Aspirin (10 mM) for 1 h and subsequently treated with Oxaliplatin at the indicated concentrations or TNF-α (10 ng/mL) for 3 h. Nuclear extracts were isolated and subjected to electrophoretic mobility shift assay with biotin labeled NF-κB enhancer DNA probes.

induced by Oxaliplatin at the designated concentrations (Fig. 4B), further indicating a NF-κB-dependent signaling pathway in Oxaliplatin activation of the HIV LTR.

To assess whether Oxaliplatin treatment provided sufficient stimulus for NF-κB nuclear translocation and DNA binding, we performed an EMSA. Nuclear extracts from C11 cells treated with Oxaliplatin or TNF-α were incubated with biotin labeled NF-κB enhancer oligonucleotides. We found that Oxaliplatin strongly increased translocation of NF-κB to the nucleus in a dose-dependent manner and that this effect was suppressed by the NF-κB inhibitor Aspirin, which also inhibited the TNF-a-induced NF-κB DNA binding (Fig. 4C). These results further confirmed that NF-κB plays an important role in Oxaliplatin-mediated regulation of HIV-1 gene expression.

4. Discussion

It is clear now that multiple processes contribute to the HIV-1 latency which were partially identified from different models of HIV-1 latency [23]. A group of anti-latency therapeutic strategies nicknamed "shock and kill" was proposed based on this molecular understanding of HIV-1 latency [24]. Reactivation of latent HIV-1 infection is considered the prerequisite for elimination of the latent HIV-1 reservoir [25]. So many agents targeting different mechanisms of HIV-1 latency have been explored, such as VPA, 5-Aza, Prostratin, etc. However, their toxicity and ineffectiveness in clinical trials and prolonged treatment limit the clinical applications of such agents [9]. Thus, to explore better and more specific latency activators are urgently needed.

In this study, we use a simple model of HIV-1 latency to research new agents that reactivate the latently infected cells. The C11 cells is latently infected Jurkat T cells encoding GFP under the control of HIV-1 LTR as a marker of HIV-1 expression, which can be easily detected by fluorescence microscopy and flow cytometry [13]. Oxaliplatin has been proved effective in reactivation of HIV-1 latency and less toxic for C11 cells (Fig. 1 and Fig. 3). We choose VPA as a reference standard because VPA has a potent effect on reactivation of latent infection in both latently infected cell lines and ex vivo primary cells [26]. We further demonstrated that Oxaliplatin activated latent HIV-1 replication in a dose- and time-dependent manner.

The reactivation potential of Oxaliplatin alone is not very high, so we next tested if synergistic activations could be obtained when Oxaliplatin was combined with VPA or 5-Aza in C11 cells. 5-Aza is a small molecule inhibitor of DNA methylation, which is a potential latency-reversing agent [9,27]. Our results showed that cotreatment with Oxaliplatin/VPA induces HIV-1 expression in a higher proportion of C11 cells than the sum of the proportions induced by the individual activators, indicating that Oxaliplatin

combined with VPA can significantly synergize reactivation of HIV-1 production in latency model systems. These results are similar to the previous reports in which the proportion of J-Lat cells displaying GFP fluorescence was synergistically increased by NF-κB inducer/HDACI cotreatments compared with treatments with the compounds alone [17,28]. We also found that Oxaliplatin showed no obviously synergistic reactivation when used in combination with 5-Aza, possibly because DNA methylation has little relevance to sustaining latent HIV-1 infection in the latently infected C11 cell lines. Further experiments are needed to investigate more clear-cut answer to the mechanisms of interaction between these two different types of stimulants.

HIV-1 gene expression depends on both viral and cellular transcription factors with binding sites present in the HIV-1 LTR. The LTR harbors several DNA-binding sites for various cellular transcription factors, including NFAT, Ap-1, Sp1, NF-κB, LEF-1, COUP-TF, Ets1, USF, etc. [29,30]. Among them the transcription factor NF-κB plays a central role in the activation pathway of the HIV-1 provirus. Thus the identification of NF-κB activities has been proved as new research strategies against HIV-1 latency [31]. As described previously, Oxaliplatin was able to enhance NF-κB transcription in some cell lines [32,33]. So we investigated whether NF-κB signaling pathway is involved in Oxaliplatin-mediated activation of the latent HIV-1 LTR in the C11 cell model. Our findings showed that Oxaliplatin effectively activated the wild-type HIV-1 LTR-luciferase reporter, but failed to activate LTR lacking the κB enhancers reporter. It has been reported that Aspirin can inhibit NF-κB activation induced by TNF-α via preventing the phosphorylation and degradation of IκBα and nuclear translocation of NF-κB [21,22]. Thus we tested this by Aspirin block experiments with TNF-α as a control. We further showed that pretreatment of C11 cells with Aspirin prevented Oxaliplatin or TNF-α-induced HIV-1 reactivation. Furthermore, we demonstrated Oxaliplatin-induced translocation of NF-κB into the nucleus by EMSA. Taken together, these data indicated that Oxaliplatin activated HIV-1 gene expression through NF-κB signaling pathway.

In summary, the persistence of latent reservoirs of HIV-1 represents a major barrier to eradicate viruses in patients undergoing HAART. At present, "shock and kill" therapy has been proposed as a promising solution to purge these reservoirs by deliberately forcing HIV-1 gene expression in presence of HAART. Therefore, the identification of potent agents is urgently required for the treatment of HIV-1 latency in combination with HAART. For the first time, we demonstrated that Oxaliplatin is a potent antagonist of HIV-1 latency with unobvious cytotoxicity and acts through NF-κB signaling pathway. We also demonstrated that Oxaliplatin synergizes with VPA in induction of HIV-1 transcription. However, it would be important to extend these observations to a wider population of latently HIV-1 infected cells from HAART-treated patients to explore Oxaliplatin as a potential drug candidate.

5. Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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