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Short communication

In vitro and in vivo anti-retroviral activity of the substance purified from the aqueous extract of *Chelidonium majus* L.

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Abstract

We have isolated a substance with anti-retroviral activity from the freshly prepared crude extract of *Chelidonium majus* L. (greater celandine) by 9-aminoacridine precipitation method and ion exchange chromatography using Dowex-50W/H+ resin followed by the gel filtration on Sephadex-75 column. Elemental and phenol/sulfuric acid method analyses as well as the mass spectrometry of the purified substance indicated that it may represent a low-sulfated poly-glycosaminoglycan moiety with molecular weight of ~3800 Da. The substance prevented infection of human CD4+ T-cell lines AA2 and H9 with HIV-1 at concentration of 25 μg/mL as well as the cell-to-cell virus spread in H9 cells continuously infected with HIV-1, as determined by the measurement of reverse transcriptase activity and p24 content in cell cultures. Furthermore, we have shown in a murine AIDS model that the treatment with purified substance significantly prevented splenomegaly and the enlargement of cervical lymph nodes in C57Bl/6 mice chronically infected with the pool of murine leukemia retroviruses. The mechanism(s) of anti-retroviral activity of this substance have to be elucidated.

Keywords: *Chelidonium*; Glycosaminoglycans; HIV-1 inhibition; Retroviruses; MAIDS

A variety of plant products are being used by Acquired Immune Deficiency Syndrome (AIDS) patients in some countries without any experimental evidence of anti-HIV-1 activity, like garlic, ginseng, shiitake mushrooms, papaya, etc. (Mills et al., 2005). Only a few plant-derived anti-HIV-1 products have been used in a limited number of patients with AIDS after in vitro experimental studies that were carried out to provide information on the anti-HIV-1 activity of the substances isolated from the plants such as *Viola yedoensis*, *Arctium lappa*, *Epimedium grandiflorum*, *Glycyrrhiza uralensis*, *Prunella vulgaris*, *Spirulina platensis* and *Castanospermum australe* (Ito et al., 1987; Tabba et al., 1989; Yao et al., 1992; Vlietinck et al., 1998). However, their effectiveness in vivo against HIV-1 has not been established as yet because of the poor bioavailability, short life time or unfavorable anti-coagulant activity.

C. majus L. (greater celandine) is being used in a traditional medicine for many centuries. The commercial drug (*Chelidonii herba*) consists of the dried aerial parts harvested when blooming. The drug is described in several European pharmacopoeias and contains various isoquinoline alkaloids, flavonoids and phenolic acids that exhibit multiple biological activities, such as antiviral, antitumor, antibacterial/antifungal or anti-inflammatory effects (Colombo and Bosisio, 1996; Jang et al., 2004; Pieroni et al., 2005). Antiviral effects of *Chelidonium* extracts are mainly due to the presence of alkaloids, like protoberberine and benzo-phenanthridine, which exhibit also some anti-reverse transcriptase activity (Tan et al., 1991; Habermehl et al., 2006).

We have collected the aerial parts of *C. majus* L. during the blooming period. The crude extract was obtained from the freshly macerated plant after pressing and removing cell detritus by several centrifugation and filtration procedures. Direct application of the crude extract on the fast protein liquid chromatography (FPLC)-gel filtration Superose-12 column revealed four main peaks that included fractions 19–20 (Pool 1), 21–22 (Pool 2), 23–30 (Pool 3) and 58–64 (Pool 4). Pooled fractions were lyophilized and kept at 4 °C until use. It was also possible to purify the active substance detected in pool 2 by mixing the crude aqueous extract with 9-aminoacridine·HCl to the final concentration of 0.5% and subsequent incubation on a shaker

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Table 1
In vitro effects of FPLC fraction pools (ChM-P) derived from the aqueous extract of *Chelidonium majus* L. on proliferation, viability and syncytium formation in CD4+ AA2 cells infected with a different $TCID_{50}$ of HIV-1 determined at 10 days post-infection

Infectious dose ($TCID_{50}$)	Non-treated control		Pool 1 (ChM-P1) (fractions 19–20)		Pool 2 (ChM-P2) (fractions 21–22)		Pool 3 (ChM-P3) (fractions 23–30)		Pool 4 (ChM-P4) (fractions 58–64)	
	cpm ± S.E.M.	% Viability/ syncytia	cpm ± S.E.M.	% Viability/ syncytia	cpm ± S.E.M.	% Viability/ syncytia	cpm ± S.E.M.	% Viability/ syncytia	cpm ± S.E.M.	% Viability/ syncytia
Non-infected control	45063 ± 3586	89/–	48005 ± 4322	92/–	44612 ± 2231	90/–	46866 ± 3281	88/–	901 ± 25	<2[a]
5×10^3	8111 ± 243	16/+	7531 ± 427	11/+	38754 ± 1938	87/–	9643 ± 379	13/+	1352 ± 14	<2[a]
10^3	7661 ± 153	12/+	6878 ± 304	9/+	39205 ± 2744	89/–	8156 ± 162	12/+	2253 ± 47	<2[a]
2×10^2	18476 ± 924	41/+	15624 ± 1608	32/+	45344 ± 2758	91/–	11266 ± 451	21/+	451 ± 19	<2[a]
50	45964 ± 1839	91/+	39864 ± 3488	83/+	47767 ± 3821	92/–	41458 ± 2902	87/+	912 ± 36	<2[a]

Different HIV-1 $TCID_{50}$'s were added to 5×10^4 CD4+ AA2 cells per well in 0.2 mL RPMI 1640 supplemented with 10% fetal calf serum and incubated for 2 h at 37 °C prior to the addition of 5 μg/well of ChM-P1, ChM-P2, and ChM-P3 pools. ChM-P4 was added at a concentration of 1 μg/well. Non-infected AA2 cell cultures were grown only in medium as a control. All combinations were tested in quadruplicates and incubation was proceeded at 37 °C for 10 days. Additional quadruplicates were set up to determine syncytium formation and the cell viability by light microscope, using trypan blue dye exclusion assay. On days 3 and 6, 50% of the cell suspension in each well was replaced with fresh culture medium. 0.5 μCi of ^{3}H-thymidine per well was added 6 h prior to the cell harvesting on a fiberglass paper. The level of incorporated ^{3}H-thymidine was measured in a direct beta-counter Matrix-96 (Packard), and the results are expressed as mean cpm ± S.E.M. for each quadruplicate. (+) Formation of syncytia; (−) no syncytia. One HIV-1 tissue culture infectious dose$_{50}$ ($TCID_{50}$) is the dose necessary to infect 50% of AA2 cell cultures in 96 wells of a Microtiter plate as detected by syncytium formation (1 $TCID_{50} \geq 10^3$ virus particles).

[a] ChM-P4 pool was highly toxic and syncytia were not determined.

at room temperature for 1 h. The mixture was centrifuged for 20 min at 20,000 rpm and the pellet was resuspended in a distilled water and shaken with Dowex-50W resin (H+ form, 100–200 mesh, Sigma) for 1 h. After centrifugation at 20,000 rpm for 15 min, pH of the supernatant was adjusted to 7.6 by addition of aqueous sodium hydroxide before being applied to a Sephadex G-75 chromatography column to remove the residual 9-aminoacridine. The substance was eluted and lyophilized, and the purity of the substance was 98% as determined by FPLC Superose-12 column.

Pooled fractions were tested in vitro for anti-HIV-1 activity using human CD4+ AA2 cell line that is extremely sensitive to HIV-1 infection. Infected cells form syncytia, undergo apoptosis and die 9–14 days post-infection. AA2 cells were infected with different HIV-1 $TCID_{50}$'s in the presence of pooled *Chelidonium* fractions and cultured for 10 days at 37 °C. Only the substance in pool 2 (ChM-P2), which was identical to the substance purified by 9-aminoacridin precipitation, prevented HIV-1 infection and virus-induced syncytium formation as well as the subsequent cell death, as determined by trypan blue dye exclusion assay and cell proliferation. Pool 1 and pool 3 exhibited no activity, whereas pool 4 was found highly toxic to AA2 cells even at lower concentrations (Table 1).

We have also demonstrated that the pre-treatment of 2.5×10^5 $TCID_{50}$ HIV-1_{IIIB} with 25 μg of ChM-P2 prior to the mixing with CD4+ H9 cells prevented infection of cells as determined by the measurement of reverse transcriptase (RT) activity and p24 level in the cell cultures on days 8, 16, 21, and 28 (Table 2). At concentrations higher than 100 μg/mL, ChM-P2 substance exhibited a substantial cytotoxic effect in cultured AA2 and H9 cells as well as significant inhibition of mitogen-induced proliferation in normal human lymphocytes (data not shown).

Table 2
Reverse transcriptase activity and p24 content determined in H9 cell cultures after 8, 16, 21, and 28 days of infection with 2.5×10^5 $TCID_{50}$ of HIV-1_{IIIB} pre-treated with different fraction pools of *C. majus* L.

2.5×10^5 $TCID_{50}$ of HIV-1_{IIIB}	RT assay ($\times 10^3$ median cpm)				p24 ELISA
	Day 8	Day 16	Day 21	Day 28	Day 28
Non-treated control	489.68	559.28	834.34	703.10	Positive
ChM-P1	508.62	406.92	768.36	782.20	Positive
ChM-P2	31.51	4.35	1.02	0.74	Negative
ChM-P3	148.95	602.76	793.12	957.38	Positive
ChM-P4[a]	6.57	ND	ND	ND	ND

The mixtures of 2.5×10^5 $TCID_{50}$ HIV-1_{IIIB} and 25 μg of each *Chelidonium* fraction pool were added to 4×10^6 CD4+ H9 cells in 1 mL RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 1 h at 37 °C. Subsequently, cells were transferred into the tissue culture flasks and incubated for 4 weeks in 5 mL medium at 37 °C. After 8 days of incubation, 50% of the cell suspension in each flask was removed every 3–4 days and replaced with fresh medium. RT activity in cell cultures was determined on days 8, 16, 21, and 28 using the method described previously by Boulerice et al. (1990). Results wae expressed as median cpm (min^{-1}). The presence of p24 antigen in cell cultures was determined on day 28 by ELISA according to the instructions of the Manufacturer (Abbott Laboratories). ND: Not done.

[a] Toxic to H9 cells.

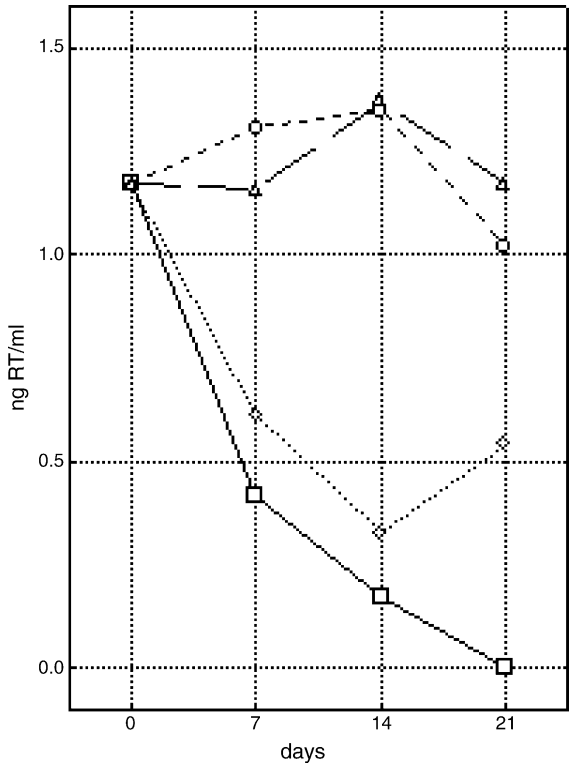

Fig. 1. The level of reverse transcriptase activity in human CD4+ H9 cells continuously infected with HIV-1_{IIIB} as determined after 7, 14, and 21 days of co-cultivation with different concentrations of *Chelidonium* fraction pool-2 (ChM-P2). 8×10^5 human CD4+ H9 T-cells per mL continuously infected with HIV-1_{IIIB} were treated with 25 (□), 10 (◊) or 2.5 μg (○) of ChM-P2/mL for 3 weeks in 5 mL RPMI 1640 medium supplemented with 10% fetal calf serum. Non-treated HIV-1-infected cells were used as a control (△). On days 7, 10, 14, and 17, 3.5 mL of each cell suspension was removed, and the equal volume of fresh medium was added. The ChM-P2 concentration was kept constant during the incubation period. The level of RT activity in cell cultures was determined on days 7, 14 and 21 by a non-radioactive assay according to the instructions of the Manufacturer (Roche Applied Science). RT level at day 0 was 1.18 ng/mL.

ChM-P2 substance was tested further at different doses in human CD4+ H9 T-cells continuously infected with HIV-1_{IIIB} and the release of virus progeny was monitored by the measurement of RT activity in cell cultures during the period of 3 weeks. Upon treatment of infected H9 cells with 25 μg ChM-P2/mL, a significant reduction of RT activity in the cell supernatants was observed, whereas at 10 μg/mL, there was a decreased inhibition, and at 2.5 μg/mL no effect was observed. This indicates inhibition of cell-to-cell virus spread, although an additional effect of the substance on virus release or stability cannot be excluded (Fig. 1).

The ChM-P2 substance was tested in vivo for anti-retroviral activity using highly susceptible C57Bl/6 strain in a mouse AIDS (MAIDS) model (Liang et al., 1996). Mice were infected intraperitoneally with 0.2 mL of the stock virus pool of defective murine leukemia retroviruses (MuLVs) LP-BM5 containing 1 U RT activity. The group of infected mice was treated intravenously with 1 mg of the ChM-P2 substance in 0.2 mL saline five times per week for a period of 2 weeks starting 4 weeks following infection. Mice were sacrificed 4 months after infection and the weight of spleens and cervical lymph nodes was measured. Significant reduction in weight of spleens and cervical lymph nodes was found in chronically infected mice treated with ChM-P2 substance as compared to non-treated animals; $p = 0.0057$ and $p < 0.001$, respectively (Table 3). In control mice receiving only the ChM-P2 substance no toxic effects were observed in liver, and the body weight was in the range of non-treated healthy animals (20.4 ± 1.73 and 21.1 ± 1.55 g, respectively; $p = 0.5584$ by two-tailed *t*-test for independent variables).

Table 3
Mouse AIDS model: in vivo effects of *Chelidonium* fraction pool-2 (ChM-P2) on the weight of spleens and cervical lymph nodes in C57Bl/6 mice chronically infected with murine leukemia retroviruses pool LP-BM5 containing one unit RT[a] activity

	Spleen (mg)		Cervical lymph nodes (mg)	
	Non-treated ($N = 10$)	ChM-P2-treated ($N = 6$)	Non-treated ($N = 10$)	ChM-P2-treated ($N = 6$)
Mean ± S.D.	964 ± 364	442 ± 176	1414 ± 247	497 ± 306
Range	510–1460	320–750	990–1730	210–960
p-Value	0.0057		<0.001	

The means were compared by two-sided *t*-test for independent samples (StatSoft Inc., 2005, STATISTICA, version 7.1; http://www.statsoft.com/).

[a] One unit is the amount of RT required for the incorporation of 1 nM of labeled dNTP in 10 min at 37 °C using poly(A)oligo$(dT)_{15}$ as a template/primer hybrid.

The mass spectrometry analysis of ChM-P2 suggested a molecular weight of 3800 Da, and the elemental analysis revealed the following values in wt.%: C = 16.65; H = 3.08; N = 3.98, and S = 0.4 (O and P elements were not analysed). The elemental and phenol/sulfuric acid (Dubois et al., 1956) analyses of the substance principally showed the characteristics of a low-sulfated poly-glycosaminoglycan moiety. However, because it strongly absorbed UV light at 280 nm, an aromatic structure must also be present. Thus, the ChM-P2 substance may be chemically different from the previously described antiviral and antitumor compounds derived from *C. majus* L. (Colombo and Bosisio, 1996; Habermehl et al., 2006).

Sulfated polysaccharides are capable of binding to different proteins with several levels of specificity (Mulloy, 2005). As highly acidic molecules, they can bind non-specifically to any basic patch on a protein surface at low ionic strength, but such interactions are not likely to be physiologically significant. However, specific interactions are described for the cell-surface glycosaminoglycan heparin sulfate (HS), in which patterns of sulfate substitution are responsible for different affinities to proteins (Bulow and Hobert, 2004), including HIV-1 gp120 (Vives et al., 2005). Unfortunately, the role of HS in the attachment of HIV-1 to the surface of target cells has been somewhat controversial (Baba et al., 1990; Moulard et al., 2000; Zhang et al., 2002). Nevertheless, there are no studies in vivo to confirm anti-HIV-1 activity of HS or other sulfated glycosaminoglycan molecules. On the contrary, the ChM-P2 substance, presumably a sulfated poly-glycosaminoglycan molecule, has shown anti-retroviral activity both in vitro against HIV-1 and in vivo against MuLV in a MAIDS model. Accordingly, it may represent a potent anti-retroviral agent with a broad anti-retroviral

specificity that overcomes the problem with the virus escape mutants, which usually appear during the standard anti-HIV-1 therapy, e.g. by using HIV protease inhibitors. Similar effects in vitro have been described for prunellin extracted from Chinese herb *P. vulgaris*, which was able to block completely HIV-1 infection and syncytium formation in MT-4 CD4+ cells (Yao et al., 1992). ChM-P2 may bind gp120 and disturb interaction with CD4 receptor as described for prunellin (Yao et al., 1992) and heparan sulfate (Vives et al., 2005), which can substantially decrease HIV-1 infectiveness and prevent cell-to-cell spread of the virus. However, the anti-retroviral activity of the substance shown in a continuously infected CD4+ H9 cell line and in the MAIDS model indicates that other mechanism(s) of inhibition could also be involved. Therefore, further studies are necessary to elucidate the mechanism(s) of ChM-P2 action as well as to determine its chemical structure.

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