Cytotoxic effect of HIV-1 coat glycoprotein gp120 on human neuroblastoma CHP100 cells involves activation of the arachidonate cascade

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HIV type-1 (HIV-1) coat glycoprotein gp120 causes necrotic death in human neuroblastoma CHP100 cells. Here, we investigated the possible role of the arachidonate cascade and membrane peroxidation in gp120-induced cell necrosis. It is shown that gp120 increases the intracellular concentrations of prostaglandin E_2 and leukotriene B_4 by up-regulating the activity and expression of the arachidonate-metabolizing enzymes prostaglandin H synthase and 5-lipoxygenase respectively. Consistent with this observation, selective inhibitors of prostaglandin H synthase (i.e.

indomethacin) and 5-lipoxygenase (i.e. MK886 and caffeic acid) protected CHP100 cells against gp120-induced necrosis. Treatment with gp120 also enhanced membrane lipid peroxidation and this may be implicated in the execution of cell damage. Interestingly, incubation with exogenous nitric oxide (NO) mimicked the effects of gp120 on necrotic death of CHP100 cells and activation of prostaglandin H synthase and 5-lipoxygenase. This suggests that NO might participate in the mechanism by which gp120 activates the arachidonate cascade.

INTRODUCTION

The entry of HIV type 1 (HIV-1) into human cells proceeds via a fusion mechanism that is initiated by binding of the viral coat glycoprotein gp120 to its primary receptors CD4 and galactosylceramide [1]. Recent findings suggest that binding of gp120 might induce membrane modifications which contribute to virus entry [2,3]. Exposure of human neuroblastoma CHP100 cells to gp120 has been shown to lead to cell necrosis [4,5] along a pathway which might involve the activity of nitric oxide synthase (E.C. 1.14.13.39), prostaglandin H synthase (PHS; E.C. 1.14.99.1) and calpain protease [4,5].

In the present study we investigated the possible role of the arachidonate cascade and membrane peroxidation in gp120-induced necrosis of CHP100 cells. Indeed, arachidonate metabolites are elevated in the central nervous system of patients with HIV-1-related dementia [6] and are involved in neurodegeneration [7]. Disturbances in the arachidonate cascade have been proposed to play a role also in HIV pathogenesis [8,9]. Arachidonic acid can be converted into biologically active compounds such as prostanoids (i.e. prostaglandins, prostacyclin, thromboxane) or leukotrienes and lipoxins, via the cyclo-oxygenase or the lipoxygenase pathway respectively [10]. The first committed step in prostanoid biosynthesis is catalysed by PHS, whereas the leukotriene and lipoxin formation is catalysed by arachidonate 5-lipoxygenase (E.C. 1.13.11.34). Therefore, the activity and expression of PHS and 5-lipoxygenase, as well as the concentration of the main products of enzyme activity, i.e. prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) respectively, were measured in gp120-treated CHP100 cells. Moreover, CHP100 cell membrane peroxidation was measured upon exposure to gp120, because membrane peroxides reduce the order of the lipid bilayer [11], and this may facilitate the entry of HIV-1 into human cells [2,3]. The possible role of nitric oxide (NO) in

mediating the effects of gp120 on the activity of PHS and 5-lipoxygenase was investigated as well, because enhanced production of NO is involved in gp120-induced death of CHP100 cells [4] and is considered one of the main triggers of neuronal cell death [12].

EXPERIMENTAL

Materials

All chemicals were of the purest analytical grade. Arachidonic (eicosatetraenoic) acid, ATP, caffeic acid, hemin, phenol, PMSF, sodium nitroprusside (sodium nitroferricyanide, SNP), soybean trypsin inhibitor, N,N,N',N' -tetramethyl- p -phenylenediamine (TMPD) and indomethacin [1-(p -chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid] were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endotoxin-free, full-length glycosylated recombinant HIV-1 glycoprotein gp120 IIIB (from baculovirus expression system; > 90 % pure by SDS/PAGE) was purchased from INTRACEL Corporation (Cambridge, MA, U.S.A.). 5-Lipoxygenase-specific inhibitor 3-[1-(p -chlorobenzyl)-5-(isopropyl)-3- t -butylthioindol-2-yl]-2,2-dimethylpropanoic acid (MK886), anti-human 5-lipoxygenase and anti-human 5-lipoxygenase activating protein (FLAP) rabbit polyclonal antibodies were a gift from Dr. A. W. Ford-Hutchinson (Merck Frosst Centre for Therapeutic Research, Canada). Anti-cyclo-oxygenase-2 rabbit polyclonal antibodies were from Cayman Chemical Company (Ann Arbor, MI, U.S.A.), whereas goat-(anti-rabbit IgG) polyclonal antibodies conjugated with alkaline phosphatase (GAR-AP) were purchased from Bio-Rad (Richmond, CA, U.S.A.). [$1-^{14}C$]Arachidonic acid (specific radioactivity = 55 mCi/mmol = 2.04 GBq/mmol) and L-[2,3,4,5- 3H]arginine (specific radioactivity = 64 Ci/mmol =

Abbreviations used: HIV-1, HIV type 1; PHS, prostaglandin H synthase; TMPD, N,N,N',N' -tetramethyl- p -phenylenediamine; SNP, sodium nitroprusside; PGE_2 , prostaglandin E_2 ; LTB_4 , leukotriene B_4 ; CSS, controlled saline solution; 5-HPETE, 5-hydroperoxyeicosatetraenoic acid; FLAP, 5-lipoxygenase activating protein; GAR-AP, goat anti-rabbit IgGs conjugated with alkaline phosphatase; RP, reverse phase.

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2.37 TBq/mmol) were from Amersham (Aylesbury, Bucks., U.K.). Authentic PGE₂ and LTB₄ were from Cayman Chemical Company (Ann Arbor).

Cell culture and treatment

Human neuroblastoma CHP100 cells were cultured as reported [4], in a 1:1 mixture of Eagle's minimal essential medium plus Earle's salts and Ham's F-12 media (Flow Laboratories Ltd., Herts., U.K.), supplemented with 15% heat-inactivated fetal bovine serum, sodium bicarbonate (1.2 g/l), 15 mM Hepes buffer, 2 mM L-glutamine and 1% non-essential amino acids. CHP100 cells were cultured at 37 °C in a 5% CO₂ atmosphere. Exposure of CHP100 cells for 20 min to 10 pM gp120 or 200 µM SNP in controlled saline solution (CSS: 120 mM NaCl/5.4 mM KCl/1.8 mM CaCl₂/10 mM glucose/25 mM Tris/HCl, pH 7.4), followed by 24 h of incubation in normal culture medium, was performed according to [5]. Paired controls were incubated for the same period of time in CSS alone (referred to as control in the Results section) or in CSS containing 10 nM BSA. The specificity of gp120 was further addressed by exposing CHP100 cells to 10 pM gp120 pre-incubated with mouse monoclonal anti-gp120 (IgG) antibodies (in the ratio 3.6 ng of gp120 to 1.0 µg of antibody) for 30 min at room temperature [4]. The effect of the PHS inhibitor indomethacin and the 5-lipoxygenase inhibitors MK886 and caffeic acid on gp120-induced cell death was assessed by adding each compound to CHP100 cultures 10 min before the addition of gp120, and leaving the substance in the medium during the 20 min exposure to gp120. Cell viability was assessed by Trypan Blue dye-exclusion, and cell death was expressed as number of stained (necrotic) cells counted per 100 cells [4].

Analysis of arachidonate metabolites

Incorporation and release of [¹⁴C]arachidonic acid was followed as reported [13]. After 20 min of treatment with 10 pM gp120, CHP100 cells were washed and resuspended in serum-free medium containing 1 µCi of [¹⁴C]arachidonic acid per flask. After 2 h of incubation at 37 °C, cells were washed again and resuspended in normal culture medium for an additional 22 h at 37 °C. Then, membrane lipids were extracted from CHP100 cells as described previously [14] and radioactivity was measured in both lipid extracts and culture media by liquid-scintillation counting. Under our experimental conditions, CHP100 cells incorporated 50 ± 5% of the [¹⁴C]arachidonic acid supplied. Arachidonate metabolites PGE₂ and LTB₄ were extracted from CHP100 cells on octadecyl-SPE columns (Baker, Deventer, The Netherlands) and were analysed by reverse-phase (RP) HPLC on a C₁₈ 3 × 3 CR column (SGE, Austin, TX, U.S.A.) [15]. RP-HPLC was performed on a Perkin-Elmer 1022 LC Plus liquid chromatograph (Norwalk, CT, U.S.A.) at a flow rate of 1.2 ml/min, using methanol/water/trifluoroacetic acid (70:30:0.07, by vol.) as mobile phase. Chromatograms were recorded at 270 nm and peak identity was assessed by comparison with authentic standards. Quantitative determinations were performed by integrating the peak area of each compound.

Analysis of membrane lipid spectra

Membrane lipids were isolated from control and gp120-treated CHP100 cells as reported [14], then absorption spectra were recorded in the wavelength range 200–300 nm, in order to measure the oxidative index, i.e. the A_{234}/A_{205} ratio [16]. Differential spectra were obtained by subtracting the absorption spectrum of controls from that of gp120-treated CHP100 cells.

Spectra were recorded at room temperature in a UV/VIS spectrometer Lambda 18 (Perkin-Elmer).

Assay of enzyme activity and expression

The activity of arachidonate 5-lipoxygenase in CHP100 cells was measured by incubating cell extracts for 10 min at 37 °C in the presence of 1 mM ATP, 2 mM CaCl₂ and 40 µM arachidonic acid [15]. 5-Lipoxygenase activity is expressed as nmol of 5-hydroperoxyeicosatetraenoic acid (5-HPETE) formed/min per mg of protein. PHS is a dual enzyme possessing cyclo-oxygenase and peroxidase activity on the same protein molecule [17]. Cyclo-oxygenase activity was measured polarographically, at 30 °C, in 0.1 M potassium phosphate, pH 7.2, 1 mM phenol and 75 µM arachidonic acid [18] and is expressed as nmol of O₂ consumed/min per µg of protein. Peroxidase activity was assayed spectrophotometrically, at 30 °C, in 0.1 M Tris/HCl, pH 8.1, 170 µM TMPD, 1 µM hemin and 75 µM arachidonic acid [19] and is expressed as m-units per µg of protein (1 unit = amount of enzyme causing a change in A_{590} of 1 unit during 5 min incubation with the substrate). The effect of the NO-donor SNP on the activity of 5-lipoxygenase and PHS was determined by adding 200 µM SNP to CHP100 cultures for 20 min, followed by 24 h of incubation in normal culture medium. Cell extracts were prepared and used to assay enzyme activity as described above. The activity of NO synthase was assayed by incubating cell extracts with the radiolabelled substrate [³H]arginine, and then isolating and quantifying the reaction product [³H]citrulline according to [20]. NO synthase activity is expressed as pmol of citrulline formed/min per mg of protein. The protein concentration of cell extracts was determined as reported [21].

ELISA of CHP100 cell extracts, prepared according to [22], were performed as reported [23], using 25 µg of protein/well. The following rabbit polyclonal antibodies were used as first antibody: anti-5-lipoxygenase (diluted 1:200), anti-FLAP (diluted 1:300) or anti-cyclo-oxygenase-2 (PHS-2) (diluted 1:400). GAR-AP was used as second antibody (diluted 1:2000). Colour development of the alkaline phosphatase reaction was followed at 405 nm.

Data analysis

The data reported in this paper are the means (± S.D.) of at least three independent determinations, each performed in duplicate. Statistical analysis was performed by Student's *t*-test, elaborating experimental data by means of the InStat programme (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Exposure of human CHP100 neuroblastoma cells to 10 pM gp120 caused significant ($P < 0.01$) cytotoxic effects, as assessed 24 h later by Trypan Blue dye-exclusion (Table 1). These effects were due to the action of the viral protein, because under similar experimental conditions cell death caused by gp120 (dose ranging from 10 pM to 10 nM) was abrogated by neutralization of the viral protein with a monoclonal anti-gp120 antibody (Table 1). The knowledge that cell death induced by 10 pM or 10 nM gp120 does not differ significantly [4] strengthens the concept that the observed cytotoxicity was due to the action of gp120 and not to the presence of contaminants; this led us to use a dose of 10 pM in subsequent experiments. In agreement with our data, concentrations of recombinant gp120 in the picomolar range have been shown to produce neurotoxic effects in cultured neurons [24–26]. Furthermore, levels of gp120 in the same range as above were detected in the brain of rat pups treated systematically with

Table 1 Effect of gp120 and the NO-donor SNP on cell death and eicosanoid content of CHP100 cells

Values in brackets represent percentage of the control, arbitrarily set to 100. ^aNumber of necrotic cells counted (at least in triplicate) in every 100 cells. **P* < 0.01, ***P* < 0.05 compared with control.

Sample	Cell death ^a	PGE ₂ (nmol/mg of protein)	LTB ₄ (pmol/mg of protein)
CHP100 (control)	7.6 ± 0.3	5.24 ± 0.50	4.63 ± 0.45
CHP100 (+ 10 pM gp120)	24.7 ± 0.4 (325)*	17.01 ± 1.70 (325)*	12.16 ± 1.20 (263%)*
CHP100 (+ 10 pM gp120 + anti-gp120 antibody)	8.6 ± 0.3 (113)**	6.10 ± 0.60 (116)**	5.43 ± 0.55 (117%)**
CHP100 (+ 200 μM SNP)	25.6 ± 1.7 (337)*	14.72 ± 1.50 (281)*	10.32 ± 1.05 (223%)*
CHP100 (+ 10 pM gp120 + 200 μM SNP)	44.1 ± 1.8 (580)*	25.20 ± 2.50 (481)*	17.64 ± 1.80 (381%)*

Table 2 Effect of gp120 and NO-donor SNP on the activity of PHS and 5-lipoxygenase in CHP100 cells

Values in brackets represent percentage of the control, arbitrarily set to 100. **P* < 0.01 compared with control.

Enzyme activity	CHP100 (control)	CHP100 (+ 10 pM gp120)	CHP100 (+ 200 μM SNP)
PHS cyclooxygenase activity (nmol O ₂ /min per μg of protein)	0.44 ± 0.05	1.20 ± 0.10 (273)*	1.00 ± 0.10 (227)*
PHS peroxidase activity (m-units/μg of protein)	1.19 ± 0.10	2.68 ± 0.25 (225)*	2.38 ± 0.25 (200)*
5-Lipoxygenase activity (nmol 5-HPETE/min per mg of protein)	0.84 ± 0.08	1.48 ± 0.15 (176)*	1.27 ± 0.13 (151)*

Table 3 Effect of gp120 on the expression of PHS-2, 5-lipoxygenase and FLAP in CHP100 cells

Values in brackets represent percentage of the *A*₄₀₅ values of the control, arbitrarily set to 100. **P* < 0.05, ***P* < 0.01 compared with control.

Enzyme	CHP100 (control)	CHP100 (+ 10 pM gp120)
PHS-2	0.35 ± 0.04	0.46 ± 0.05 (131)*
5-Lipoxygenase	0.50 ± 0.05	0.70 ± 0.07 (140)**
FLAP	0.65 ± 0.07	0.98 ± 0.10 (151)**

the viral protein, which also caused retardation of behavioural development and a pattern of brain damage similar to that found *post mortem* in the brains of AIDS patients [27]. Treatment with gp120 did not affect the ability of human neuroblastoma CHP100 cells to incorporate arachidonate into membrane lipids or to release it into the culture medium, indeed incorporation and release of arachidonate in CHP100 cells treated with gp120 were 99 % or 92 % of the control values respectively. RP-HPLC analysis clearly indicated that gp120 significantly up-regulated the cascade itself, leading to a 2.6–3.2-fold increased concentration of the arachidonate metabolites LTB₄ and PGE₂ (Table 1). PGE₂ is the main product of PHS *in vivo* [17], whereas LTB₄ is the product of 5-lipoxygenase [28]. Therefore, the activity of both enzymes was measured after treating CHP100 cells with gp120. Furthermore, both cyclo-oxygenase and peroxidase activities of PHS were assayed, because they catalyse two different steps of prostanoid biosynthesis [17,18]. Table 2 shows that treatment with gp120 enhanced both 5-lipoxygenase (up to 176 % of the control) and PHS (273 % for cyclo-oxygenase and 225 % for peroxidase activity) in CHP100 cells. The increased enzyme activity was paralleled by a seemingly lesser increase in enzyme protein content (Table 3). Interestingly, the expression of the FLAP, which is needed to translocate 5-lipoxygenase to the nuclear membranes [29], was up-regulated by gp120 treatment,

Table 4 Effect of the PHS inhibitor indomethacin and the 5-lipoxygenase inhibitors MK886 and caffeic acid on gp120-induced cytotoxicity

Values in brackets represent percentage of the control, arbitrarily set to 100. ^aNumber of necrotic cells counted (at least in triplicate) in every 100 cells. **P* < 0.01, ***P* > 0.05, ****P* < 0.05 compared with control. #*P* < 0.01 compared with gp120-treated sample.

Sample	Cell death ^a
CHP100 (control)	7.6 ± 0.3
CHP100 (+ 10 pM gp120)	24.7 ± 1.4 (325)*
CHP100 (+ 10 μM indomethacin)	8.9 ± 0.5 (117)**
CHP100 (+ 10 μM MK886)	12.5 ± 0.5 (164)*
CHP100 (+ 10 μM caffeic acid)	9.5 ± 0.4 (125)***
CHP100 (+ 10 pM gp120 + 10 μM indomethacin)	9.6 ± 0.6 (126)***,#
CHP100 (+ 10 pM gp120 + 10 μM MK886)	14.5 ± 0.7 (191)*,#
CHP100 (+ 10 pM gp120 + 10 μM caffeic acid)	10.5 ± 0.5 (138)*,#

reaching 151 % of the control value (Table 3). On the other hand, the 5-lipoxygenase inhibitors caffeic acid [30] and MK886 [31] were able to counteract the cytotoxic effect of gp120 on CHP100 cells (Table 4). In line with our previous data [4], the PHS inhibitor indomethacin [32] also prevented the cell death otherwise produced by the viral protein (Table 4). In pilot experiments it was found that each inhibitor was effective in protecting cells against gp120 toxicity, with a dose-dependence in the concentration range 0–10 μM. The maximum concentration of 10 μM completely neutralized gp120 (Table 4).

Interaction of CHP100 cells with HIV-1 coat protein gp120 led also to a significant increase in membrane lipid peroxidation, as shown by the significant increase in UV absorption (Figure 1A). Exposure to gp120 increased the absorption of CHP100 membrane lipids in the region around 230 nm (Figure 1B), where the main contributors to the spectrum are conjugated hydroperoxides. Consistent with this, the oxidative index of membrane lipids, i.e. the *A*_{234/205} ratio [16], was increased by gp120 treatment to up to 159 % of the control level (from 0.108 ± 0.010 to 0.172 ± 0.015; *P* < 0.01 compared with control). It is noteworthy

Figure 1 Absorption spectra of membrane lipids of CHP100 cells

(A) Membrane lipids were extracted from 1.0×10^7 CHP100 cells, untreated (spectrum a) or exposed to 10 pM gp120 for 20 min and then cultured for 24 h (spectrum b). (B) Difference spectrum, calculated by subtracting spectrum a from b in (A).

that HPETEs, which are products of lipoxygenase activity, are among the most important conjugated hydroperoxides in cellular biomembranes [16].

CHP100 cells treated with gp120 showed increased activity of NO synthase, which reached 128% of the control level (from 5.76 ± 0.29 to 7.39 ± 0.17 pmol of citrulline/min per mg of protein; $P < 0.05$ compared with control). Interestingly, incubation of CHP100 cells with the NO-donor SNP, under the same conditions as those used for gp120 treatment, increased cell death dose-dependently in the concentration range 0–200 μ M (results not shown). Exposure of CHP100 cells to 200 μ M SNP yielded almost the same increases in cell death (up to 337% of the control) and PGE₂ (281%) and LTB₄ (223%) levels as those induced by 10 pM gp120 (Table 1). Remarkably, the effects of gp120 and SNP were additive (Table 1). Incubation of CHP100 cells with 200 μ M SNP also enhanced the cyclo-oxygenase and peroxidase activity of PHS (up to 227% and 200% of the control respectively), and 5-lipoxygenase activity (up to 151% of the control value), which resembles the effect of 10 pM gp120 (Table 2).

DISCUSSION

The present investigation shows that necrotic death of human neuroblastoma CHP100 cells induced by HIV-1 coat glycoprotein gp120 involves the activation of the arachidonate cascade and peroxidation of cell membranes. The increased formation of the arachidonate metabolites PGE₂ and LTB₄ (Table 1) was not

attributable to increased availability of the substrate arachidonate, because gp120 did not affect the ability of CHP100 cells to incorporate arachidonate into membrane lipids or to release it in the culture medium. Approximately 25% of incorporated arachidonic acid was released during the 22 h following incubation with arachidonic acid, the remaining 75% being detected in the membrane lipid extracts. RP-HPLC analysis indicated that arachidonate was not present in membrane lipids as a free fatty acid (results not shown), suggesting that it was incorporated into the different (phospho)lipid classes which constitute human cell membranes [14]. Instead, the increased formation of arachidonate metabolites was due to augmented activity and expression of PHS and 5-lipoxygenase (Tables 2 and 3), the two enzymes which catalyse the cyclo-oxygenase and the lipoxygenase pathways of the arachidonate cascade [10]. Remarkably, gp120 treatment enhanced the expression of the type-2 isoenzyme of PHS (cyclo-oxygenase-2 or PHS-2), which is the inducible form sensitive to environmental factors [17,30]. Up-regulation of PHS and 5-lipoxygenase was linked to gp120-induced cytotoxicity by a cause-effect relationship, because specific enzyme inhibitors of PHS, such as indomethacin [32], or of 5-lipoxygenase, such as caffeic acid [30] and MK886 [31], completely prevented the gp120 cytotoxic effects (Table 4). It is noteworthy that all inhibitors were able to counteract the gp120 effect at micromolar concentrations, which is the same concentration range where they effectively inhibit enzyme activity *in vitro* [30–32]. These results give biochemical background to previous observations suggesting that disturbances in the arachidonate cascade might be involved in HIV neuropathogenesis [8,9]. The ability of PGE₂ to induce necrosis in neuroblastoma cells [4] and that of lipoxygenase metabolites to regulate cell survival and death [33], also in HIV-infected cells [34], strengthen this concept. In particular, 5-lipoxygenase plays a critical role in lipid-mediated signal transduction [35] and its metabolites were recently found to be active in the nucleus of human cells [29]. Enhancement of FLAP expression, observed in gp120-treated CHP100 cells (Table 3), speaks in favour of a nuclear activity of 5-lipoxygenase, because FLAP translocates 5-lipoxygenase to the nucleus [29]. 5-Lipoxygenase might play its role in gp120-induced cytotoxicity, not only by generating arachidonate metabolites, but also by directly oxygenating cell membranes [16,36]. Peroxidation of outer membranes (Figure 1) could facilitate the entry of HIV-1 into human cells, because hydroperoxide groups in the fatty acid moieties of membrane (phospho)lipids perturb tail-to-tail contacts, thus reducing the stiffness of the lipid bilayer [11]. These alterations of membrane properties might be critical for the interaction of gp120 with membrane lipids, such as the primary receptor of the viral protein, galactosylceramide [1], or lipid moieties of secondary receptors [3]. Finally, the interplay between gp120 neurotoxicity, the arachidonate cascade and NO has been investigated, because NO has been associated with gp120-induced cytotoxicity [4] and is considered one of the main triggers of neuronal cell death [12]. Here it is shown that gp120 enhanced the NO synthase activity of CHP100 cells, and that 20 min incubation of cells with the NO-donor SNP mimicked the effects of gp120 on cell death and arachidonate metabolites (Tables 1). Remarkably, the effects of NO and gp120 were additive. Since exogenous NO enhanced the activity of PHS and 5-lipoxygenase in the same way as gp120 (Table 2), it can be suggested that NO may participate in gp120-induced cytotoxicity by activating these arachidonate-metabolizing enzymes, which in turn release toxic arachidonate metabolites and promote peroxidation of cell membranes. These findings are in keeping with recent studies on the *in vitro* interaction of NO with the purified enzymes, showing that NO enhances the cyclo-oxygenase and

peroxidase activity of PHS [37], while short pulses (< 5 min) of NO inhibit [38,39] and long pulses (> 5 min) activate lipoxigenase [39,40].

In conclusion, results reported here clearly show that the cytotoxic effect of HIV-1 coat protein gp120 on human neuroblastoma CHP100 cells involves activation of the arachidonate cascade, along a pathway which might involve NO.

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