Flupirtine protects both neuronal cells and lymphocytes against induced apoptosis *in vitro*: Implications for treatment of AIDS patients

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Abstract

In the present study we demonstrate that flupirtine, an already clinically used, centrally acting, non-opiate analgesic agent, protects rat cortical neurons against HIV-gp120 induced apoptotic cell death. The drug was active at concentrations between 1 and 10 μg/ml. Furthermore we show inhibition of *in vitro* induced apoptosis in human blood mononuclear cells, using flupirtine. Induced apoptosis in peripheral blood mononuclear cells from healthy individuals and HIV-1 infected patients was reduced to approximately 50% after *in vitro* preincubation with flupirtine at concentrations between 0.1 and 10 μg/ml. The anti-apoptotic effect of flupirtine was restricted to CD3+ lymphocytes and in particular to CD4+ cells. Flupirtine does not affect uninduced apoptosis in human lymphocytes *in vitro*. The selective potential of flupirtine to reduce apoptosis without influencing uninduced apoptosis may qualify this compound as a potential drug in the therapy of HIV-1 infected patients.

Keywords: flupirtine, apoptosis, apoptosis (uninduced), apoptosis (induced), HIV, AIDS, lymphocytes, neurons

Abbreviations: CDC, Centers for Disease Control; HIV-1, human immunodeficiency virus type 1; HX, hypoxanthine; LNAC, N-acetyl-L-cysteine; MNC, Mononuclear cells; NMDA, N-methyl-D-aspartate; XOD, xanthine oxidase

Introduction

It remains a fundamental paradoxon of human immunodeficiency virus type 1 (HIV-1) caused disease that a progressive diminution in CD4+ lymphocytes occurs despite the fact that only a small fraction of the cells is infected (Schattner and Laurence, 1994). In addition to lymphocytes, other cells such as human astrocytes (Rytik *et al*, 1991) and neuronal cells (Li *et al*, 1990) can be infected by HIV-1 *in vitro*. Primary neurons are very rarely, if at all infected by HIV-1 (Cheng-Meyer *et al*, 1987). The finding that exposure of rat cultured neurons to HIV-1 gp120 leads to apoptotic cell death was unexpected (Brenneman *et al*, 1988; Müller *et al*, 1992).

It has been argued that the mechanism for T-cell depletion in HIV-1 infection includes immune and autoimmune destruction of both infected and uninfected cells by apoptosis (Schattner and Laurence, 1994; Müller *et al*, 1989). In addition, HIV-1 causes apoptosis in neurons (Müller *et al*, 1992; Dreyer *et al*, 1990). Due to the fact that the clinically safe drug flupirtine-maleate [2-amino-3-ethoxycarbonylamino-6- (4-fluoro-benzylamino)-pyridine maleate], a member of the class of triaminopyridines, displays an anti-apoptotic effect on neurons *in vitro* (Perovic *et al*, 1994), we have screened for its potential anti-apoptotic activity also in the lymphocyte system.

Flupirtine previously found to be a centrally acting, non-opiate analgesic agent (Szelenyi *et al*, 1989) is applied in the clinics under the trademark of Katadolon® (Friedel and Fitton, 1993). Recently we established flupirtine as an anti-apoptotic drug for neurons *in vitro* (Perovic *et al*, 1994). It acts like an N-methyl-D-aspartate (NMDA) receptor antagonist but until now no binding at this receptor has been identified (Osborne *et al*, 1994; Schwarz *et al*, 1994; Osborne *et al*, 1996). Moreover, it was found that flupirtine enhances basal levels of ATP (Osborne *et al*, 1996) and reduces the NMDA-induced intracellular Ca^{2+} increase *in vitro* (Rupalla *et al*, 1995). In addition, flupirtine acts neuroprotectively at doses of 1–10 mg/kg in animal models of focal cerebral ischemia (mouse) (Rupalla *et al*, 1995) and global cerebral ischemia (rat) (Block *et al*, 1995) as well as of ischemia (rat, rabbit) (Block *et al*, 1994; Osborne *et al*, 1996).

Here we describe that flupirtine substantially abolishes the induced apoptotic cell death of lymphocytes both from AIDS patients and HIV-1 uninfected individuals. In addition it is shown that apoptosis, caused by HIV-1 gp120 in rat cortical cells, is completely blocked by flupirtine, while this drug displays no effect on cell death of permanent lymphocyte lines, infected by HIV-1. Important for future clinical trials is the finding, that flupirtine displays this effect only on induced and not on uninduced apoptosis. As an inducer for apoptosis in lymphocytes we used free oxygen radicals (Muschel *et al*, 1995), generated by the hypoxanthine/xanthine oxidase system (Bruck *et al*, 1994) as well as ionomycin (Gougeon *et al*, 1993) and for neurons the gp120 protein (Müller *et al*, 1992, 1996).

Results

Adjustment of the hypoxanthine/xanthine oxidase system

The staining pattern for apoptosis, using 7-AAD, is shown in Figure 1 (right column). Apoptotic cells show a fluorescence intensity of > 10 arbitrary channel numbers. At 0 mU/ml of XOD only $6.6 \pm 2.1\%$ of the cells are above this borderline, indicating apoptosis. With increasing concentrations of XOD the percentage of apoptotic cells rose and reached at 80 mU/ml a value of $93.2 \pm 6.8\%$. Applying 10 mU/ml of XOD approximately 50% ($54.6 \pm 6.1\%$) of the cells underwent apoptosis.

In Figure 1 (left column) it is shown that in the absence of XOD the cells showed a FSC/SSC distribution pattern as follows; size 349 ± 27 (arbitrary channel number) versus granularity 112 ± 15. At an enzyme concentration of 80 mU/ml the size decreased to 256 ± 22 whereas the degree of granularity increased to 180 ± 29. These changes are also indicative for apoptotic morphological alterations of cells. At 10 mU/ml of XOD the cells show a distribution pattern which is approximately in the middle between these two extremes. If not mentioned otherwise, 10 mU/ml of XOD were used for the following experiments.

Effect of flupirtine on rate of uninduced apoptosis in human MNC

Peripheral blood MNC both from healthy individuals and from HIV-1 infected patients were analyzed for uninduced apoptosis one day after drawing blood. $6.32 \pm 0.82\%$ of MNC from controls showed apoptosis, while $28.42 \pm 7.84\%$ of those taken from HIV-1 individuals underwent this process (Figure 2). MNC from both groups were treated with flupirtine within a concentration range of 0.1 to 30 μg/ml for 24 h. As summarized in Figure 2 the rate of uninduced apoptosis of MNC from uninfected and HIV-1 infected individuals did not change significantly.

Reduction of apoptosis, induced by reactive oxygen, in human MNC by flupirtine

MNC from healthy as well as HIV-1 infected individuals had been treated with 1 mM HX and 10 mU/ml XOD to induce apoptosis. Flupirtine was added at different concentrations to the cells 6 h prior to the inducer. As summarized in Figure 3, flupirtine at concentrations between 0.1 and 3 μg/ml significantly reduced the extent of apoptotic death ($p < 0.001$). At 10 μg/ml the protective effect was seen significantly ($p < 0.001$) only in the assays with MNC from infected individuals. At concentrations between 0.3 and 3 μg/ml the protection of the cells against induced apoptosis was most pronounced; the reduction of induced apoptosis in the assays with MNC from healthy individuals was $\approx 40\%$ and in those from HIV-1 infected patients $\approx 60\%$.

Apoptosis in MNC from HIV-1 infected individuals was also traced by DNA fragmentation. After incubation of the cells with the HX/XOD system DNA was extracted from the cells, size-separated in an agarose gel and blot transferred. As shown in Figure 4a, the DNA degraded in a ladder-like

pattern with fragments of multiples of ≈ 180 base pairs. Such a pattern is characteristic for apoptotic DNA fragmentation (Wyllie, 1980). In cells treated with 1 μg/ml of flupirtine together with the inducer such a degradation pattern could not be seen (Figure 4b).

In control studies it was determined that flupirtine does not inhibit the enzyme activity of XOD at a concentration of 50 μg/ml (not shown); the assay was performed as described by Bergmeyer (1974).

Reduction of ionomycin-induced apoptosis in mononuclear cells by flupirtine

Ionomycin (1 μg/ml) was used as a second inducer of apoptosis in MNC from healthy individuals. As shown in Figure 5, induced apoptosis was significantly ($p < 0.001$) reduced by flupirtine within the concentration range of 0.1 to 3 μg/ml. The degree of induced apoptosis decreased from $44.3 \pm 6.2\%$ (measured in the controls without flupirtine) to $22.4 \pm 5.6\%$ if cells had been preincubated with 0.3 μg/ml of flupirtine.

Effect of flupirtine on induced apoptosis in T-lymphocytes

In order to investigate if the protective effect of flupirtine is restricted to a special T-lymphocyte population, a two colour flow cytometric assay was performed. Fragmented DNA was labeled with biotinylated dUTP using the enzyme TdT and visualized by FITC-avidin. The lymphocyte subpopulations were identified with specific antibodies. The results with anti-CD3 antibodies are shown in Figure 6. In untreated cells (left blot) $> 95\%$ of the CD3$^+$ as well as non-CD3 lymphocytes are non-apoptotic. If cells were treated with HX/XOD $46.4 \pm 6.2\%$ ($46.2 \pm 5.9\%$) of CD3$^+$ (non-CD3) became apoptotic (blot in the middle). The degree of induced apoptosis decreased significantly ($p < 0.001$) if those cells were treated with 1 μg/ml of flupirtine. Under these conditions only $18.9 \pm 2.1\%$ ($32.3 \pm 4.9\%$) of the CD3$^+$ (non-CD3) lymphocytes remained apoptotic (blot to the right).

Parallel experiments were performed with antibodies directed against CD4$^+$ and CD8$^+$ lymphocytes. These experiments demonstrated that flupirtine displayed also an anti-apoptotic effect against induced apoptosis in these lymphocyte subpopulations. It was determined that in the absence of flupirtine $22.9 \pm 4.9\%$ of CD4$^+$ ($28.3 \pm 5.2\%$ CD8$^+$) underwent apoptotic cell death, if the cells were induced with HX/XOD. If MNC had been pretreated with 1 μg/ml of flupirtine, the extent of apoptosis was reduced significantly ($p < 0.001$) to $11.8 \pm 1.8\%$ ($14.3 \pm 2.6\%$)

Neuroprotective effect of flupirtine on gp120 induced apoptosis in cortical cells

For induction of apoptosis, rat cortical cells were treated with gp120. In the presence of 20 ng/ml of gp120 (incubation time of 18 h) cell viability dropped from 75.9% (control) to 16.3% as checked by the MTT assay system (Table 1). DNA fragmentation (application of the sedimentation technique) increased from 13.1% (in the absence of gp120) to 92.2% (in
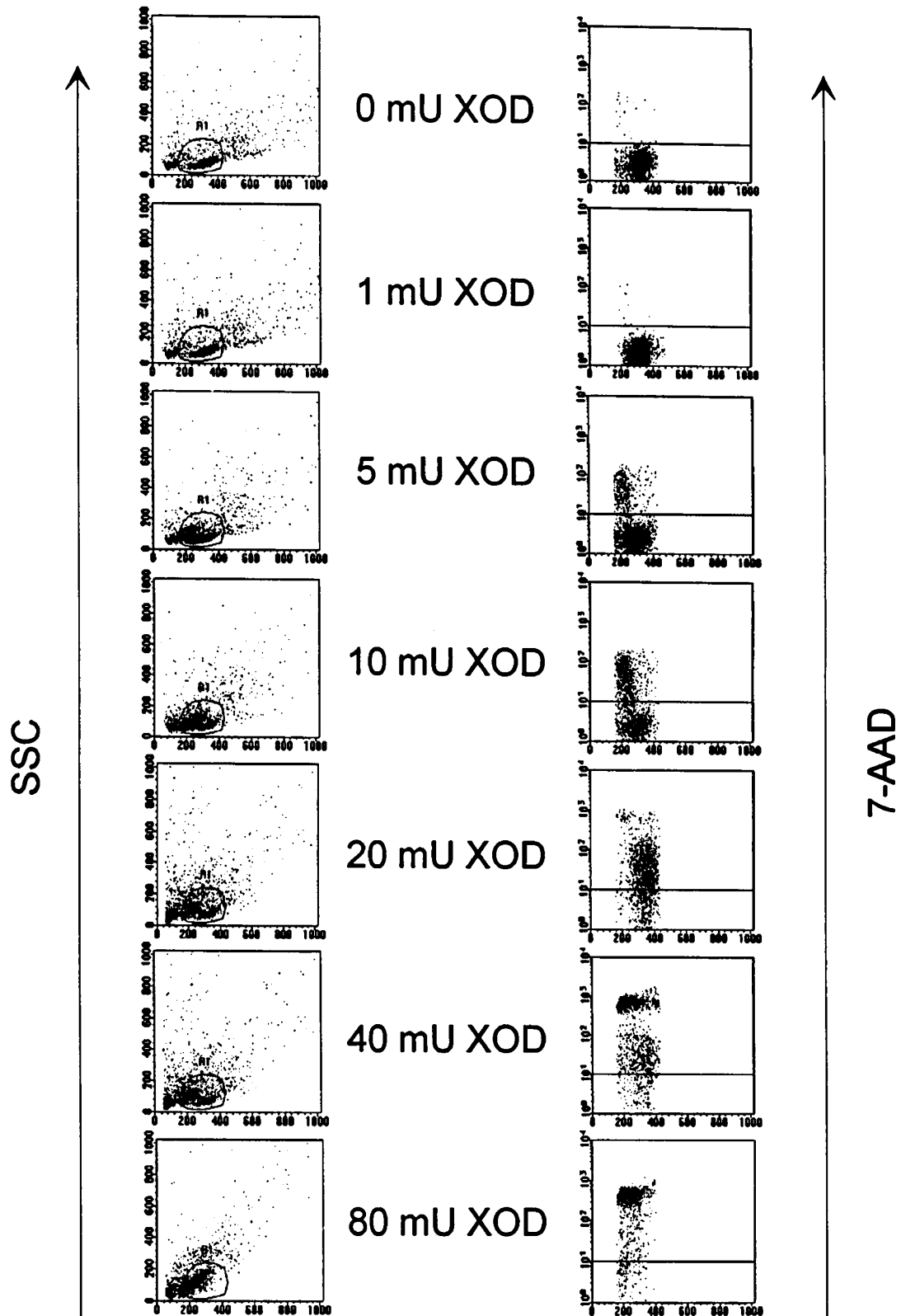
Figure 1 Induction of apoptosis by incubation of mononuclear cells (MNC) from healthy donors at a fixed concentration of hypoxanthine (HX; 1 mM) and increasing concentrations of xanthine oxidase (XOD; mU/ml). MNC were gated for analysis. Left column; the cells were analyzed by flow cytometry with respect to cell size (abscissa: FSC, forward light scatter) and cell granularity (ordinate: SSC, sideward/right angle light scatter). Right column; the cells were stained with 7-AAD and red fluorescence was analyzed by FACScan flow cytometer for cell size (abscissa) and fluorescence (ordinate).
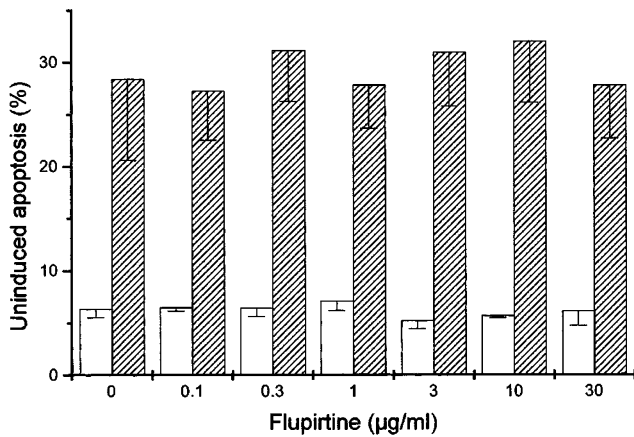
Figure 2 Effect of flupirtine on uninduced apoptosis of MNC from uninfected-(open bars) and HIV-1 infected individuals (hatched bars); after 1 day the cells were analyzed by flow cytometry. MNC from 12 infected and eight uninfected individuals were studied; the mean values and the SD are indicated.
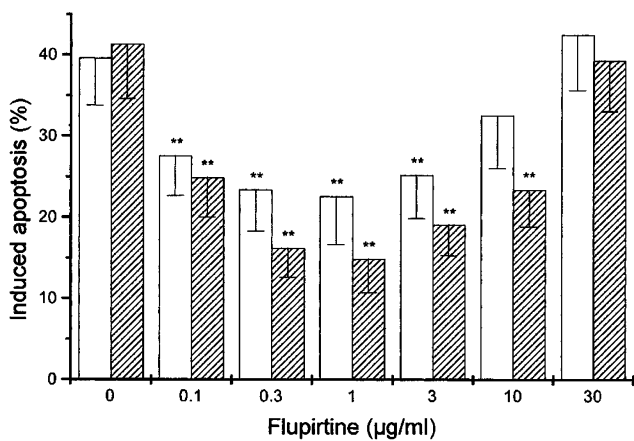

Figure 3 Reduction of induced apoptotic cell death of MNC by treatment with flupirtine. MNC from uninfected- (open bars) and HIV-1 infected individuals (hatched bars) were induced to apoptosis with HX/XOD. Flupirtine was added to the cells 6 h prior to the oxygen radical-generating system. One day later the cells were analyzed by flow cytometry. The values for uninduced apoptosis have been subtracted from the numbers measured. MNC from 12 infected and eight uninfected individuals were studied; the mean values and the SD are indicated. **p < 0.001 (versus assay with HX/XOD alone).
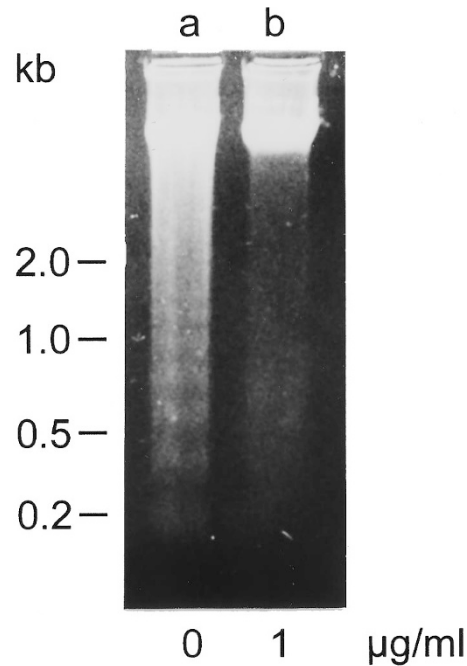

Figure 4 HX/XOD-induced apoptotic fragmentation of DNA in MNC from HIV-1 infected individuals. Cells were treated with the radical generating system and remained either without flupirtine (lane **a**) or were treated with 1 μg/ml of flupirtine (lane **b**) for 1 day. Then DNA was extracted from the cultures and analyzed by agarose (1.2%) gel electrophoresis. The molecular masses are shown.
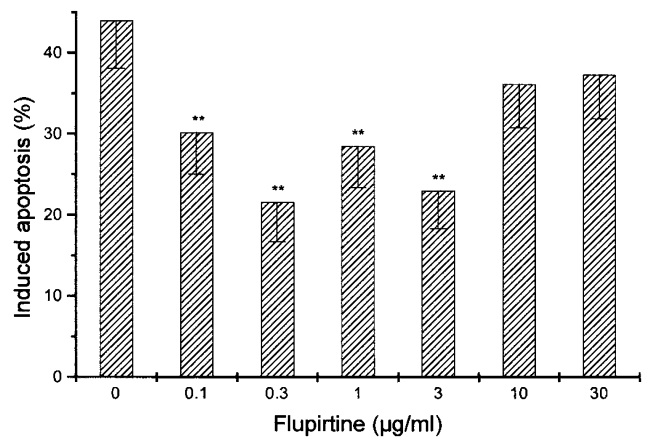

Figure 5 Effect of flupirtine on ionomycin-induced apoptotic cell death of MNC. MNC from uninfected individuals were induced to apoptosis with 1 μg/ml of ionomycin. Six hours prior to the addition of ionomycin flupirtine was added to the cells. One day later the cells were analyzed by flow cytometry. **p < 0.001 (versus assay with ionomycin alone).

the presence of gp120) (p < 0.001). Incubation of cortical cells with different concentrations of flupirtine resulted in a dose-dependent inhibition of the rate of the gp120-induced apoptosis (Table 1). DNA isolated from cultures incubated with 20 ng/ml of gp120 together with 1 μg/ml to 10 μg/ml of flupirtine showed a significant reduction in the percentage of DNA fragmentation (p < 0.001). Concomitantly with the decrease of DNA fragmentation the percentage of viable cells increased progressively (p < 0.001).

Under the conditions used a significant amount of cortical cells die – from 100% at the beginning of the incubation period to 75.9% – during the 18 h incubation period. This reduction can be significantly prevented by incubating the cells with flupirtine at concentrations higher than 3 μg/ml (p < 0.001) (Table 1). Simultaneously the

degree of DNA fragmentation is reduced (Table 1). This finding indicates that flupirtine acts neuroprotective also in the absence of the viral protein.

Discussion

Apoptosis is one major mechanism which balances homeostasis between cell proliferation and cell death in multi-
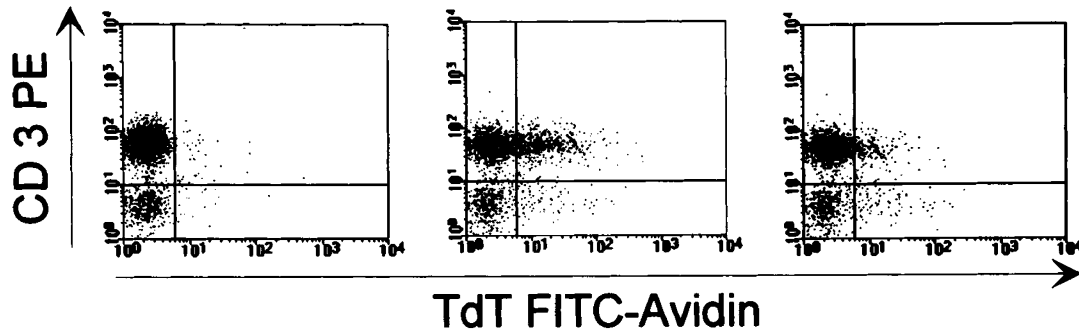
Figure 6 Effect of flupirtine on the degree of induced apoptosis in CD[3+]lymphocytes. The cells were treated with biotinylated dUTP and TdT and subsequently incubated with FITC-avidin to identify fragmented DNA; in addition the cells were labelled with anti CD3 monoclonal antibodies conjugated to phycoerythrin (PE). Left dot blot: control cells; middle: cells treated with 1 mM HX and 10 mU/ml XOD; right: cells treated with HX/XOD in the presence of 1 μg/ml of flupirtine. The upper-left quadrant contains CD[3+], non-apoptotic cells; the lower-left shows the CD3-negative, non-apoptotic cells. The right quadrants show the corresponding apoptotic fractions.

Table 1 Effect of flupirtine on gp120-induced DNA fragmentation and cell viability. Rat cortical cell cultures were incubated in the absence or presence of 20 ng/ml of gp120

Addition of gp120 (ng/ml)	Flupirtine (μg/ml)	DNA fragmentation (%)	Viable cells (%)
0	0	13.1 $\pm$ 3.3	75.9 $\pm$ 7.6
0	0.1	10.8 $\pm$ 3.8	77.0 $\pm$ 7.3
0	1	8.4 $\pm$ 2.4	85.5 $\pm$ 8.7
0	3	5.0 $\pm$ 2.3*	94.3 $\pm$ 5.2*
0	10	6.5 $\pm$ 2.2*	91.5 $\pm$ 6.1*
20	0	92.2 $\pm$ 4.7	16.3 $\pm$ 2.6
20	0.1	82.4 $\pm$ 7.5	22.1 $\pm$ 3.4
20	1	22.3 $\pm$ 4.9**	31.3 $\pm$ 5.6**
20	3	18.2 $\pm$ 4.0**	87.2 $\pm$ 7.4**
20	10	10.1 $\pm$ 2.6**	83.8 $\pm$ 7.7**

Where indicated flupirtine was added to the cultures 2 h prior to the addition of gp120. The incubation time was 18 h as described under Materials and Methods. DNA fragmentation was determined by the sedimentation technique and total cell viability was assayed using the MTT test. Twelve parallel experiments were performed. *$p < 0.001$ (Student's t -text versus assay without flupirtine); **$p < 0.001$ (versus assay with gp120 alone).

cellular organisms. The induction of apoptotic cell death can emanate from endogenous signals, or can be induced by extracellular signals (reviewed in: Vermes and Haanen, 1994; Thompson, 1995). Both (i) failure of cells to undergo apoptosis, e.g. in cancer patients, and (ii) excess of apoptotic cell death can be the basis of disorders, e.g. in AIDS. A variety of extrinsic signals have been described to induce apoptosis (reviewed in: Vermes and Haanen, 1994); in our group we identified (i) physiological activators, neurotransmitters (Müller *et al*, 1992), (ii) damage-related inducers, heat shock (Batel *et al*, 1993) and (iii) toxins, lead and prion protein (Perovic *et al*, 1995) as inducers of apoptosis. In AIDS patients excessive cell death has been described both for neurons and lymphocytes, a process which is assumed to be the major cause of this disease (Ameisen, 1992). *In vitro*, lymphocytes from HIV-1 infected individuals undergo uninduced apoptosis; a significant amount of lymphocytes taken from HIV-1 infected patients dies within 48 h in *in vitro* culture

(Gougeon, 1993). In addition, a series of extrinsic factors have been implicated in the initiation of 'induced' apoptosis in AIDS patients (Kabelitz *et al*, 1996).

It is very important not to interfere with the program causing uninduced apoptosis since this might result in proliferation disorders, e.g. cancer formation. One major task for a therapeutic intervention on cells undergoing apoptosis in AIDS patients is to block extracellularly induced apoptosis. Following this rationale, it was demonstrated that N-acetyl-L-cysteine (LNAC) prevents trophic factor-induced apoptosis of neruonal cells (Ferrari *et al*, 1995) and also induced apoptosis in blood cells, e.g. lymphocytes (Sandstrom 1994) *in vitro*. Because LNAC acts also as an oxygen radical scavenger (Blanco *et al*, 1995) like the oncogen *bcl-2* which is known to be (i) an antidote for apoptotic cell death (reviewed in: Tsujimoto, 1996) and (ii) downregulated in lymphocytes after HIV-1 infection (DeRossi *et al*, 1994), it was proposed to use this compound as a chemotherapeutic agent for AIDS patients. Unfortunately, data revealed that LNAC enhances HIV-1 replication (Nottet *et al*, 1994).

Flupirtine was effective against induced apoptosis in two cell systems: in lymphocytes and in neurons. Apoptosis in lymphocytes was induced by the oxygen radical forming HX/XOD system. The advantage of this inducer is the fact that it does not affect the expression of cytokines (to be published). Furthermore, the extent of apoptosis in a given cell system, here in lymphocytes, can be adjusted. Under the culture conditions used in the present study a ratio of 1 mM of HX and 10 mM of XOD caused apoptosis in approximately 50% of the lymphocytes. Using this system flupirtine significantly abolished the induced apoptosis at concentrations between 0.1 and 10 μg/ml.

In another set of experiments MNC were treated with ionomycin to undergo apoptosis. This effect of ionomycin was described earlier (Gougeon *et al*, 1993). Flupirtine at 0.1 to 3 μg/ml acted also anti-apoptotic in this system. In parallel, we have performed experiments with HL-60 cells and induced them to apoptosis with 0.5 μg/ml actinomycin D within an incubation period of 18 h. In this system

flupirtine displayed no protective effect within the concentration range of 0.1 to 10 μg/ml (to be published).

In a further series of experiments the anti-apoptotic effect displayed by flupirtine, was also demonstrated for T-lymphocyte subpopulations. The data showed that flupirtine protects CD3^{+}, as well as CD4^{+} and CD8^{+} lymphocytes, against apoptosis induced by the HX/XOD system.

Previously we reported that flupirtine displays an anti-apoptotic effect on cortical cells from rat embryos induced to undergo apoptosis by intact HIV-1 particles (Perovic *et al*, 1994). Now we show, in a more defined system, that apoptosis of neuronal cells caused by purified HIV-1 gp120 is also prevented by flupirtine. The optimal concentration range which protected the cells was 1 to 10 μg/ml. These doses are lower than those required in the earlier study using intact HIV-1 where at least 40 μg/ml had to be applied for 90% protection. From this finding we conclude that further molecules of the virus, other than pure protein gp120 are involved in the initiation of neuronal apoptosis.

Neuronal cells are known to undergo cell death *in vitro*, very likely due to the lack of neurotrophic agent(s) (reviewed in: Lo *et al*, 1995). It remains open, whether gp120 causes directly, or via a further cell type neuronal apoptosis (discussed in: Ushijima *et al*, 1995). It was found that neuronal apoptosis is almost completely blocked by flupirtine (Perovic *et al*, 1994). The anti-apoptotic mechanism displayed by flupirtine is not yet fully understood. As outlined in Introduction, flupirtine acts like an NMDA antagonist without binding to the NMDA receptor. Due to the fact that (i) the NMDA receptor is present exclusively at the postsynaptic membrane of excitatory synapses in the brain (Ehlers *et al*, 1995) and (ii) 1 mM of glutamate does not significantly induce apoptosis in human lymphocytes (to be published), it appears likely that flupirtine displays its anti-apoptotic effect via a second mechanisms, e.g. increase of ATP level (Osborne *et al*, 1996).

Flupirtine is clinically safe; adverse reactions are minimal in incidence, nature and degree, with drowsiness as the most frequently reported reaction (approximately 10% of the patients (McMahon *et al*, 1987). Important for future therapeutical applications of the drug is the finding that after a single oral application of 200 mg of flupirtine a peak concentration (c_{max}) of 2.4 μg/ml is reached in the plasma after 90 min; the level of $\geqslant 1$ μg/ml of flupirtine which was found to act anti-apoptotically *in vitro* remains there for 12 h (Hlavica and Niebch, 1985).

From these data we conclude that flupirtine is a potent anti-apoptotic compound in *in vitro* systems with lymphocytes which have been induced to undergo apoptosis by extracellular agents, such as the oxygen radical inducing system HX/XOD and ionomycin. In addition flupirtine acts anti-apoptotically on neurons against the deleterious effect caused by HIV-1 gp120. Furthermore, the neuroprotective effect of flupirtine has been proven in animal models of cerebral and retinal ischemia (Osborne *et al*, 1996; Rupalla *et al*, 1995; Block *et al*, 1995). Therefore, the results presented strongly suggest that flupirtine is a promising drug to treat not only (i) neurodegenerative disorders in general and AIDS-associated encephalopathia in particular

but also (ii) induced apoptotic death of lymphocytes in AIDS patients.

Materials and Methods

Materials

Flupirtine maleate [2-amino-3-ethoxycarbonylamino-6-(4-fluoro-benzylamino)-pyridine maleate] (M_r: 420,41) was obtained from ASTA Medica Corp. (Frankfurt/M, Germany).

Purified gp120 was prepared from HIV-infected H9 cells as described (Robey *et al*, 1986). The HIV-1 isolate IIIB was used (Popovic *et al*, 1984). The preparation was >95% pure as checked by polyacrylamide gel electrophoresis (Müller *et al*, 1988).

Blood samples

Human peripheral blood samples were obtained from 12 HIV infected homosexual males (mean age 35 years, range 22 – 43 years) and from eight healthy HIV seronegative and 'risk-matched' males of about the same age (mean age 30 years, range 23 – 35 years). Patients were staged according to the Centers for Disease Control (CDC) surveillance definition (CDC, 1992). The study of HIV seropositive patients was performed with blood samples from asymptomatic carriers (CDC stages: A1 and A2, mean CD4^{+} cell count 407/μl, range 209 – 698/μl). None of the patients had malignancies, symptomatic infections or any immunosuppressive treatment i.e. corticosteroids 10 weeks before sampling (Voth *et al*, 1988).

Cell preparation and treatment

Mononuclear cells (MNC) were isolated from heparinized blood by centrifugation on a Ficoll Hypaque density gradient (Voth *et al*, 1988). 0.5×10^6 cells were cultured in 500 μl medium composed of MEM supplemented with 10 mM HEPES buffer, 2.6% sodium bicarbonate, 0.1 U/ml penicillin, 0.1 U/ml streptomycin, 10% FCS and 1 mM glutamine. Where indicated, the cells were treated immediately after isolation with flupirtine.

Induction of apoptosis in MNC by reactive oxygen species and ionomycin

Reactive oxygen species were generated using hypoxanthine (HX) and xanthine oxidase (XOD) (Bruck *et al*, 1994). To induce apoptosis MNC were cultured for 24 h in a total volume of 500 μl MEM medium containing 0 to 80 mU/ml of XOD and 1 mM of HX. At 80 mU/ml of XOD (Sigma) >90% of MNC underwent apoptosis. If not mentioned otherwise, 10 mU/ml of XOD and 1 mM HX have been used for the studies. Under these experimental conditions approximately 50% of the MNC underwent apoptosis. This degree is desirable for studies in which a given compound is tested for potential inhibition and/or stimulation of apoptotic cell death. As a second inducer of apoptosis we applied ionomycin at a concentration of 1 μg/ml.

Flupirtine was applied to the cells 6 h prior to the addition of the oxygen radical producing system or ionomycin and incubation was terminated after 1 day.

Analysis of cells by flow cytometry

Apoptosis was measured in a flow cytometer FACScan (Becton Dickinson) with an argon laser turned to 488 nm, according to the

technique described by Schmid *et al* (1994). MNC were gated for analysis by a combination of forward light scatter (a measure for the size of cells, FSC) and right angle light scatter (granularity; SSC) properties. To stain apoptotic cells MNC were incubated with 20 μg/ml of 7-aminoactinomycin D (7-ADD) (Sigma) in PBS for 20 min at 4°C under light protection. Then the cells could be analyzed by the flow cytometer in the staining solution. The red fluorescence of 7-AAD was filtered through a 650 nm long pass filter. 7-AAD penetrates into apoptotic cells due to loss of membrane integrity and stains DNA by intercalation. The data analysis was performed using LYSYS II software. Mean channel numbers were converted to mean intensity of fluorescence, cell size and granularity.

Fluorescence *in situ* TdT assay and cell surface staining

In order to define the cell subsets undergoing apoptosis the terminal desoxynucleotidyltransferase (TdT) assay was applied, as described by Gorczyca *et al* (1993) with minor modifications. Cells in suspension were washed twice in PBS and fixed in 1% paraformaldehyde (Riedel de Haen) for 10 min at 4°C. Then cells were washed twice in PBS (supplemented with 5% AB serum and 0.5% bovine serum albumin (BSA)), pelleted and resuspended in 50 μl of 2.5 mM cobalt chloride solution, containing 0.5 nM of biotin-16-dUTP and 25 U of TdT in a reaction buffer containing 200 mM potassium cacodylat, 25 mM Tris-HCl and 0.25 mg/ml BSA; incubation was proceeded at 37°C for 30 min. Cells were washed twice in PBS (5% AB serum and 0.5% BSA) and resuspended in 100 μl of staining buffer, which contained 5 μg/ml of fluorescein isothiocyanate (FITC)-labeled avidin (cell sorter grade) (Serva). For simultaneous cell surface staining 20 μg/ml of phycoerythrin-labeled monoclonal anti-CD3-, anti-CD4- or anti-CD8-antibodies (Becton Dickinson) were added. Fluorescence was assessed on a flow cytometer (Halliwell, 1987).

Rat cortical cells

Primary cortical cells were prepared from the brains of 18 days old Wistar rat embryos by dissociation as described previously (Perovic *et al*, 1994, 1995). Briefly, cells were dissociated with 0.025% of trypsin, placed into polylysine-coated plastic flasks and cultivated in growth medium (Dulbecco's modified Eagle's medium (DMEM), supplemented with 30 mM glucose, 100 mU/l of insulin, 2 mM glutamate, 0.1 U/ml penicillin, 0.1 U/ml streptomycin) with 10% FCS. To enrich the percentage of neurons the cultures were incubated for 24 h in the presence of 10 μM of cytosine arabinoside. The cultures contained > 90% of neurons; the other cells were astrocytes (Boehringer detection kit) and macrophages (Perovic *et al*, 1994). One day later the cells were used for the experiments.

Treatment of cortical cells with gp120

Cells were treated with 20 ng/ml of gp120 in phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free; containing 25 mM Tris/HCl (pH 7.4) and 15 mM glucose). Flupirtine was added to cells 2 h prior to the viral protein; the total incubation period was 18 h.

DNA fragmentation was determined as follows: firstly, fragmentation was determined qualitatively by electrophoresis on horizontal agarose gels. The modified highly sensitive method (labelling of extracted DNA with biotin-dUTP – size separation on agarose gels – blot-transfer – visualization by streptavidin alkaline phosphatase-BCIP/NBT) was applied (Lauc *et al*, 1994). Secondly, for quantitative analysis of DNA fragmentation the sedimentation technique was applied (Wyllie, 1980).

The viability of total cells was determined with the MTT colorimetric assay system (Scudiero *et al*, 1988), followed by evaluation with an ELISA plate reader (BioRad 3550, equipped with the program NCIMR IIIB).

Statistical analysis

The results were analyzed by paired Student's t-test (Sachs, 1984).

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