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INHIBITORY EFFECT OF COUMARINS ON HIV-1 REPLICATION AND CELL-MEDIATED OR CELL-FREE VIRAL TRANSMISSION

A. S. BOURINBAIAR[1], X. TAN[2], R. NAGORNY[3]

[1]Department of Pediatrics, Division of Infectious Diseases, NYU Medical Center, 550 First Avenue, New York, NY 10016; [2]The Population Council, New York, NY 10021; and [3]Synaptic Pharmaceutical Co., Paramus, NJ 07652, U. S. A.

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Summary. – A single dose of coumarin derivatives, warfarin, 4-hydroxycoumarin and umbelliferone, added at the time of inoculation either by free virus or by contact with U1 monocytes exhibited a dose-dependent inhibitory effect on viral replication in target MOLT-4 lymphocytes observable even at 5 days post infection. In addition, marked decrease of HIV-1 *gag* p24 release and reduction in reverse transcriptase activity was observed when chronically HIV-infected ACH-2 lymphocytes were treated with coumarins ($ED_{50\%}$ range 10^{-6} –10^{-9} mol/l). However, the intracellular composition of HIV-1 core proteins in drug-exposed cells was not modified. Results suggest that although no complete inhibition of viral production has been observed *in vitro* this class of drugs may present potential interest as antiviral agents.

Key words: HIV; coumarins; lymphocytes; monocyte; antivirals

Introduction

Coumarin and common simple derivatives are found in a variety of plants (e. g., coriander, sweet clover, lavender) and due to pleasant flavor properties have been used widely in the food and cosmetic industries (Soine, 1964; Opdyke, 1974). Hydroxycoumarins are considered potent immunomodulators and have been successfully used in therapy of malignant metastases (Thornes *et al.*, 1982). Coumarins, mechanism-based serine protease inhibitors (Mor *et al.*, 1990), are major clinical anticoagulants acting as blockers of proteolytic conversion of prothrombin into thrombin through a vitamin K-dependent mechanism (Soine, 1964; Fasco, 1982). Another effect of coumarins is displayed by their effect on cysteine metabolism resulting in depletion of intracellular glutathione (GSH) – a naturally occurring tripeptide – which regulates cellular activities such as sulfhydryl-dependent enzyme reactions, protection from oxidation, initiation of DNA synthesis (Lake *et al.*, 1989) as well as HIV replication *in vitro* (Staal *et al.*, 1990). In preliminary investigation we have demonstrated that nontoxic, pharmacological doses of the synthetic coumarin, warfarin, manifest anti-HIV

activity (Bourinbaiar *et al.*, 1993). In this study we evaluated further the effect of diverse coumarin derivatives on HIV infectivity and replication.

Materials and Methods

Drugs. Warfarin (3-[alpha-acetonylbenzyl]-4-hydroxycoumarin), cyclocumarol (4-hydroxycoumarin) and umbelliferone (7-hydroxycoumarin) were purchased from Sigma Chemical Co., St Louis, MO.

Infection assay in virus-cell system. Fresh viral inoculum from supernatant of ACH2 lymphocyte culture (subclone of CEM T cell line, courtesy of AIDS Research and Reference Reagent Program, NIAID, Rockville, MD) (Folks *et al.*, 1989), containing 10^6 viral particles/ml (quantitated on the basis of p24 concentration according to Bourinbaiar (1991); Bourinbaiar *et al.* (1991); Bourinbaiar (1992)) was incubated with uninfected MOLT-4 lymphocytes (10^6 cells/ml, provided by Dr. J. Minowada, Fujisaki Cell Center, Okayama, Japan) (Minowada *et al.*, 1972) for 1 hr at 37 °C in the presence of tenfold dilutions of coumarins ranging from 10^{-10} to 10^{-4} mol/l. Target cells were washed three times and were cultured further in coumarin-free medium for 5 days until tested for virus production. All cultures were maintained in RPMI 1640 medium with 10 % foetal calf serum (Whittaker, Walkerville, MD) in humidified 5 % CO_2 incubator at 37 °C.

Infection assay in cell-cell system. HIV-1 carrying unstimulated U1 monocytes (subclone of U937 cells was provided by AIDS Research and Reference Reagent Program, NIAID, Rockville, MD) (Folks *et al.*, 1987) were incubated at a ratio of 1:10 with uninfected MOLT-4 lymphocytes (10^6 cells/ml) for 5 days in the continuous presence of dilutions of coumarins added at day 1 and the resulting culture medium was tested for viral production either by measuring HIV-1 core protein p24 by ELISA or by reverse transcriptase (RT) assay.

p24 antigen capture assay. Productive infection was evaluated by commercial ELISA kit for viral core *gag* 24 K protein according to manufacturer's instructions (Coulter, Hialeah, FL) as follows. Detergent-lysed samples of medium of cultures that were incubated in wells (96-well format) precoated with anti-p24 antibody were screened for *gag* antigen by adding biotin-labelled anti-p24 antibody followed by streptavidine-peroxidase conjugate. The amount of captured p24 was measured by comparing the absorbancy at 470 nm of TMB substrate with supplied standards containing known amounts of p24. The lower limit of reliable sensitivity of this kit is 7.8 pg/ml.

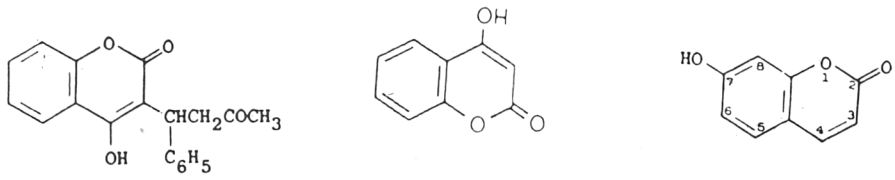
RT assay. HIV-associated RT activity was measured as described previously (Bourinbaiar and Nagorny, 1992) with modifications as follows: 10 μl of culture supernatant was mixed with 50 μl of an RT reaction mixture (Pharmacia LKB, Gaithersburg, MD) containing [^{32}P]dTTP (Amersham, Arlington Heights, IL). After 90 min at 37 °C, 10 μl of the reaction mixture was spotted onto DE.81 ion-exchange paper (Whatman, Hillsboro, OR), washed four times in 2× SSC, and then twice in 95 % ethanol to remove unincorporated [^{32}P]dTTP. Dried filters were counted in a scintillation counter. The background counts of the reaction mixture with culture medium which does not contain HIV-1 served as negative values.

Proliferation assay. Target ACH-2 cells were grown for 5 days in the presence or absence of dilutions of coumarins. Eight hours before assay, cells were placed in a leucine-deficient medium supplemented with 50 μCi/ml of [^{3}H]-leucine (Amersham, Arlington Heights, IL) and incorporation of the labelled precursor was determined by scintillation spectroscopy.

Western blot analysis. Drug-exposed and control ACH-2 cells were lysed and protein extracts were separated by SDS-PAGE in 8 % gel gradient, transferred to nitrocellulose membranes, and incubated with mouse anti-*gag* biotin-labelled antibody (1:1000) (Coulter). Immunoreactive proteins were visualized by ECL Western blot detection system (Amersham, Arlington Heights, IL).

Results

To determine whether coumarin derivatives warfarin, cyclocumarol and umbelliferone (Fig. 1) present during brief period of virus inoculation (1 hr) may



(a) (b) (c)

Fig. 1
Structure of coumarins tested for their effect on HIV transmission and replication
(a) warfarin (3-[alpha-acetonylbenzyl]-4-hydroxycoumarin), (b) 4-hydroxycoumarin, (c) umbelliferone (7-hydroxycoumarin).

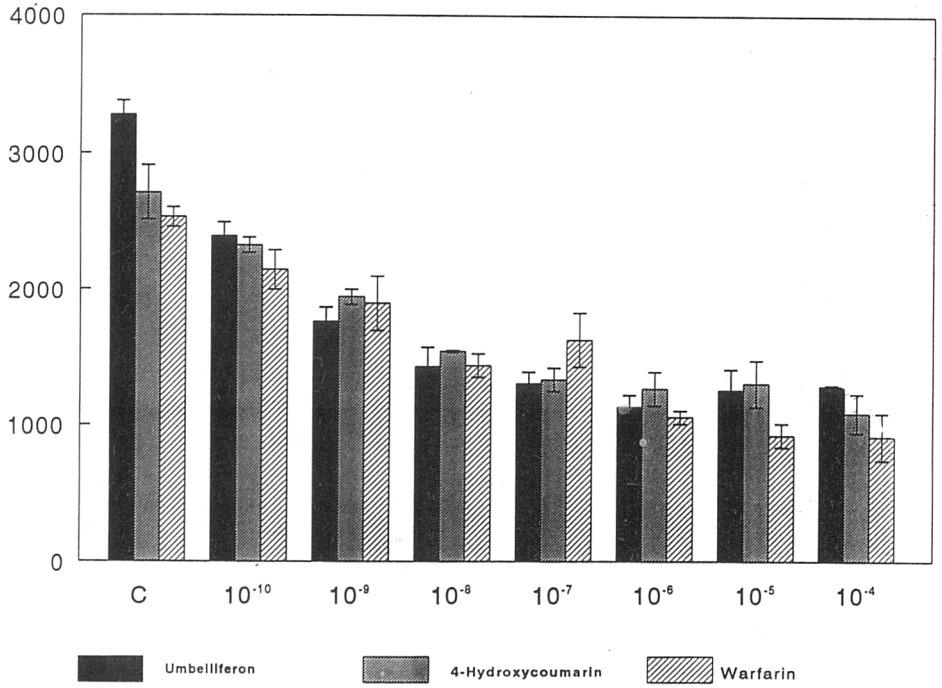


Fig. 2
Direct effect of coumarins on HIV infectivity
Abscissa: concentration of coumarins (mol/l), control(s); ordinate: p24 concentration (pg/ml). Averages and ranges of results of three independent experiments are shown.

affect HIV infectivity we evaluated their effect in an infectivity assay using free virus (Fig. 2). In separate experiments we examined the effect of coumarins present continuously for 5 days on the transmission of HIV via cell-cell contact by coincubating HIV-1 infected U1 monocytes with target MOLT-4 lymphocytes (Fig. 3). The results seem to indicate that even low doses of these drugs can protect from both cell-free and cell-mediated HIV infection.

We then tested the effect of coumarins on viral production from chronically infected cells. HIV-infected ACH-2 lymphocytes were grown in a 96-well Multiscreen filtration plate with a gravity-permeable membrane at the bottom (Millipore, Bedford, MA) in the presence of various concentrations of coumarins for 48 hr. Thereupon the culture supernatants were collected into a 96-well ELISA plate by centrifugation and were tested for p24 protein or RT activity. The inhibitory dose-effect of coumarins is illustrated in Figs. 4 and 5. All three compounds demonstrated a similar antiviral effect. The dose-effect was observable starting from 10^{-10} mol/l. According to the values from virus measurement

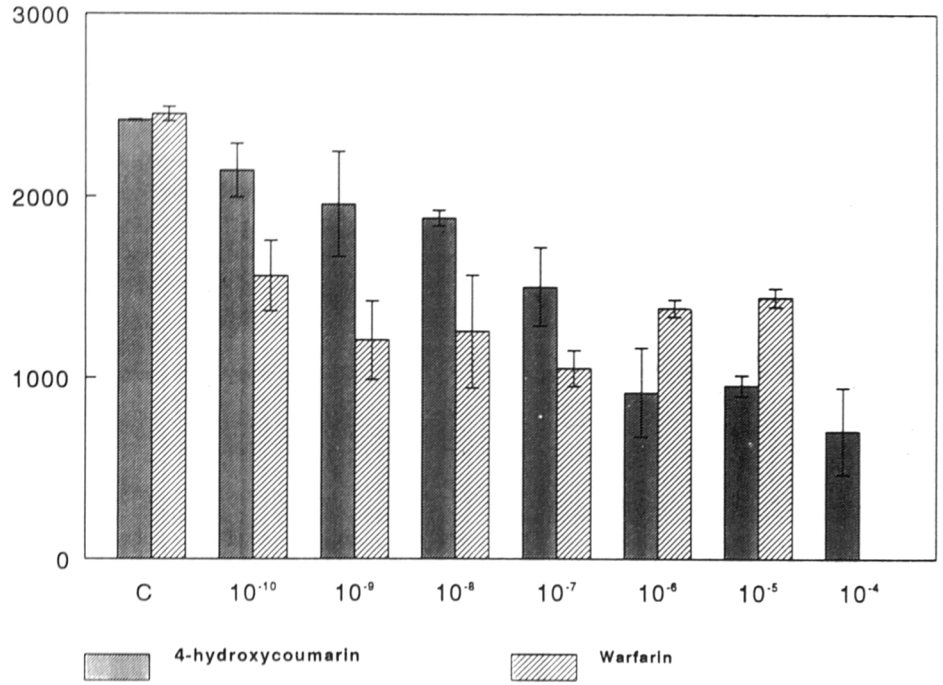


Fig. 3
Effect of coumarins on cell-to-cell transmission of HIV
For legend see Fig. 2.

assays there is a 50 % reduction ($ED_{50\ \%}$) in RT activity and p24 release resulting from exposure to coumarins at doses 10^{-9} and 10^{-6} mol/l, respectively. Discrepancy between two assays can be explained by the excessive defective particle production reflected in the prevalence of p24 over replication-competent virions with active viral RT (Bourinbaiar *et al.*, 1991).

To determine whether the observed inhibition of virus production was not related to growth suppression of used cells we evaluated the effect of coumarins on incorporation of [^{3}H]-leucine (Fig. 6). Results indicate that observed reduction in RT activity and inhibition of p24 production from HIV-infected lymphocytes is not correlated to cell growth pattern except for the highest concentration of umbelliferone. Cell viability of drug-treated population as determined by trypan blue exclusion did not differ from the control cells (95 % - 97 % versus 93 % - 97 %, respectively). It is, therefore, likely that coumarins have a specific antiviral effect which is unrelated to the suppression of cell proliferation.

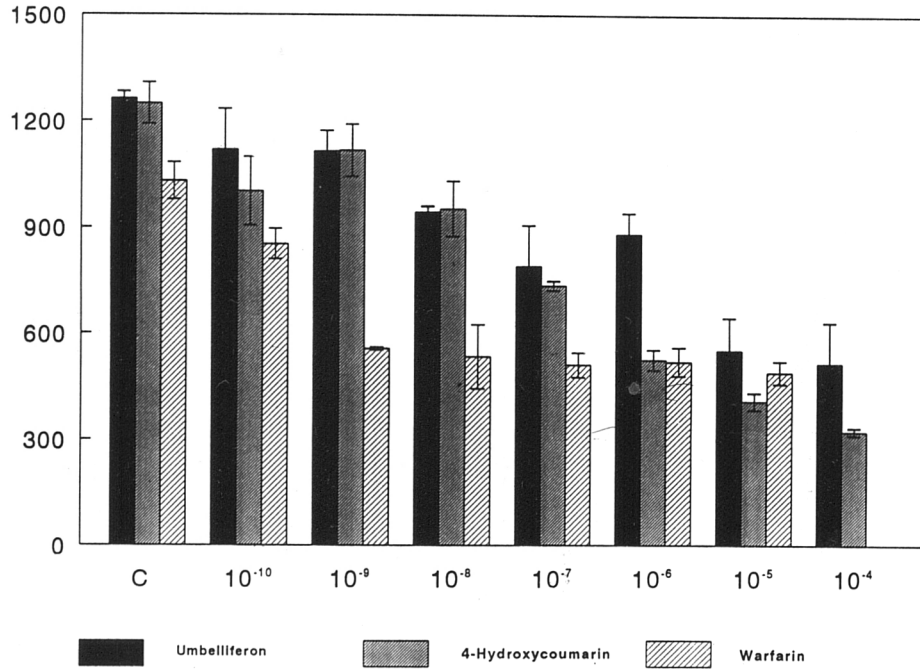


Fig. 4
Effect of coumarins on HIV production from ACH-2 lymphocytes assayed by p24 release
For legend see Fig. 2.

Western blot analysis of retroviral core proteins from lysed drug-exposed ACH-2 cells did not demonstrate a detectable difference from the untreated control cells (Fig. 7). The bands corresponding to HIV gag proteins in treated cells appear to be of similar density and size as in control untreated cells.

Discussion

We report here the inhibitory effect of coumarin derivatives on cell-mediated and cell-free viral spread *in vitro* as well as suppression of viral production from chronically infected cells with doses that could be easily achieved in vivo. All three agents, except for plant-extracted crude umbelliferone with a low degree of purity, displayed negligible cytotoxicity acceptable even at the highest tested concentration of 10^{-4} mol/l. As the ratio of cytotoxic dose to effective dose

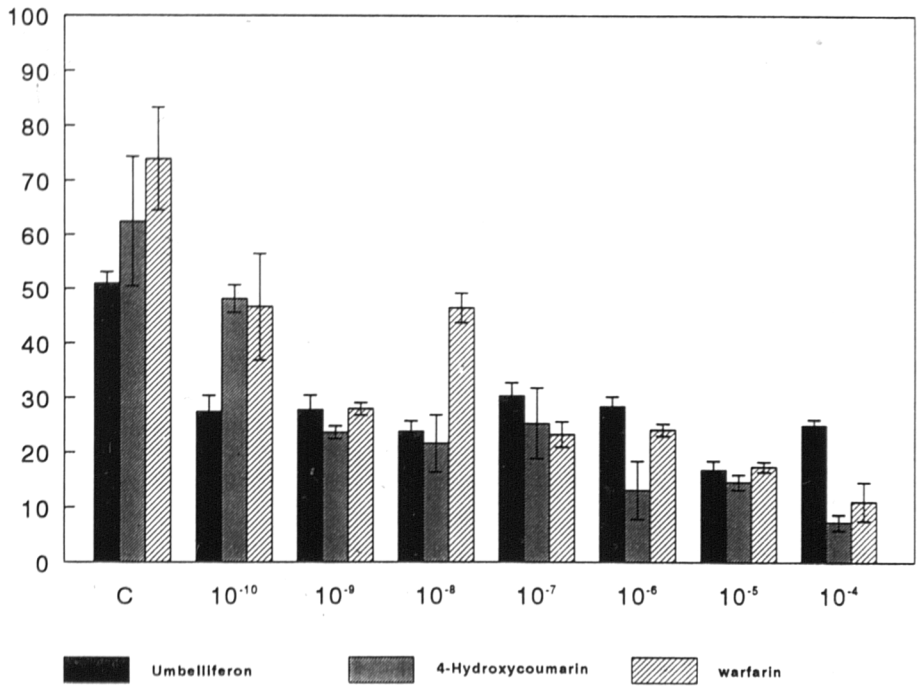


Fig. 5
Effect of coumarins on HIV production from ACH-2 lymphocytes assayed by RT activity
Abscissa: see Fig. 2; ordinate: cpm $\times 10^{-3}$.

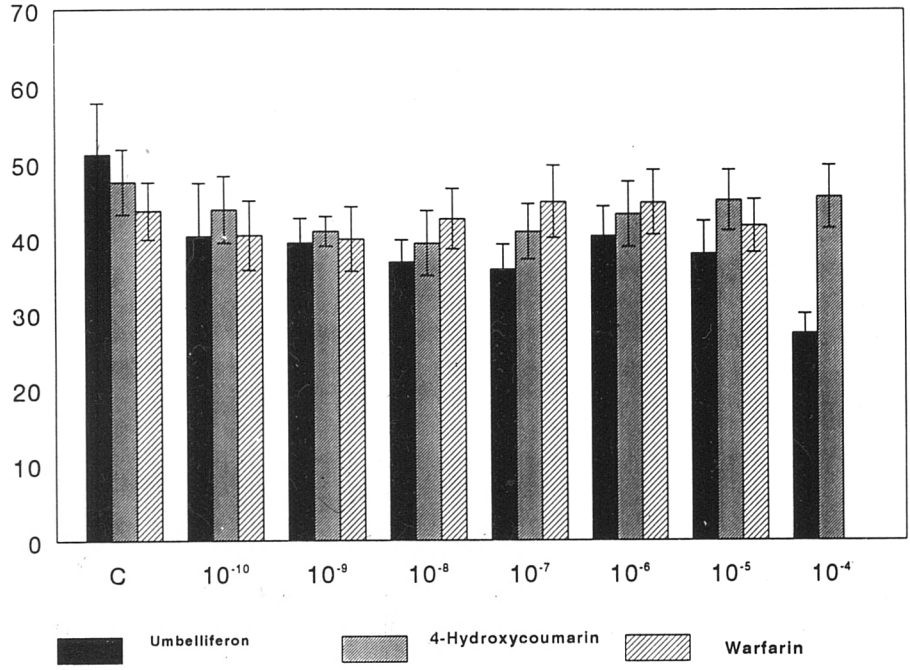


Fig. 6
For legend see page 248.

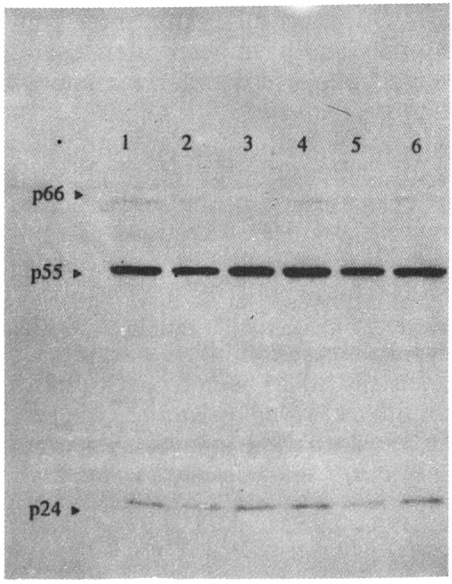


Fig. 7
Effect of 4-hydroxycoumarin on HIV core proteins in ACH-2 lymphocytes (Western blot analysis)
4-hydroxycoumarin concentrations 10^{-8}–10^{-4} mol/l (lanes 1–5). Control (lane 6).

determined by RT assay was higher than 5 $\log_{10}$ units, it is possible that these drugs act on the host cell metabolism rather than on the virus itself (Ito *et al.*, 1990). This is supported by Western blot analysis demonstrating an insignificant effect of 4-hydroxycoumarin on intracellular viral protein composition. It seems that the HIV-specificity of coumarins is similar or better than the specificity of RT-selective nucleoside inhibitors such as AZT (Mitsuya and Broder, 1987), since $ED_{50\%}$ starts from concentrations as low as 10^{-9} mol/l without being cytotoxic at the highest dose. It may appear, at first sight, that despite an impressive $ED_{50\%}$ record the overall effect of coumarins is weak, since no 100 % inhibition was observed. This drawback could be attributed to the decline of pharmacological activity of coumarins, since the antiviral effect was measured five days after exposure to a single dose of drug. The antiviral effect of coumarins has been demonstrated in both types of infectivity assays, i. e., by cell-free virus when the drug was present for only 1 hr at the inoculation and by cell-cell contact in the continuous presence of coumarins for 5 days. The latter results are particularly encouraging since it appears that AZT does not inhibit effectively cell-to-cell transmission of HIV (Gupta *et al.*, 1989). In addition the effect of coumarins on HIV production from already infected cells, though also incomplete, is remarkable, since few, if any, of the available antiviral drugs can achieve inhibition of viral production from steady producing cells without being cytostatic (Bourinbaiar *et al.*, 1992*b*).

It is interesting that coumarin- unrelated polysaccharide anticoagulants such as dextran sulfate and heparin have also been shown to have an anti-HIV effect *in vitro* (Baba *et al.*, 1991; Bourinbaiar and Nagorny, 1992*c*), and despite low biological availability they are currently tested *in vivo*. As opposed to polysaccharide anticoagulants, clinically used coumarins such as warfarin are more convenient alternatives since they can be taken orally and have been safely used for many years in the treatment of blood coagulation disorders (Lancaster *et al.*, 1991). The mechanism of coumarins' action is relatively well established: they inhibit the vitamin K-dependent carboxylation of specific glutamic acid residues to gamma-carboxyglutamic acid, required for conversion of prothrombin to an active form as well as the enzymatic activity of thrombin, the serine protease (Mor *et al.*, 1990; Fasco, 1982). Coumarins serve as suicide substrates (Zimmerman *et al.*, 1976) and irreversible inhibitors of trypsin-like proteases such as lymphocyte granule cytolytic serine proteases (Hudig *et al.*, 1991). The effect of synthetic peptide inhibitors of HIV protease is extensively investigated but little is known of the interaction of coumarin -type protease inhibitors with retroviral aspartyl enzyme (Scharpe *et al.*, 1991). Incidentally, it has been recently reported that classic (Bourinbaiar and Nagorny, submitted) as well as Kunitz-type serine protease inhibitors (Hattori *et al.*, 1989) can prevent HIV-1 infection, apparently

Fig. 6
Effect of coumarins on the growth of ACH-2 lymphocytes
Abscissa: see Fig. 2; ordinate: cpm × 10^{-3}.

through an aspartyl protease-independent mechanism. However, it is not known whether coumarins can interfere with viral infectivity through serine or aspartyl protease inhibitory pathways.

In addition, some variants of coumarins known as inhibitors of poly (ADP-ribose) polymerase - a chromatin-bound enzyme activated by free DNA ends - were reported to suppress *tat*-independent activation of HIV-1 expression at the posttranscriptional level (Yamagoe *et al.*, 1991; Cole *et al.*, 1991). Moreover, the fact that coumarins affect the levels of GSH (Soine, 1964; Lake *et al.*, 1989) may imply a related mechanism upon intracellular metabolism whereby HIV production can be suppressed (Staal *et al.*, 1990).

In conclusion, reported preliminary findings require further multidisciplinary investigations with the goal of establishing the antiviral mechanism of coumarins and their potential for clinical applications.

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