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Short communication

The additive *in vitro* anti-HIV-1 effect of chloroquine, when combined with zidovudine and hydroxyurea

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Abstract

The 4-aminoquinoline chloroquine and its analogue hydroxychloroquine are endowed with anti-HIV-1 activity both *in vitro* and *in vivo*. We previously reported that the addition of CQ (chloroquine) to the combination of HU (hydroxyurea) and ddI (didanosine) provides additive anti-HIV-1 activity. We here extended this *in vitro* investigation by studying whether the addition of CQ also resulted in additive anti-HIV-1 activity when combined with HU plus AZT (zidovudine). The same effect was found, whether CQ was added to HU plus AZT or to HU plus ddI, in recently infected H-9 and U-937 cells or primary T cells and monocytes, as well as in immunologically or oxidatively stimulated ACH-2 and U-1 cells. At concentrations where CQ exerts its anti-HIV-1 effect in combination with the other drugs, CQ addition does not result in either cell toxicity or apoptosis.

Keywords: Chloroquine; Zidovudine; Didanosine; Hydroxyurea; HIV-1

1. Introduction

The antimetabolite HU exerts anti-HIV-1 activity *in vitro*. This effect is substantially increased when the drug is used in combination with one of the NRTIs [1]. Indeed, by inhibiting ribonucleotide reductase, HU reduces the concentration of the natural substrates utilized during reverse transcription, so that lower concentrations of dNTPs will compete with NRTIs [1,2]. *In vitro* studies showed a potentiation of the anti-HIV-1 effect by HU in combination with either AZT, zalcitabine, or ddI, the combination with the latter being the most potent [1,2]. In the case of AZT, the enhanced anti-HIV-1 effect in the presence of HU is not fully explained by a decreased dTTP concentration, and HU has been suggested to potentiate AZT by increasing the cellular uptake and phosphorylation of the drug [3]. Most clinical studies on the combination of HU with an NRTI drug have been performed with ddI [2].

Both CQ and its analogue hydroxychloroquine have been reported by our group and others to have anti-HIV-1 activity *in vitro* [4–7] as well as *in vivo* [8,9]. Furthermore, we found that CQ enhances the anti-HIV-1 activity of the ddI and HU combination *in vitro* [10,11]. CQ is extremely inexpensive and HU is also inexpensive. It is to be anticipated that the cost of AZT will substantially decrease, at least in the developing world, where there is urgent need for effective and affordable anti-HIV-1 combination therapies. We therefore examined whether CQ might exert *in vitro* additive anti-HIV-1 activity when associated with the combination of AZT plus HU, as we had previously observed when CQ was associated with the combination of ddI plus HU [10,11].

2. Materials and methods

Methods used were as previously reported [11]. In brief, the following cells were used for the infection protocol: U-937 and U-1 (promonocytic) as well as H-9 and ACH-2 (T lymphocytic) cell lines grown in "com-

Abbreviations: AZT, zidovudine; CQ, chloroquine; ddI, didanosine; HU, hydroxyurea; LPS, lipopolysaccharide; NRTI, nucleoside reverse transcriptase inhibitor; PMA, phorbol myristate acetate; and RT, reverse transcriptase.

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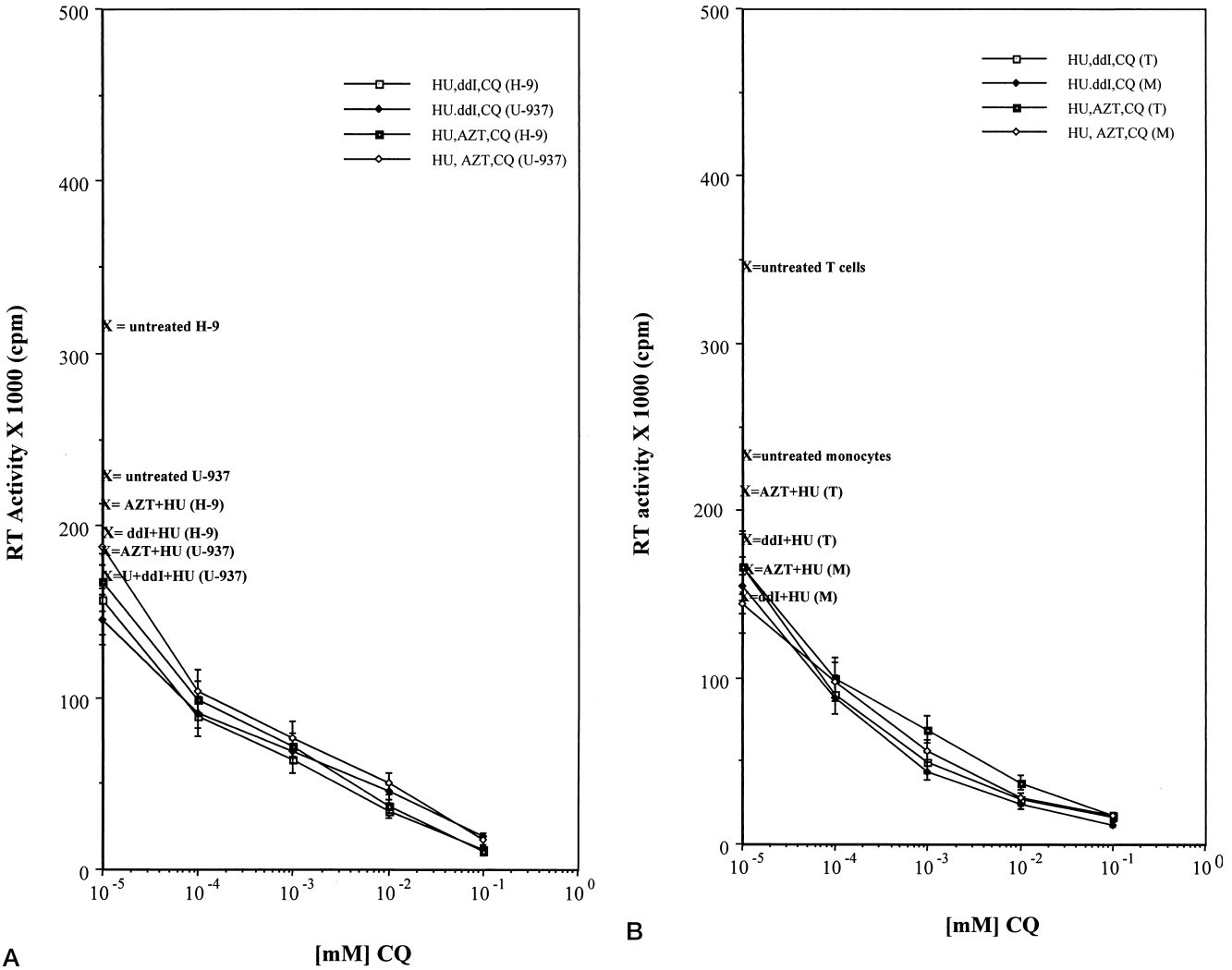

Fig. 1. Effect of chloroquine on HIV-1 infection of T cell and promonocytic cell lines (A) as well as of primary T cells and monocytes (B). H-9 and U-937 cells were untreated (x) or treated with HU (0.2 mM) and either AZT (1 μM) or ddI (1 μM), and varying concentrations of CQ. They were infected with HIV-1_{IIIB} and then maintained in culture. Primary T cells and monocytes ("T" and "M" in B) were isolated from normal blood donors and either untreated (x) or treated with the same drugs. The T cells were stimulated with PHA (phytohemagglutinin, 1 μg/mL) and then infected with HIV-1_{IIIB}, while the monocytes were infected with HIV-1_{BaL}. In all cases, RT activity in the culture supernatant was measured 4 days after infection. The values represent the means of 3 separate experiments.

plete medium" [11]; peripheral blood mononuclear cells (PBMC) from normal blood donors at 5.0×10^6 cells/mL in complete medium; and T cells at 0.5×10^6 cells in complete medium supplemented with 10% interleukin-2. These cells were infected with HIV-1_{IIIB} and HIV-1_{BaL} [11]. One-half million cells were infected by co-cultivation of an infectious supernatant, standardized to 80,000 cpm/mL, for 90 min at 37°. After washing, the cells were cultured to a final concentration of 0.5×10^6/mL in complete medium at 37°. In some experiments, cells were pretreated with HU (0.2 mM), either AZT (1 μM) or ddI (1 μM), and varying concentrations of chloroquine or sterile PBS before infection.

To study HIV-1 reactivation from chronically infected U-1 and ACH-2 cell lines, these cells were incubated with the above drugs before being stimulated for 16 hr with either LPS (10 μg/mL) for U-1 cells and with PMA (1 ng/mL) for ACH-2 cells. When stimulation was done by oxidative stress, H_2O_2 (hydrogen peroxide) was added at different concentrations (200 μM for ACH-2 and 2 mM for U-1 cells) and after 30 min the cells were incubated with the above drugs. RT activity was measured in the culture supernatant 4 days after stimulation, as previously reported [11]. Two independent experiments were done in triplicate.

Toxicity of the above drugs to the above cells was measured by [^{3}H] thymidine incorporation, and apoptosis was measured by FITC (fluorescein isothiocyanate)-labeled annexin V and flow cytometry, gating on the live cells [11]. Data from the interaction of CQ with HU, ddI, and ZDV were analyzed by the non-parametric response surface methodology [11]. The results in the figures are expressed as means ± standard deviation.

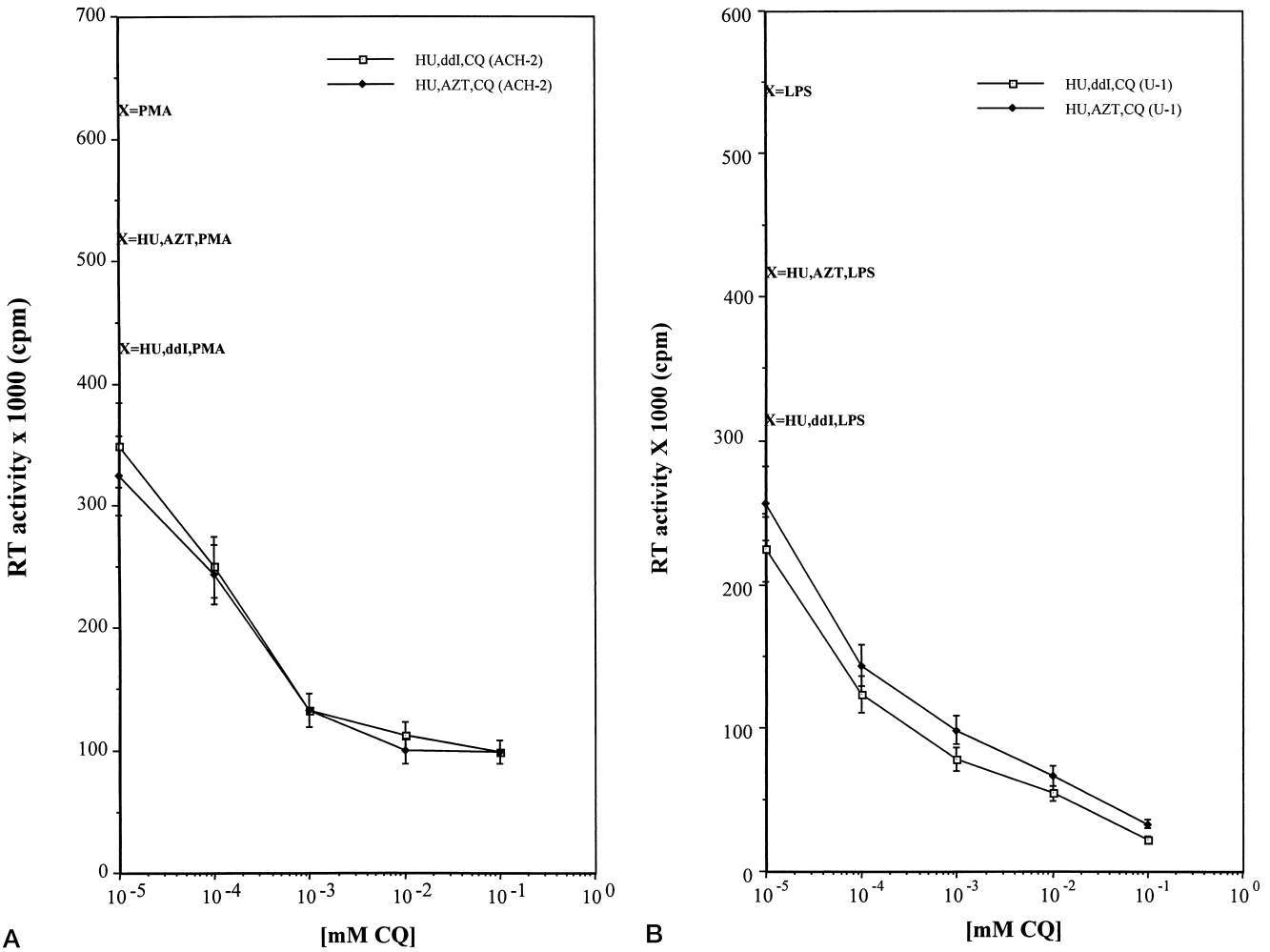

Fig. 2. Effect of chloroquine on ACH-2 (A) and U-1 (B) cells, stimulated immunologically. ACH-2 and U-1 cells were treated with HU (0.2 mM), either AZT (1 μM) or ddI (1 μM), and varying concentrations of CQ and then stimulated with PMA (1 ng/mL) or LPS (10 μg/mL), respectively, for 16 hr. RT activity was measured in the culture supernatant 4 days after stimulation. The values represent the means of 3 separate experiments.

3. Results

We confirmed that the combination of HU at 0.2 mM with either AZT or ddI at 1 μM suppressed HIV-1 replication in both H-9 and U-937 cell lines. Using the non-parametric response method, the effect of CQ on the HU + ddI or HU + ZDV combination was additive, and not synergistic or subsynergistic, in both the recently and chronically infected T cells and monocytes. This additive effect was dependent on its concentration and was the same whether AZT or ddI was used as nucleoside (Fig. 1A). The same was observed with primary T cells and primary monocytes from 3 healthy donors. The mean effective dose (EC_{50}) of CQ to inhibit HIV-1_{IIIB} in the H-9 cells and primary T cells was 0.9 μM, in the U-937 cells 0.4 μM, and in primary macrophages 0.2 μM. Using primary clinical isolates of HIV-1 from 3 HIV-1-infected patients led to the same results (data not shown).

When using both the PMA-stimulated ACH-2 T cell line and the LPS-stimulated U-1 promyelocytic cell line, the addition of CQ to HU plus either AZT or ddI resulted in a further reduction of the RT activity (Fig. 2, A and B). When H_2O_2 was used to reactivate HIV-1 in both cell types, reactivation decreased to a similar degree with AZT plus HU as with ddI plus HU. Moreover, CQ at increasing concentrations, added to both drug combinations, again led to a further decrease in the RT activity that was similar in both cases (Fig. 3, A and B).

To study toxicity and apoptosis, H-9 and U-937 cells were first treated with HU at 0.2 mM, either AZT or ddI at 1 μM, and CQ at varying concentrations. The addition of CQ at concentrations that exerted an antiviral activity did not result in an inhibition of cell proliferation, as assessed by thymidine incorporation (data not shown). Similarly, the addition of CQ at the same concentrations did not result in an enhanced annexin V staining (data not shown). Results are the same, whether the drug combination was with AZT or ddI.

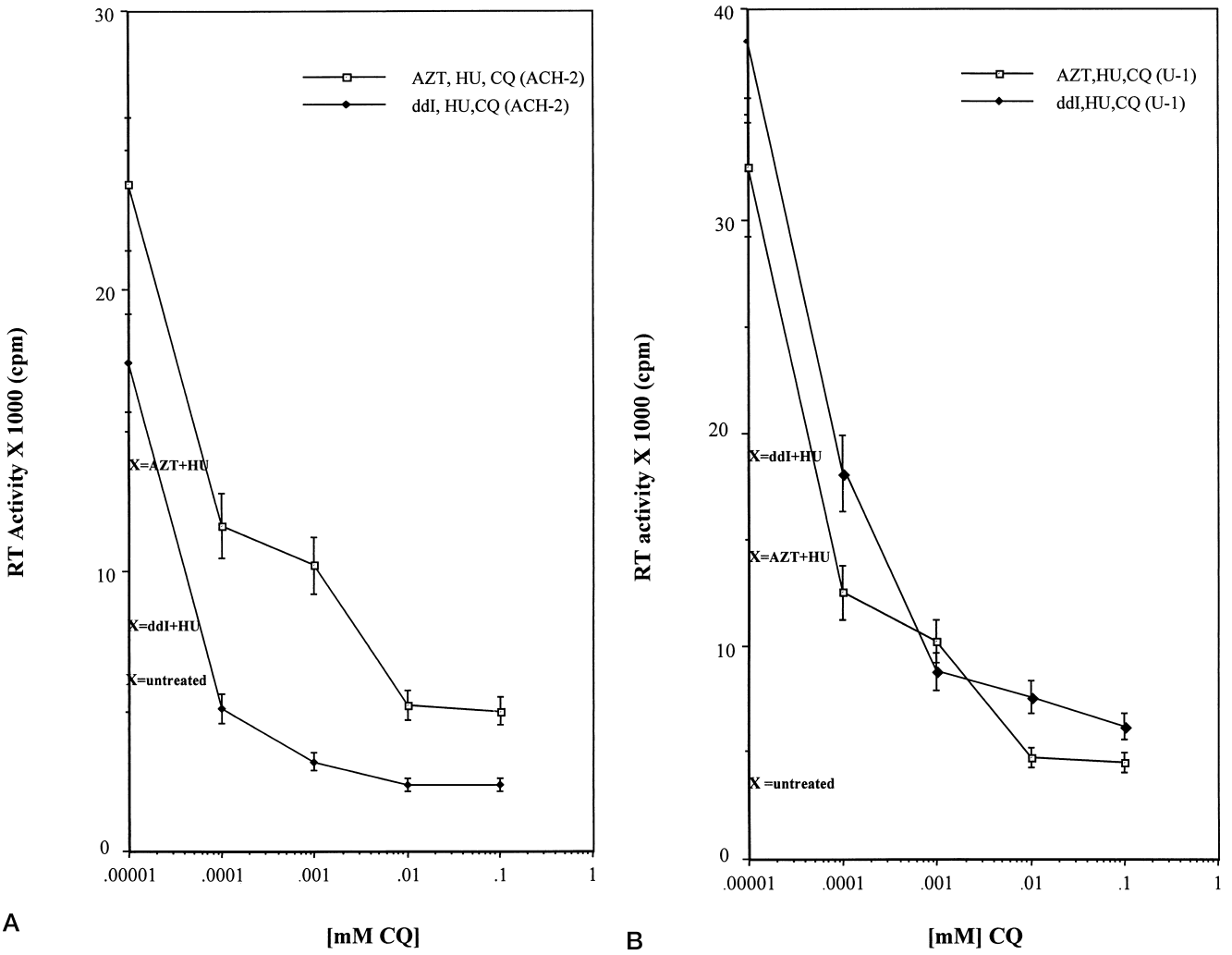

Fig. 3. Effect of chloroquine on ACH-2 (A) and U-1 (B) cells, stimulated by oxidative stress. ACH-2 and U-1 cells were treated with H_2O_2 (0.2 mM for ACH-2 and 2 mM for U-1) for 30 min, and cells were washed and incubated with HU (0.2 mM), either AZT (1 μM) or ddI (1 μM) and varying concentrations of CQ. RT activity was measured in the culture supernatant 4 days after stimulation. The values represent the means of 2 separate experiments.

4. Discussion

There are several general properties of CQ that may make its use in HIV-1-infected patients attractive. First, the drug presented antimicrobial properties against several AIDS opportunists when studied on macrophage infection or *in vivo* in mice [12]; second, it may limit the deposition of iron in the reticuloendothelial system [13], which may be beneficial in view of the negative role that iron accumulation seems to have on the outcome of HIV-1 infection [14]; third, it is an anti-inflammatory drug that inhibits the synthesis of several proinflammatory cytokines [15,16]. More importantly, both CQ and its analogue hydroxychloroquine have been reported by our group and others to have anti-HIV-1 activity *in vitro* and in HIV-1-infected patients [4–9]. It is likely that CQ exerts its anti-HIV-1 activity posttranscriptionally, but the exact mechanism of the drug action is unknown. The target may well be a cellular rather than a viral one, which should decrease the likelihood of the emergence of viral resistance to the drug. We have previously reported an additive effect between AZT and hydroxychloroquine in inhibiting HIV-1 replication in T cells and monocytes [5]. Likewise, there is an additive anti-HIV-1 effect between NRTIs, such as AZT and particularly ddI, and HU [1–3]. We recently reported that CQ exerts an additive *in vitro* anti-HIV-1 activity when combined with HU and ddI [10,11]. Here, we show that the addition of CQ to either HU plus AZT or to HU plus ddI further and equally inhibits HIV-1 replication as determined by RT activity. This further reduction in HIV-1 replication due to CQ was observed both in recently and chronically HIV-1-infected T cells and monocytes, both in cell lines and in primary cells. The concentration of CQ that provides this additional activity (EC_{50} value of 0.2–0.9 μM for the primary T cells and monocytes) is in the range of plasma concentrations that are reported in patients chronically treated with (hydroxy)chloroquine for either rheumatic diseases or the prevention of malaria [17]. Using higher doses of CQ might increase the

antiviral effect of the drug, but should be avoided in view of the enhanced risk of ocular toxicity.

CQ is a non-patent drug that is widely produced and available in the developing world. It is shown here to exert a similar additive antiretroviral effect *in vitro* when combined either with HU plus AZT or HU plus ddI. Theoretically, combining HU and AZT may enhance the risk of bone marrow depression more than the HU plus ddI combination. Although most HU-containing regimens associate ddI rather than AZT, some patients receive the latter drug combination with an acceptable hematological tolerance.[1] Both CQ and HU are inexpensive drugs. If AZT becomes more affordable to patients of the developing world, we suggest that, based on the present *in vitro* data, clinical studies should determine the efficacy and assess the toxicity of the CQ/AZT/HU combination administered to asymptomatic HIV-1-infected patients. We recently reviewed the properties that make CQ potentially interesting in the treatment of HIV-1 infection: its anti-HIV-1 activity (as discussed above), its anti-inflammatory effect, its inhibitory activity toward several AIDS-opportunistic pathogens, and its potential to reduce the tissular accumulation of iron [18]. Finally, CQ may play a role in the prophylaxis of mother-to-child transmission of HIV-1, whether given as adjuvant to antiretovirals during pregnancy and peripartum or administered to the breastfeeding mother to prevent the breastfeeding-associated viral transmission [18]. We are presently studying the latter aspect.

Note

1. De Wit S, Brussels, Belgium. Personal communication.

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