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In Vitro Assessment of Antiretroviral Drugs Demonstrates Potential for Ototoxicity

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Abstract

Several studies have reported an increased incidence of auditory dysfunction among HIV/AIDS patients. We used auditory HEI-OC1 cells in cell viability, flow cytometry and caspases 3/7-activation studies to investigate the potential ototoxicity of fourteen HIV antiretroviral agents: Abacavir, AZT, Delavirdine, Didenosine, Efavirenz, Emtricitabine, Indinavir, Lamivudine, Nefinavir, Nevirapine, Tenofovir, Ritonavir, Stavudine and Zalcitabine, as well as combinations of these agents as used in the common anti-HIV cocktails Atripla™, Combivir™, Epzicom™, Trizivir™, and Truvada™. Our results suggested that most of the single assayed anti-HIV drugs are toxic for HEI-OC1 auditory cells. The cocktails, on the other hand, decreased auditory cells' viability with high significance, with the following severity gradient: Epzicom ~ Trizivir ≫ Atripla ~ Combivir > Truvada. Interestingly, our results suggest that Trizivir- and Epzicom-induced cell death would be mediated by a caspase-independent mechanism. L-Carnitine, a natural micronutrient known to protect HEI-OC1 cells against some ototoxic drugs as well as to decrease neuropathies associated with anti-HIV treatments, increased viability of cells treated with Lamivudine and Tenofovir as well as with the cocktail Atripla, but had only minor effects on cells treated with other drugs and drug combinations. Altogether, these results suggest that some frequently used anti-HIV agents could have deleterious effects on patients hearing, and provide arguments in favor of additional studies aimed at elucidating the potential ototoxicity of current as well as future anti-HIV drugs.

Keywords

anti-HIV drugs; HAART; ototoxicity; HEI-OC1 auditory cells; apoptosis; L-Carnitine

1. Introduction

HIV/AIDS patients are generally treated with combinations of nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTI), and protease inhibitors (PIs). This clinical approach, known as High-Activity Anti-Retroviral Therapy (HAART), is currently considered the most effective weapon against

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HIV infections. A potential side effect of these interventions, however, is drug-induced hearing loss, ranging from moderate loss to complete deafness.

Currently, the cocktails most frequently used in clinical therapies are Atripla™ (Efavirenz +Emtricitabine+Tenofovir), Combivir™ (Lamivudine+AZT), Epzicom™ (Abacavir +Lamivudine), Trizivir™ (Abacavir+Lamivudine+AZT), and Truvada™ (Emtricitabine +Tenofovir). Although these antiretroviral therapies have reduced AIDS morbidity and mortality, prolonged treatment may cause a number of toxicities that result in auditory dysfunction and peripheral neuropathies, among other ailments. About 30% of HIV-positive patients receiving antiretroviral treatment experience hearing loss, and the association between hearing loss and antiretroviral therapy is significant for patients older than 35 years (Marra et al., 1997)]. A recent study on multidrug-resistant tuberculosis reported that the incidence of deafness nearly doubles in HIV-positive (receiving anti-retroviral treatment) compared to HIV-negative patients (Harris et al., 2012). In addition, eight cases have been reported suggesting that NRTIs can cause deafness (Simdon et al., 2001), irreversible ototoxicity has been associated with zalcitabine (Monte et al., 1997) and didanosine (Vogeser et al., 1998) treatment, and it has been presumed that these effects might be associated with mitochondrial DNA damage caused by the drugs (Marra et al., 1997). Studies in mice suggested a synergistic relationship between anti-HIV drugs and noise, with animals pre-treated with Lamivudine+AZT (Combivir) and then exposed to noise showing a decrease in DPOAE activity consistent with drug-induced damage of outer hair cells (Bektas et al., 2008). As the population infected with HIV survives longer because of the success of potent antiretroviral therapy, more cases of antiretroviral-associated ototoxicity will likely occur.

Peripheral neuropathies can develop in HIV-infected individuals during exposure to NRTIs resulting from the drugs' disruptive effect on neuronal mitochondrial DNA synthesis. The use of NRTIs has been associated with decreased intracellular levels of L-carnitine (LCAR) and acylcarnitines (De Simone et al., 1994). Acetyl-L-carnitine (ALCAR), abundant in mitochondria and crucial for energy generation in this cellular organelle, has been shown to be very useful in ameliorating peripheral neuropathies in HIV patients treated with NRTIs (De Simone et al., 1993; Hart et al., 2002; Hart et al., 2004; Herzmann et al., 2005; Moretti et al., 2002; Moretti et al., 1998; Youle, 2005; Youle, 2007; Youle et al., 2007). Since LCAR treatment is generally safe and well tolerated, is not associated with any progression of HIV infection and it has been shown to significantly reduce a variety of validated pain ratings, any potential protective effect of LCAR against anti retroviral-induced damage of auditory cells would open a door to increasing the safety of anti-HIV treatments.

In this work we treated HEI-OC1 auditory cells, an *in vitro* system widely used for screening the potential ototoxicity or otoprotective properties of pharmacological drugs (Kalinec et al., 2003), for 24 and 48 hours with fourteen different anti-HIV drugs alone and in combinations. We found that most of the tested drugs, both alone and in combinations as used in HAART interventions, decreased viability of HEI-OC1 cells as measured by the MTT assay, and their cytotoxic effects were only slightly modified by LCAR. Fluorescence activated cell sorting (FACS) technique, in turn, indicated that Epzicom and Trizivir induced highly significant cell death by activating signaling pathways not involving executioner caspases 3/7.

2. Materials and Methods

2.1. Cells

Auditory HEI-OC1 cells were grown at permissive conditions (33 °C, 5% CO_2 in DMEM supplemented with 10% FBS) in plastic cell culture dishes as previously described (Kalinec

et al., 2003) for 24 and 48 h. HEI-OC1 cells were first trypsinized, counted and concentrations adjusted using Cellometer Auto T4 (Nexelcom Bioscience, Lawrence, MA). The cells were then seeded on a 96-well flat bottom plate (100 μL per well), and incubated at 33°C overnight before being used in the experiments described below.

2.2. Anti-HIV drugs

Fourteen individual anti-retroviral agents were assayed: Abacavir, AZT, Efavirenz, Emtricitabine and Tenofovir (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); Delavirdine, Lamivudine, Nelfinavir and Nevaripine (Sigma-Aldrich Corp., St. Louis, MO); Didenosine,Indinavir, Ritonavir, Stavudine and Zalcitabine (Shanghai Tech, China). In addition, combinations of these agents, as used in the cocktails Atripla™ and Truvada™ (Gilead Sciences, San Dimas, CA), and Combivir™, Epzicom™, and Trizivir™ (GlaxoSmithKline, Middlesex, UK), were also assayed.

HEI-OC1 cells were transferred to 96-well flat bottom plates (100 μL each well), incubated again at 33°C overnight, and then exposed to single anti HIV drugs at different concentrations (Fig. 1) or drug combinations at a fixed concentration. The doses used in the cocktails were: Efavirenz 100 μM + Emtricitabine 33.3 μM + Tenofovir 50 μM (Atripla), Lamivudine 500 μM + AZT 1 mM (Combivir), Abacavir 750 μM + Lamivudine 375 μM (Epzicom), Abacavir 750 μM + Lamivudine 375μM + AZT 750 μM (Trizivir), and Emtricitabine 200 μM + Tenofovir 300 μM (Truvada). We selected the concentrations of the drugs used in combinations following two considerations: *i*) single drug concentrations in which cell viability in our experiments was around 50% of the untreated control condition, and *ii*) FDA's guideline for proportions of antiretroviral drugs used in the treatment of HIV infection (FDA, 2012).

2.3. MTT Assay

HEI-OC1 cells concentration was adjusted to 2.5×10^5 cells/mL (24 h experiments) or 2.0×10^5 cells/mL (48 h experiments). At these time points TACS® MTT Cell Proliferation Assay (Trevigen, Gaithersburg, MD) was performed following the manufacturer's protocol. Absorbance was measured with Spectra Max 5 Plate Reader with Soft Max Pro 5.2 Software (Molecular Devices, Sunnyvale, CA), and average OD in control cells was taken as 100% of viability.

2.4. FACS Assay

Cells were incubated in DMEM alone (negative control) or DMEM plus the drug combinations corresponding to Atripla, Combivir, Epzicom, Trizivir and Truvada (experimental conditions as described in 2.2.), or 250 μM cisplatin (positive control), for 24 and 48 h. At the end of the treatment, cells were washed three times with phosphate buffer saline (PBS, pH 7.4. Gibco-Life Technologies, Grand Island, NY) and detached using a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO). After detaching, cells were spin down, the supernatant was removed, and the cells were stained for 15 min in the dark with CellTox™ Green Cytotoxicity Assay (Promega, Madison, WI) at a concentration of 2 μL of 1X CellTox™ per mL of medium. Green positive HEI-OC1 cells were determined using flow cytometry with 488 nm laser excitation (FACS AriaIII®, BD Biosciences, Erembodegem, Belgium). In all cases, 10,000 cells were analyzed by using FACS Diva software (BD Biosciences, Erembodegem, Belgium).

2.5. Caspase Assay

HEI-OC1 cells concentration was adjusted to 2.0×10^5 cells/mL. At the 24 and 48 h time points, Caspase-Glo® 3/7 Assay (Promega,Madison, WI) was performed following the

manufacturer's protocol. Luminescence was measured using Spectra Max 5 Plate Reader with Soft Max Pro 5.2 Software.

2.6. LCAR treatment

Following the literature (Kalinec et al., 2005; Kalinec et al., 2003), HEI-OC1 cells were pre-incubated for 24 h with 2 μg/mL LCAR (Sigma), and then collected and used in MTT and caspase 3/7 assays just as described above.

2.7. Data Analysis

Statistical analysis of the data was performed by Two-way ANOVA using JMP 9 software (SAS Institute, Cary, NC), with p   0.05 as the criterion for statistical significance.

3. Results

3.1. Single Drugs

As expected, cell viability decreased (drug cytotoxicity increased) with time of exposure to most of anti-HIV drugs (normalized mean for all drugs, 24 h = 0.755±0.007, 48 h = 0.675±0.005; p 0.0001). Didenosine, Indinavir and Zalcitabine, however, showed a lower effect on cell viability at 48 h than at 24 h. Actually, the response of HEI-OC1 cells to different drugs showed interesting trends (Fig. 1). For instance, cytotoxicity of Abacavir, Delavirdine, Efavirenz, Nelfanivir and Tenofovir increased with drug concentration, but whereas with Abacavir, Nelfinavir and Tenofovir dose-dependent impairment of cell viability was gradual, with Delavirdine and Efavirenz it increased significantly for concentrations above 100 μM. This response was particularly dramatic with Efavirenz, where concentrations below 100 μM killed less than 50% of the cells and those above 100 μM killed practically 100% of them. The cytotoxic effect of AZT, Didenosine, Emtricitabine, Lamivudine, Nevaripine, Stavudine or Zalcitabine, on the other hand, was generally weaker and showed less significant changes with dose and time than the observed with the other drugs. Finally, whereas the effect of Ritonavir increased with time of exposure as expected, cytotoxicity of Indinavir was lower at 48 h than at 24 h.

3.2. Drug combinations

Our MTT results with five of the most commonly used anti-viral therapeutic cocktails indicated that each of them decreased HEI-OC1 cells' viability with high significance (P 0.0001), and the effects showed the severity gradient Epzicom ~ Trizivir ≫ Atripla ~ Combivir > Truvada (Fig. 2). Interestingly, Atripla, Combivir and Truvada exhibited similar cytotoxicity at 24 and 48 h, whereas the effects of Epzicom and Trizivir were significantly greater at 48 h (Fig. 2).

Given the concentrations of Atripla components used in our experiments (Efavirenz 100 μM, Emtricitabine 33.3 μM and Tenofovir 50 μM), and referencing the results reported in Fig. 1, we speculate that its effect was mostly associated with Efavirenz cytotoxicity. In support of this idea Efavirenz, like Atripla, showed similar toxicity at 24 and 48 h. Likewise, we speculate that Epzicom and Trizivir effects could be linked to Abacavir cytotoxicity, whereas Truvada's would be associated with Tenofovir cytotoxicity. However, the effect of Combivir on HEI-OC1 cells is not easily explained by its components, 500 μM Lamivudine and 1 mM AZT, and in this case cytotoxicity might be associated with the combination of these drugs rather than to either of them alone.

Although MTT assay is a widely used technique to investigate cell viability, it cannot discriminate between a decrease in the rate of cell proliferation and an increase in cell death. Therefore, we performed FACS studies to estimate the number of dead cells in cultures

treated with the five anti-viral therapeutic cocktails as well as in control (untreated), negative control (unstained), and positive control (treated with cisplatin, a well-known ototoxic drug) cultures. As shown in Figure 3, only cisplatin, Epzicom and Trizivir induced significant more cell death than the control condition. Thus, flow cytometry experiments confirmed that the significant toxicity of Epzicom and Trizivir to HEI-OC1 cells observed with MTT assays was mostly associated with induction of cell death, and suggested that, in contrast, the toxic effects of Atripla, Combivir and Truvada were probably more related to impairment of cell proliferation (Fig. 3).

3.3. Cell Death Pathways

We investigated whether cell death induced by these drugs cocktails was mediated by activation of executionary caspases 3/7, as is typical in caspase-dependent apoptotic cell death in mammals (Parrish et al., 2013). As a positive control we used cells exposed to 5% DMSO, which increased simultaneously caspases 3/7 activation and cell death. As shown in Fig. 4, only Combivir and Truvada activated caspases 3/7, and Combivir effect was significant only at 24 h (1.43 ± 0.06 *re* Control 1.00 ± 0.06, p 0.0001, *re* Combivir 48 h 1.01 ± 0.06, p 0.0001). Neither Combivir at 48 h nor Truvada at either time point induced significantly different Caspase 3/7 activation that the control condition.

Interestingly, activation of the caspases 3/7-mediated pathway was significantly lower in cells treated with Epzicom and Trizivir than in all the other conditions both at 24 and 48 h, even though FACS results indicated that these anti-retroviral combinations induced a significant increase in cell death (compare Figs. 3 and 4).

3.4. Effect of LCAR

Finally, we investigated the potential protective effect of LCAR on the cytotoxicity of anti-HIV drugs either alone, at a single representative dose (Fig. 5), or combined in cocktails as described before (Fig. 6). At 24 h, whereas LCAR alone did not affect viability of HEI-OC1 cells (Figs. 5 & 6, control+LCAR), it significantly improved viability of auditory cells treated with Lamivudine, and Stavudine but decreased viability of those treated with Indinavir and Ritonavir (Fig. 5A). At 48 h, in contrast, the only significant effect of LCAR was a decrease in Stavudine cytotoxicity (Fig. 5B). When we tested the effect of LCAR on the cocktails, we found a significant LCAR-associated improvement in viability only in cells treated with Atripla at 24 h (Fig. 6A). Interestingly, LCAR significantly increased caspases 3/7 activation in control cells at 24 h and cells treated with Truvada at 24 and 48 h (Fig. 7), results not correlated with a similar increase in levels of cell death (compare Figs. 6 and 7).

4. Discussion

Our results indicated that all the fourteen different anti-HIV drugs included in this study, seven NRTIs, three NNRTIs, three PIs and one RTI, decreased viability of HEI-OC1 cells, most in a dose-dependent manner. Moreover, five of the most common drug combinations used in HAART therapies were significantly cytotoxic for this auditory cell line, and two of them, those commercialized under the brand names of Epzicom and Trizivir, induced significant cell death in a caspases-independent manner, suggesting that their action do not involve activation of traditional apoptotic pathways. It should be emphasized, however, that immortalized auditory HEI-OC1 cells are not cochlear hair cells, and that the results reported in this study should be confirmed in animal models before investigating the molecular mechanisms of cell death stimulated by these drugs and drug combinations.

4.1. Effects of individual drugs

Of the single drugs investigated, those decreasing the most HEI-OC1 viability as measured by MTT were Abacavir, Delavirdine, Efavirenz, Indinavir, Nelfinavir, Ritonavir and Tenofovir. In contrast, AZT, Didenosine, Emtricitabine, Lamivudine, Nevirapine, Stavudine and Zalcitabine had only a moderated effect (Fig. 1). These results differ from reports in the literature with other cell lines. For example, working with human hepatoma cells, Venhoff and coworkers reported minimal toxicity of Tenofovir and Lamivudine, a moderate effect of emtricitabine, and strongly impaired cell proliferation with abacavir and AZT (Venhoff et al., 2007).

An interesting response resulted from Efavirenz exposure, which killed practically 100% of the cells at concentrations above 150 μM. Efavirenz is an NNRTI, therefore it does not inhibit DNA polymerase-γ and it is not expected to elicit mitochondrial toxicity, a major suspected cause of cytotoxicity. Since Efavirenz has shown better antiretroviral efficacy than PIs in pivotal clinical trials (Riddler et al., 2008; Staszewski et al., 1999), it is one of the preferred third agents in antiretroviral regimes (Gazzard et al., 2008; Hammer et al., 2008). Interestingly, another NNRTI, Delavirdine, is also very cytotoxic at concentrations similar to those of Efavirenz. These results suggest that Efavirenz and Delavirdine should be used at low concentrations in order to minimize potential ototoxic effects on auditory cells.

The most cytotoxic of the PIs investigated in our study was Nelfinavir, with significant effects on cell viability at concentrations as low as 25 μM (Fig. 1). On the other hand, Abacavir and Tenofovir, components of the therapeutic cocktails Epzicom™, Trizivir™ and Truvada™, were the most toxic NRTIs for auditory HEI-OC1 cells.

It should be noted, however, that all these results were obtained using the MTT assay. This assay, which measures the reduction of tetrazolium salts by metabolic active cells, is widely accepted as a reliable way to examine cell proliferation and, in presence of apoptosis or necrosis, the reduction in cell viability. MTT advantages include rapid semi-automated reading, objective assessment, comparative low cost, and high-reproducibility. However, it cannot discriminate between, for example, a treatment that decreases cell proliferation while maintaining constant the rate of cell death, and other that increases cell death while maintaining constant the rate of cell proliferation. Properly speaking, this is not a "problem" but rather a limitation of the technique. Both cell death and changes in proliferation rate indicate a cytotoxic effect of the treatment. However, in some cases is convenient to identify the precise cytotoxic effect of a given compound as an initial step to investigate the molecular mechanisms involved in the response. The FACS technique, in contrast to the MTT assay, estimates the percentage of drug-induced cell death without providing any information about proliferation rate. Thus, both techniques are complementary. Therefore, we used both to evaluate more precisely the cytotoxic effect of the combinations of anti-retroviral drugs more frequently used in HAART interventions.

4.2. Effects of drug combinations

Our results with the MTT assay indicated that all drug combinations investigated decreased auditory HEI-OC1 cells' viability with high significance (Fig. 2). FACS studies, on the other hand, showed that only Epzicom and Trizivir significantly increased death of HEI-OC1 cells, whereas no significant effects were detected with the other three drug combinations (Fig. 3). Altogether, these results suggest that treatment with Epzicom and Trizivir induced cell death, whereas the decrease in cell viability caused by Atripla, Combivir and Truvada was probably associated with impaired cell proliferation. As mentioned in Results, Epzicom and Trizivir effects could be linked to Abacavir cytotoxicity, Atripla and Truvada effects could be associated mostly with Efavirenz and Tenofovir cytotoxicity, respectively, whereas

Combivir effect could be explained by our results by assuming either additive or synergic effects of Lamivudine and AZT. This possibility is not implausible, since unpredicted interactions of NRTIs have been reported in the literature (Venhoff et al., 2007).

The results of caspases 3/7 activation assays were quite unexpected. DMSO, the positive control, showed the expected significant increase in caspases 3/7 activation both at 24 and 48 h, suggesting a similar increase in cell apoptosis. In contrast, Epzicom and Trizivir, which induced significantly more cell death than the other three drug combinations included in our study, showed a striking decrease in caspases 3/7 activation with respect to all other conditions tested. Since activation of these executioner caspases is considered the hallmark of caspase-dependent apoptosis (Parrish et al., 2013), our results suggest that Trizivir- and Epzicom-induced cell death was mediated by a caspase-independent mechanism. Interestingly, the similar decrease in caspases 3/7 activation by Abacavir, Epzicom and Trizivir, provides additional support to the idea that Abacavir may be the main responsible agent for Epzicom and Trizivir cytotoxicity. The effects of Atripla, Combivir and Truvada on caspases 3/7 activation, although significant in some cases, could be considered minor under the lack of significant changes in cell death associated with these treatments indicated by the FACS studies.

4.3. Effects of LCAR

Pre-incubation of HEI-OC1 cells with LCAR provided a significant protective effect against only a couple of NRTI drugs (Lamivudine, Stavudine) and one anti-retroviral combination (Atripla). Curiously, LCAR significantly decreased viability of cells treated with the PIs Indinavir and Ritonavir and increased caspases 3/7 activation in HEI-OC1 cells treated with Truvada, but we did not find clear correlation between LCAR positive and negative effects and activation or inactivation of caspases 3/7. These results suggest that LCAR effects may not be associated with the traditional apoptotic process.

4.4. Mechanism of antiretroviral drugs-induced cell death

During the last decade, an extensive effort has been put forth to study HAART toxicity. There is general agreement that toxicity of NRTIs may be related to their effects on the mitochondrial DNA polymerase gamma (Pol γ), responsible for mitochondrial DNA replication, leading to cell apoptosis(Feng et al., 2001; Marra et al., 1997). PIs, on the other hand, are known to induce endoplasmic reticulum stress, followed by apoptosis or autophagy (Bruning et al., 2009; Zha et al., 2011; Zhou, 2011). However, it has been noted that toxicity of different anti-HIV drugs is cell-, tissue-, and organ-specific (Benbrik et al., 1997), and consequently the response of auditory HEI-OC1 cells could be different to that observed in other cell lines from different organs.

Recent data points to the existence of multiple non-apoptotic, regulated cell death mechanisms, some of which overlap or are mutually exclusive with apoptosis (Degterev et al., 2008). Moreover, multiple types of cell death, including apoptosis, apoptosis-like, oncosis, oncosis-like and autophagy, can be observed simultaneously in cell cultures exposed to the same stimulus (Fink et al., 2005; Maiuri et al., 2007). In our experiments we did not observe evident signals of cell autophagy or oncosis (although no specific tests were assayed for detecting them) but, as already mentioned, the decrease in caspases 3/7 activation induced by some of the assayed compounds suggests stimulation of caspase-independent mechanisms of cell death. Experiments aimed at identifying the alternative death pathways activated by antiretroviral drugs are currently being performed in our laboratory.

4.5. Conclusions

Altogether, our results indicate that some frequently used anti-HIV agents are toxic for auditory HEI-OC1 cells, either by impairing cell proliferation or inducing cell death. Although HEI-OC1 cells were originally proposed, and are generally considered, as a good model for the screening of ototoxic drugs, it must be emphasized that they are not cochlear hair cells, and further studies with animal models are required to confirm the ototoxicity of the anti-retroviral agents as predicted by this in vitro system. Nevertheless, this study strongly suggests that some frequently used anti-HIV agents could have deleterious effects on the hearing of human patients, and provide arguments in favor of additional studies aimed at elucidating the potential ototoxicity of current as well as future anti-HIV drugs.

Acknowledgments

Abbreviations

FACS	fluorescence activated cell sorting
NRTI	nucleoside analogue reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
PI	protease inhibitor
HAART	High-Activity Anti-Retroviral Therapy

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Highlights

> In vitro studies suggest that several common anti-HIV drugs are likely ototoxic

> Cocktails used in High-Activity Anti-Retroviral Therapy are toxic for HEI-OC1 cells

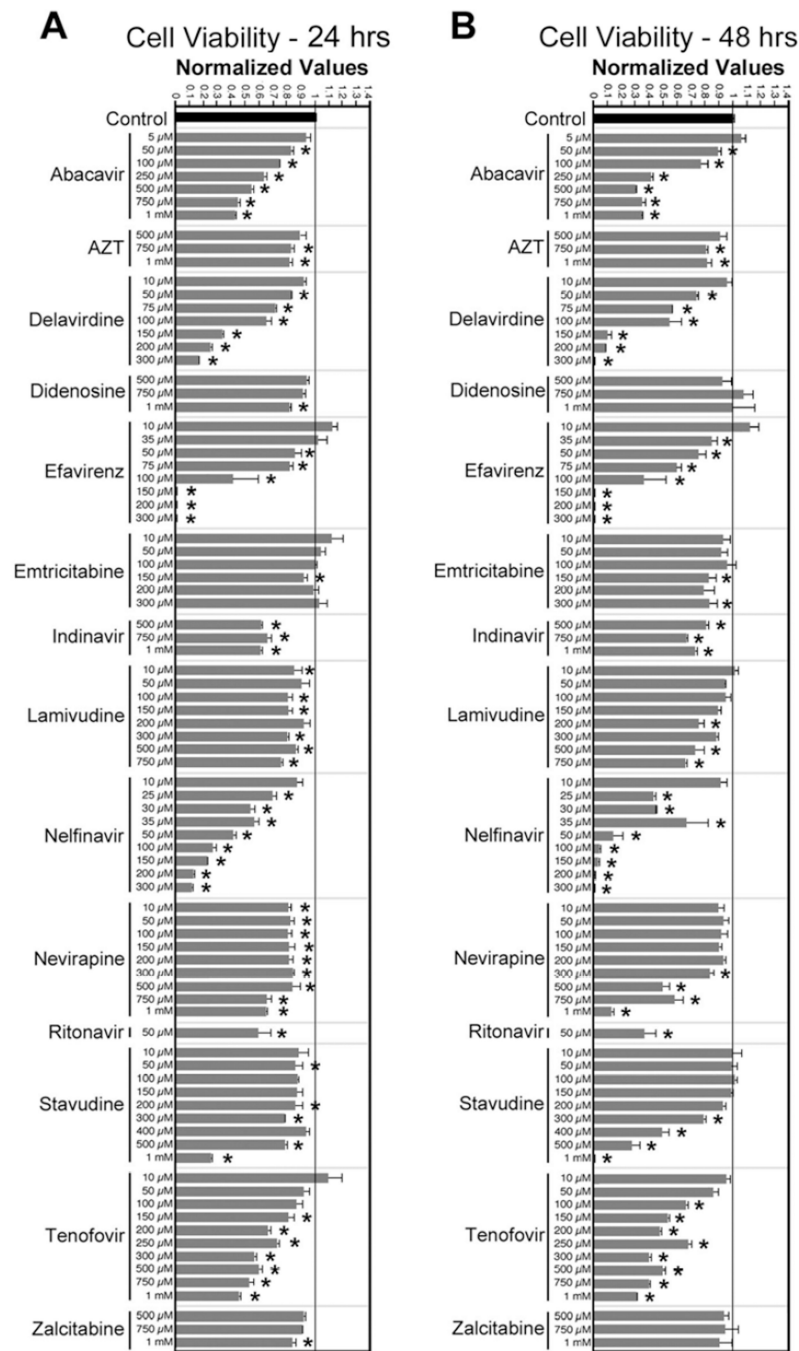> Anti retrovirals-induced cell death was associated with caspase-independent mechanisms

Fig. 1.
Viability of HEI-OC1 cells exposed to anti-HIV drugs at different concentrations, as measured with the MTT assay, at 24 hours (**A**) and 48 hours (**B**). Absorbance values were normalized to Control by making the mean for this group = 1.0. *= p 0.05 *re* Control.
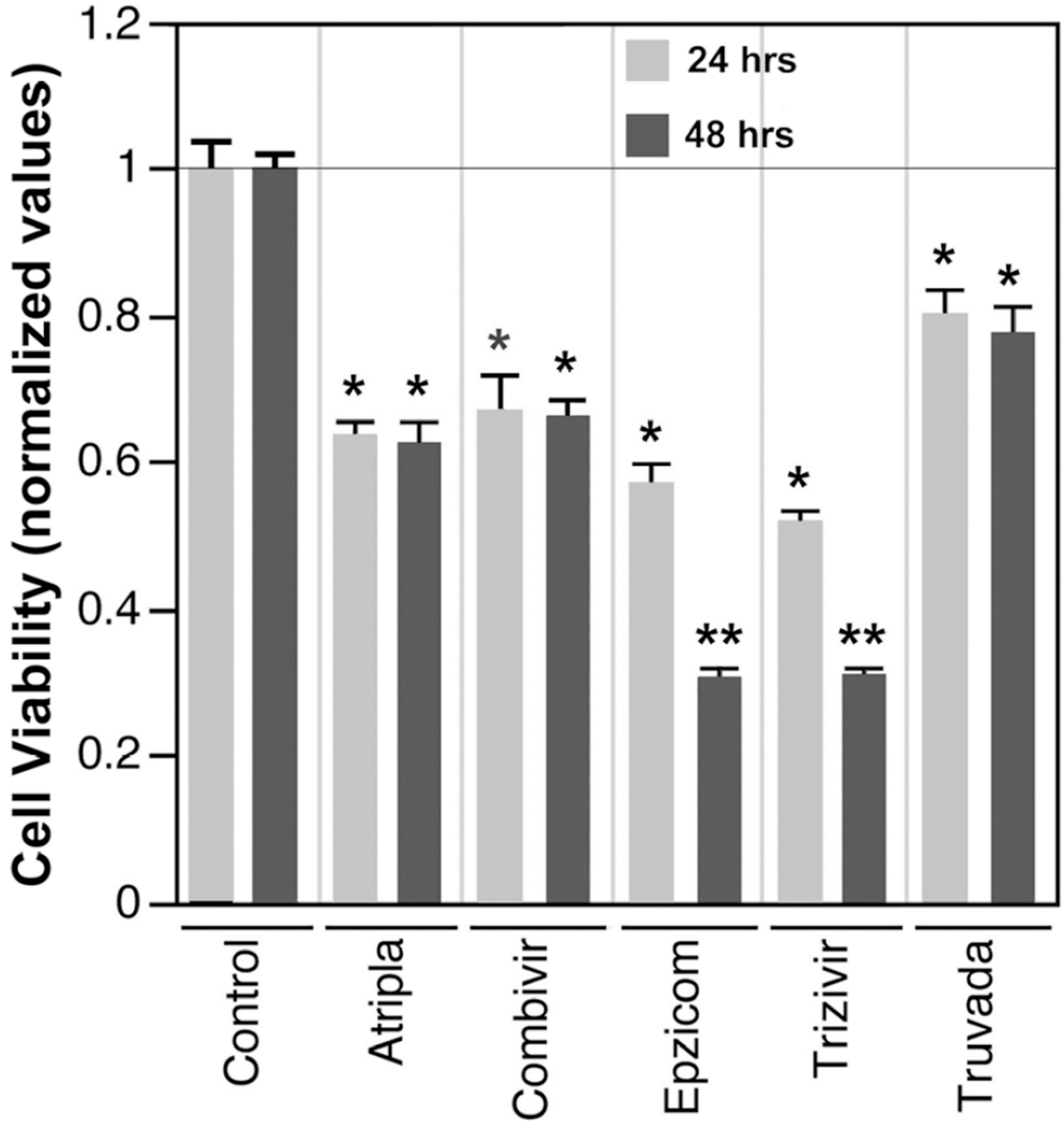
Fig. 2.
Viability of HEI-OC1 cells exposed to anti-HIV drugs in five different combinations for 24 and 48 hours, as measured with the MTT assay. All the assayed drug combinations significantly decreased viability of HEI-OC1 cells. The effects were similar at 24 and 48 h except for Epzicom and Trizivir. *=p 0.05 *re* Control; **= p 0.05 *re* Control and same combination at 24 or 48 h.
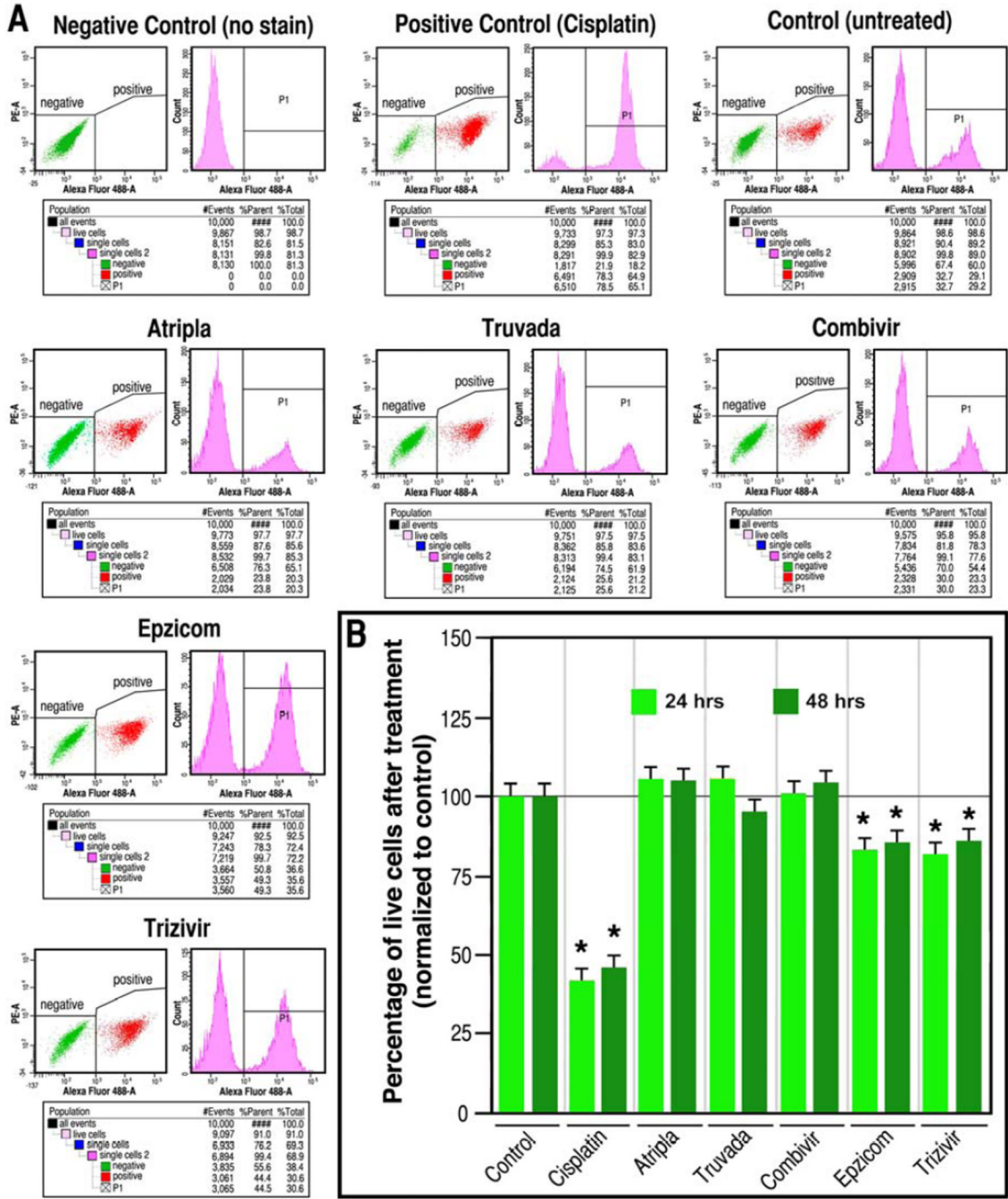
Fig. 3.
Fluorescence-assisted Cell Sorting studies of drug-induced death of HEI-OC1 cells. (**A**) Typical results provided by the FACS experiments. Data corresponds to one (out of three) independent experiments performed at 24 h. Note the use of unstained samples as a negative control and cisplatin as a positive control. For every treatment a sample of 10,000 cells was investigated, the number of HEI-OC1 cells dead (red) and alive (green) was counted, and the full information was provided in form of a table. (**B**) Statistical comparison of the effect of five different HAART cocktails plus a positive control (cisplatin) and a normal control (untreated cells). Experiments were performed at 24 (n=3) and 48 h (n=3), respectively. The

primary data consisted of the percentages of live cells after each treatment as provided by the FACS instrument in the correspondent tables ("negative" values under "%Parent" column). Data was analyzed by two-way ANOVA using arcsin transformation of the percentages normalized to control values. Only cisplatin (0.41 ± 0.04 at 24 h, p 0.0001, and 0.47 ± 0.04 at 48 h, p 0.0001), Epzicom (0.84 ± 0.04 at 24 h, p 0.002, and 0.88 ± 0.04 at 48 h, p 0.014), and Trizivir (0.83 ± 0.04 at 24 h, p 0.001, and 0.89 ± 0.04 at 48 h, p 0.02) induced significant more cell death than control condition at the same time point. No significant different responses between 24 and 48 h were observed. $*=p$ 0.05.
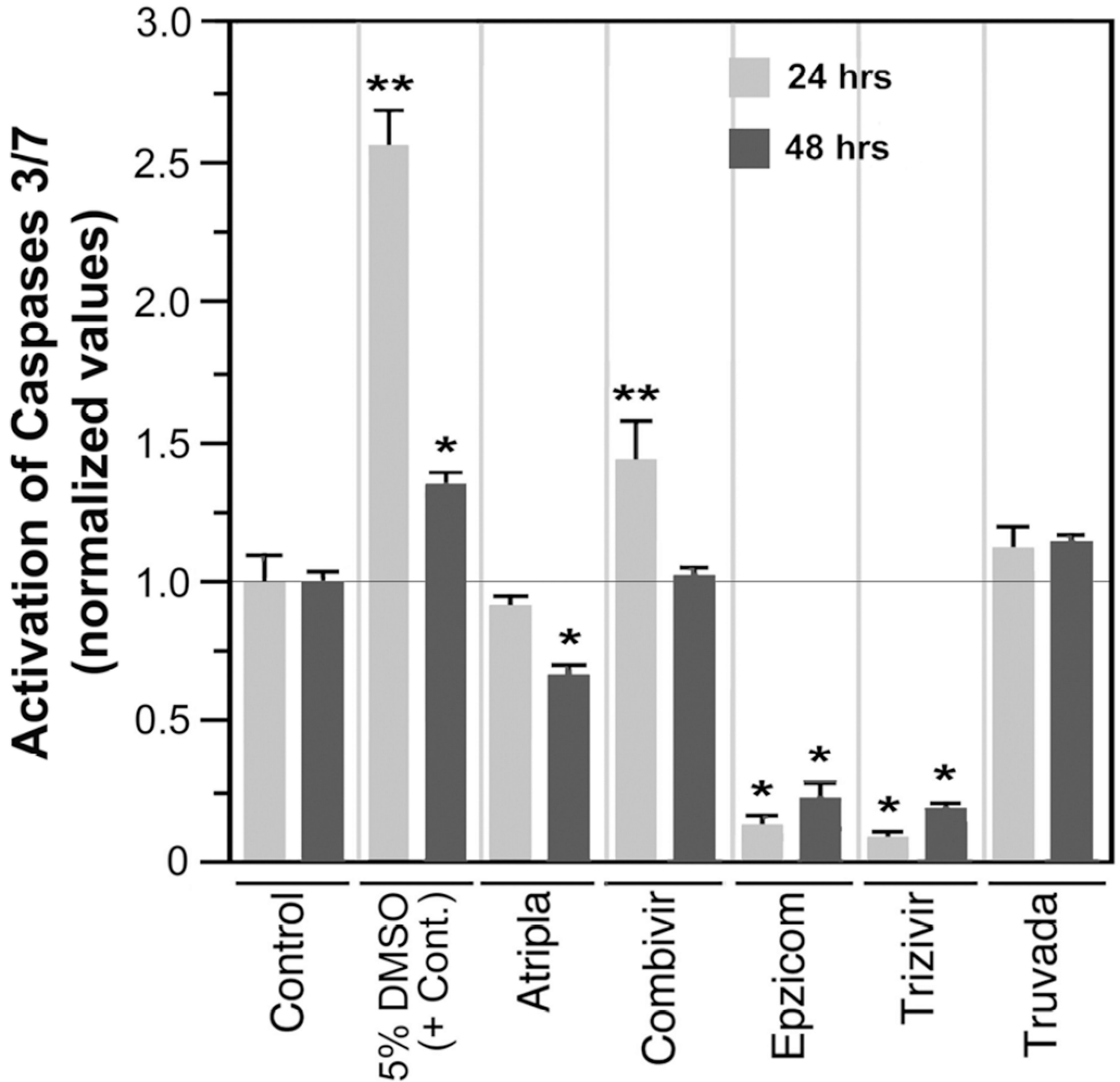
Fig. 4.
Activation of Caspases 3/7 in HEI-OC1 cells exposed to five different combinations of anti-HIV drugs for 24 and 48 h. Only DMSO (positive control) and Combivir at 24 h activated caspases 3/7 more than the Control condition. *=p 0.05 *re* Control; **= p 0.05 *re* Control and same combination at 24 or 48 h.
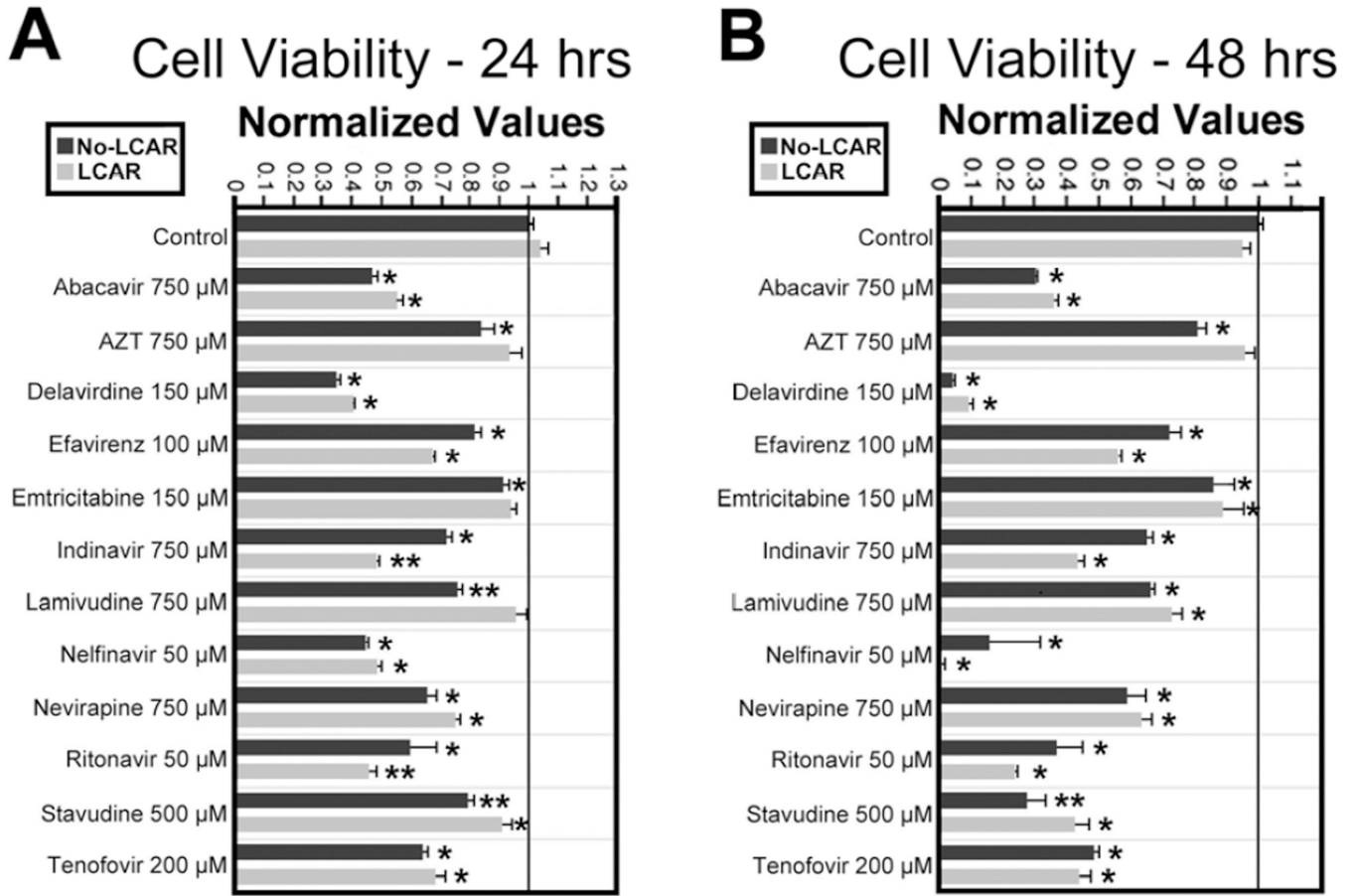
Fig. 5.
Effect of LCAR on cytotoxicity of anti-HIV drugs as measured with the MTT assay at 24
(**A**) and 48 h (**B**). LCAR significantly decreased cytotoxicity of Lamivudine at 24 h and
Stavudine at 24 and 48 h, but increased cytotoxicity of Indinavir and Ritonavir at 24 h.
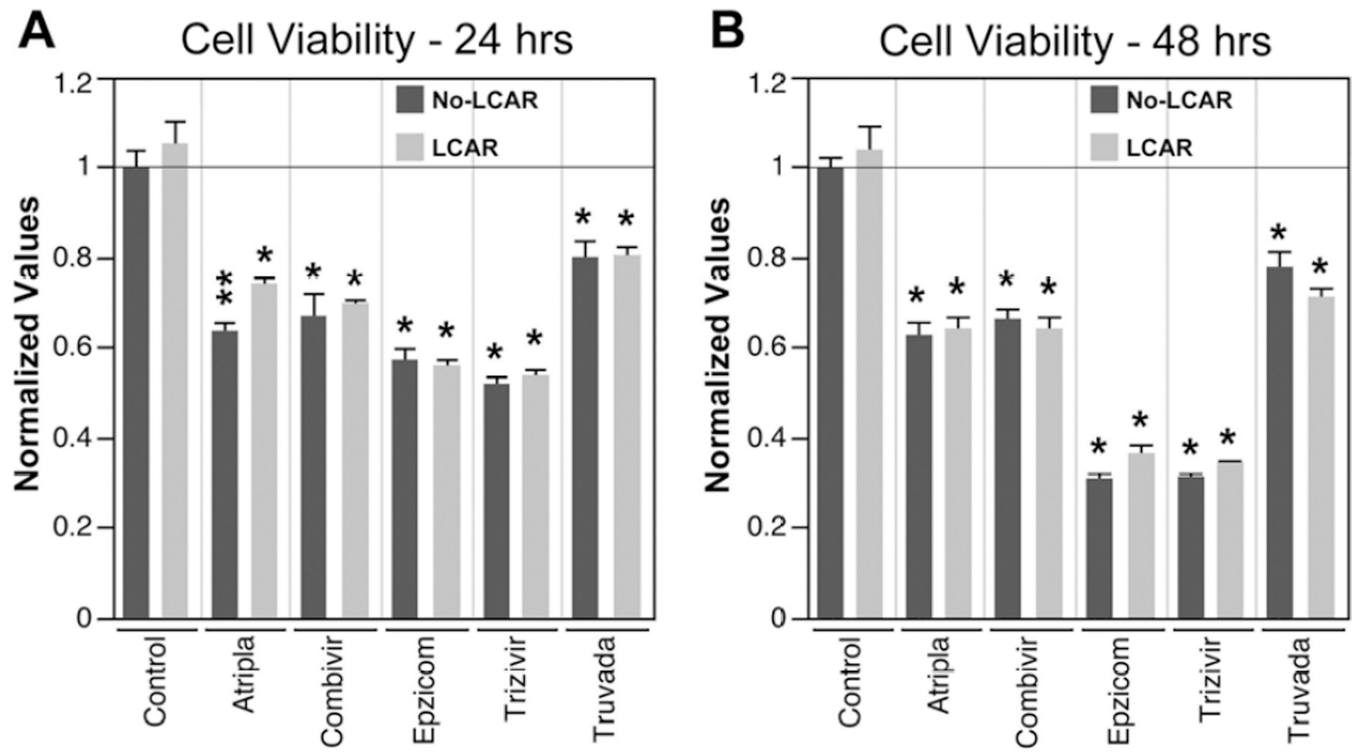*=p 0.05 *re* Control; **= p 0.05 *re* Control and same drug without LCAR.

Fig. 6.
Effect of LCAR on cytotoxicity of five different combinations of anti-HIV drugs at 24 (**A**) and 48 h (**B**). LCAR significantly decrease Atripla cytotoxicity at 24 h, but no effects were detected with other combinations or at 48 h *=p 0.05 *re* Control; **= p 0.05 *re* Control and same combination without LCAR.
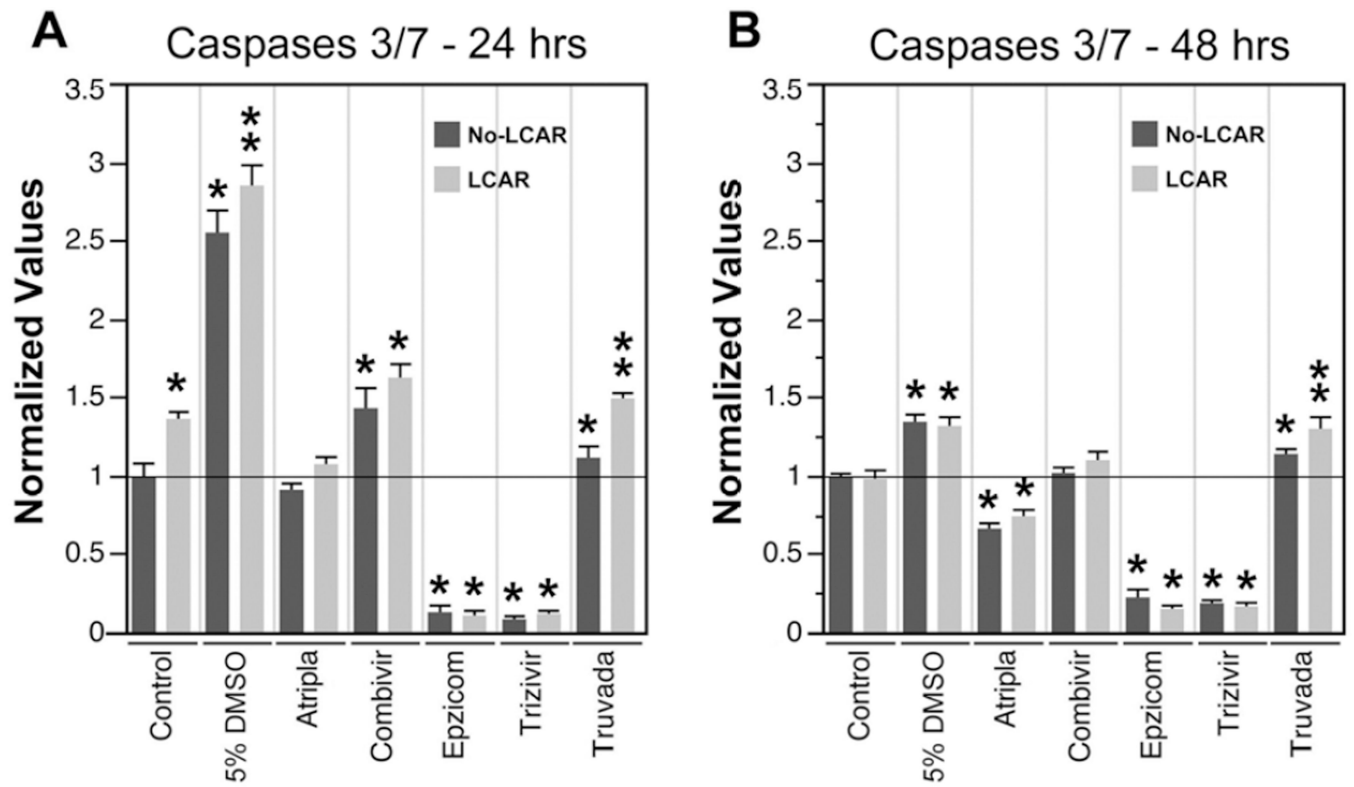
Fig. 7.
Effect of LCAR on activation of caspases 3/7 in HEI-OC1 cells treated with five different combinations of anti-HIV drugs at 24 (**A**) and 48 h (**B**). DMSO was used as positive control.
*=p 0.05 *re* Control; **= p 0.05 *re* Control and same combination without LCAR