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Inhibition of Epstein-Barr Virus by the Triterpenoid Betulin Diphosphate and Uvaol

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Abstract Betulin, a pentacyclic triterpenoid isolated from the bark of *Betula papyrifera*. Laboratory synthesized structural analogs were tested for antiviral activities against Epstein-Barr Virus (EBV) by immunofluorescent antiviral assay. Among the several analogs tested, betulin 3,28-diphosphate and uvaol exhibited significant antiviral activities against EBV. The EC_{50} of betulin 3,28-diphosphate and uvaol was found to be 0.6 μM and 0.7 μM, respectively.

Key words: Epstein-Barr Virus (EBV), triterpenoid, antiviral

Terpenoids of diverse structural types are widely distributed in both prokaryotes and eukaryotes. Essentially, they are derived from the basic 5-carbon isoprene unit, biosynthetically as isopentyl pyrophosphate, which is itself derived from acetate via mevalonic acid [8]. The majority of triterpenes possess the conventional skeletal structure arising from the cyclization of sequalene-2,3-epoxide to produce fused polycyclic products [10]. Triterpenoids occur abundantly in plants, where their function is generally believed to be a chemical defense against pathogens and herbivores. Triterpenoid compounds have been reported to have different biological properties. Several triterpenoids are known to possess antitumor, antiinflammatory, antibacterial, and antiviral properties [7, 10]. Betulin, a pentacyclic triterpenoid, is primarily isolated from barks via solvent extraction or sublimation, and purified by chromatography or recrystallization [7]. Several derivatives of betulin have been reported to possess potential antiviral activities against HIV and herpes viruses [1, 4, 9, 11, 13].

The result presented here shows the *in vitro* effectiveness of the diphosphate derivative of betulin and uvaol against Epstein-Barr Virus (EBV) (Fig. 1). EBV is the etiological agent of infectious mononucleosis, and has a worldwide distribution. Primary infection occurs during the first decade of life in areas with crowded living conditions and poor hygiene. Childhood infections are usually asymptomatic, as compared to the 50 to 75% of young adults who undergo primary EBV infection, with the illness ranging from mild to severe.

For this study, betulin was isolated from Minnesota White Birch bark by the chloroform extraction. The compound was purified by filtration and passing through silica with chloroform. The purity of betulin (>98%) was determined by GC/MS-spectrometry, and H^1 and C^{13} NMR, and was identical to the compound received from a commercial supplier (Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.). Structural analog betulin 3,28-diphosphate was prepared by vigorous stirring of betulin (5 g, 11.4 mM) and 25 ml of dry pyridine. To this mixture, 9.5 ml of $POCl_3$ was added at 60°C and stirred continuously for 1.5 h. This reaction mixture was kept cold at 5–8°C for 10 h. White precipitates were filtered and dissolved in boiling dioxane-water (9:1). The dioxane-water solution was filtered and reprecipitated with 50 ml cold water. After keeping at 5°C for 3 h, white crystals of betulin 3,28-diphosphate were filtered, washed with water, and dried in vacuum at 60°C for 4 h. Stock solutions of structural analogs of betulin synthesized in our laboratory, uvaol (Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.), and control drugs for antiviral assay were prepared in distilled water and filtered through 0.2 μm filter (Gelman Sciences, Ann Arbor, MI, U.S.A.). Compounds solubilized in dimethyl sulfoxide (DMSO) were also used for the antiviral assay. The P3HR-1 cell line derived from nontransforming early antigen (EA) expressing Epstein-Barr Virus (EBV) was used in our antiviral assay. Raji, a Burkitt's lymphoma cell line was used for the primary screening, and Daudi cells, which spontaneously express EBV (EA) in 0.25–0.5% of the cells, were used in the

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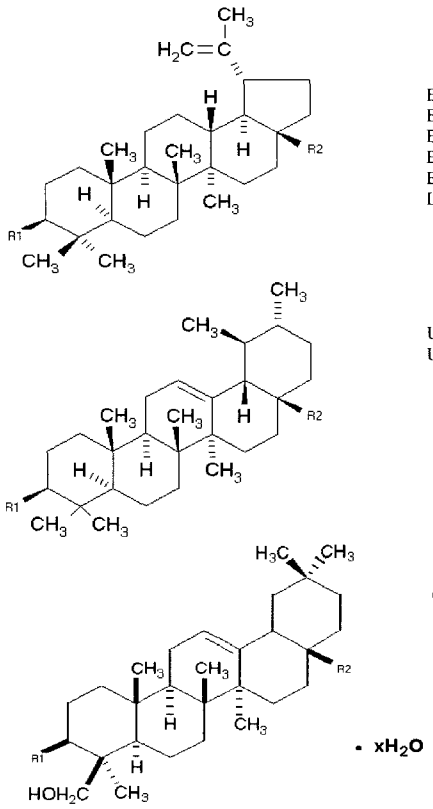

	R_1	R_2
Betulin	OH	CH_2OH
Betulin 28-succinate	OH	$OOCCH_2CH_2COOH$
Betulin 3,28-disuccinate	$OOCCH_2CH_2COOH$	$OOCCH_2CH_2COOH$
Betulin 3,28-diphosphate	OPO_3H_2	OPO_3H_2
Betulin 3,28-dioxalate	OOCCOOH	OOCCOOH
Lupeol	OH	OH

	R_1	R_2
Ursolic acid	OH	COOH
Uvaol	OH	CH_2OH

	R_1	R_2
α-Hederine Hydrate	Rahmnose (1→2) Arabinose (1→2)	COOH

Fig. 1. Structures of triterpenoids.

follow-up studies. These cell lines respond to superinfection by EBV by expressing EA-diffuse component EA (D), EA restricted component EA (R), and viral capsid antigen (VCA). All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and 100 μg/ml gentamicin at 37°C in the presence of 5% CO_2.

Cells were infected with the P3HR-1 strain of EBV at a multiplicity of infection (MOI) of 0.1. The antiviral compounds at different concentrations were added after 45 min of virus adsorption and the cultures were incubated for two days in RPMI-supplemented medium to allow for viral antigen expression. Following incubation, the number of cells of each sample was counted and smears were prepared. To detect EBV-specific antigens, cells were fixed in acetone at room temperature for 10 min, and then washed in phosphate buffered saline (PBS) supplemented with FCS for 20 min. Monoclonal antibodies to different EA components and VCA were then added to the cells and incubated for 1 h in a humidified chamber. After incubation, the cells were washed in PBS and incubated for 45 min with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG antibody (Sigma Chemical Co., St. Louis, MO, U.S.A.). All stained preparations were mounted in PBS:glycine (1:1) and were examined under a fluorescent microscope. The total number of cells in the cultures positive for EA or VCA were then calculated and compared, and the 50% antiviral effective concentration (EC_{50}), i.e., the concentration of the test antiviral compound required to reduce the number of virus infected cells by 50%, were determined. The cytotoxicity was determined by trypan blue staining method and the results were expressed as CC_{50} (50% cell cytotoxic concentration), i.e., the concentration of test antiviral compound required to reduce the growth of uninfected cell by 50%.

A significant antiviral effect of betulin diphosphate and uvaol was observed against EBV. The EC_{50} and CC_{50} of betulin 3,28-diphosphate was found to be 0.6 μM and >77.9 μM, respectively, with the selectivity index of >129.8. The EC_{50} and CC_{50} of uvaol were found to be 0.7 μM and 100.1 μM, respectively, with the selectivity index of 143. The EC_{50} and CC_{50} for Acyclovir was 6.2 μM and >444.0 μM, respectively, with the selectivity index of >71.6 (Table 1). There are several reports that natural and laboratory synthetic derivatives of triterpenoid compounds exhibit antiviral activities. A series of simple modifications of the triterpenoid have produced several biologically active molecules. Betulinic acid and derivatives have been known to possess potential antiviral activity against HIV-1 virus [9, 15]. Triterpenoid compounds are also known to possess antiviral activities against several enveloped viruses [13]. We have reported the antiviral activities and the use of betulin and analogs to treat herpes virus infection

Table 1. Antiviral activities of triterpenoids, betulin 3,28-diphosphate, and uvaol against Epstein-Barr Virus.

Compound	CC_{50} (μM)	EC_{50} (μM)	SI
Betulin	6.1±1.3	3.8±0.9	1.6
Betulin 28-succinate	92.1±7.8	65.3±10.1	1.4
Betulin 3,38-disuccinate	43.5±11.1	22.4±6.7	1.9
Betulin 3,28-diphosphate	>77.9±9.6	0.6±0.3	>129.8
Betulin 3,28-dioxalate	>83.2±10.8	12.0±3.7	>6.9
Lupeol	117.2±16.8	97.2±11.1	1.2
Ursolic acid	>109.5±18.9	67.3±9.3	>1.6
Uvaol	100.1±18.1	0.7±0.4	143.0
Hederine hydrate	5.1±2.1	1.9±0.3	2.6
Acyclovir	>444.0±19.8	6.2±0.4	>71.6

Values are means±(S.D.) of three separate experiments.

CC_{50} (50% cell cytotoxic concentration) is the concentration of test antiviral compound required to reduce the growth of uninfected cell by 50%.

EC_{50} (50% antiviral effective concentration) is the concentration of the test antiviral compound required to reduce the number of virus infected cells by 50%.

Selectivity Index (SI): Ratio of CC_{50} for cell growth to EC_{50} for viral CPE, i.e., (CC_{50}/EC_{50} ratio).

[1, 4]. A recent study indicated the antiviral activities of betulin and betulinic acid against influenza, ECHO 6, and herpes simplex viruses [2, 11]. Besides antiviral activities, triterpenoids are also known to possess antitumor [6, 14], antiinflammatory [12], and antimalarial properties [3]. Natural triterpenoids and triterpenoid saponins are well known for their inhibitory effect on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced EBV-EA activation, and thus are potential antitumor promoting agents [2, 11].

The antiviral mechanism of action of triterpenoid compounds is not known. The selective activity against enveloped viruses indicates that these compounds might interfere with protein synthesis and posttranslational modifications of membrane components, e.g., glycosylation and sulfation, during the virus assembly and maturation, resulting in the interference of the normal processing and maturation of the HSV envelop. Thus, although viruses are synthesized, they are not active and not able to be released from the infected cells [5].

The present *in vitro* antiviral study outlines the significance of antiviral activities of triterpenoid compounds against herpes viruses including EBV. Detailed work on the antiviral activity of different triterpenoids against EBV is underway.

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