ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. LIX SECTIO C 2004

TERESA KAMIŃSKA, JÓZEF KACZOR, WOJCIECH RZESKI,
KATARZYNA WEJKSZA, MARTYNA KANDEFER-SZERSZEŃ,
MARIA WITEK

Department of Virology and Immunology, Maria Curie-Skłodowska University,
Akademicka 19, 20-033 Lublin, Poland

A comparison of the antiviral activity of three triterpenoids isolated from *Betula alba* bark

Porównanie aktywności przeciwwirusowej trzech trójterpenów
otrzymanych z kory *Betula alba*

SUMMARY

Three triterpenoids derived from the bark of *Betula alba,* betulin, betulinic acid and oleanolic acid, have been evaluated for antiviral activity against vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV). The virucidal activity of all the compounds was poor (concentrations of triterpenoids effective in inactivation of 50% of viral particles above 26 μg/ml), but all the three triterpenoids examined inhibited replication of both viruses with EC_{50} values below 1.3 μg/ml and therapeutic index (TI) higher than 18.

STRESZCZENIE

Przebadano cytotoksyczność wobec fibroblastów ludzkiej skóry i aktywność *in vitro* przeciw wirusom: EMCV (wirus zapalenia mózgu i mięśnia sercowego) oraz VSV (wirus pęcherzykowego zapalenia błony śluzowej jamy ustnej) trzech trójterpenów otrzymanych z kory *Betula alba*: betuliny, kwasu betulinowego i kwasu oleanolowego. Aktywność wirusobójcza tych związków była niska — stężenie powodujące inaktywację 50% cząstek wirusowych wynosiło około 26 μg/ml. Jednakże wszystkie trzy trójterpeny hamowały replikację *in vitro* obydwu wirusów z EC_{50} poniżej 1,3 μg/ml i indeksem terapeutycznym (TI) powyżej 18.

K e y w o r d s: antiviral activity, betulin, betulinic acid, encephalomyocarditis virus, oleanolic acid, vesicular stomatitis virus.

ABBREVIATIONS

B — betulin, Ba — betulinic acid, CPE — cytopathic effect, EMCV — encephalomyocarditis virus, HAV — hepatitis A virus, HIV — Human Immunodeficiency Virus, HSF — human skin fibroblast, Oa — oleanolic acid, VSV —- vesicular stomatitis virus.

INTRODUCTION

Triterpenes are aliphatic compounds based on a skeleton with 30 carbon atoms, and they are present in all parts of plants such as roots, pollen, fruit and seeds (16). Anti-inflammatory, antitumor, liver-protective and immunoregulatory effects have been reported as biological effects of triterpenes (7, 16–21). In the last years, the antiviral activity of several triterpene compounds has been described. Moronic and betulonic acids have been detected to possess anti-herpes (*Herpes simplex*) activity, and betulin and betulinic acid as well as several of their derivatives have been described as very active anti-HIV (Human Immunodeficiency Virus) agents affecting virus-cell fusion, reverse transcriptase activity, virion assembly and/or budding (1, 8, 9–11, 20). However, there are few papers concerning the activity of triterpene compounds against other viruses, members of families other than *Herpesviridae* and *Retroviridae* (2–5, 13–15).

Therefore, the aim of this paper is to compare the virucidal and virostatic activity of betulin, betulinic acid and oleanolic acid (three triterpenoids isolated from the bark of *Betula alba* and purified nearly to homogeneity) against viruses belonging to *Picornaviridae* (encephamomyocarditis virus — EMCV) and *Rhabdoviridae* (vesicular stomatitis virus — VSV*)* families.

MATERIAL AND METHODS

The compounds

Betulin and oleanolic acid had been isolated from the bark of *Betula alba* and purified nearly to homogeneity ($>95\%$) by Stanisław Piela, Sylveco, Jesionka, Poland. Betulinic acid as well as the standards of betulin, betulinic acid and oleanolic acid had been obtained from Sigma Aldrich, St. Louis, MO, USA. The betulin, and oleanolic acid obtained from Sylveco were subjected to thin-layer chromatography on a Kiselgel 60 (Merck, Darmstadt, Germany) plate 100×200 mm, eluent: chloroform/ethyl acetate (10 : 1) and compared with those obtained from Sigma Aldrich. Rf (0.46) of both betulins was identical, as was Rf (0.28) of both oleanolic acid preparations. For further experiments betulin and oleanolic acid from Sylveco and betulinic acid from Sigma Aldrich were used. Triterpenes were dissolved in dimethylosulfoxide (DMSO) at a concentration of 1 mg/ml as stock solution.

The cells and viruses

Human skin fibroblasts (HSF) had been obtained by a routine method of trypsynization of adult human skin fragment and cultured in Eagle's Minimal Essential Medium (MEM, Gibco, BRL) supplemented with 10% foetal calf serum (FCS, Gibco), 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Vesicular stomatitis virus (VSV), Indiana strain, member of *Rhabdoviridae* family and encephalomyocarditis virus (EMCV), Col MM strain, the member of *Picornaviridae* family, had been obtained from the Institute of Immunology and Experimental Therapy in Wrocław. Virus stocks were prepared from infected L929 cells and titrated in HSF cells. Their titers were: VSV $10^{5.67 \pm 0.37}$ $TCID_{50}$/ml and EMCV $10^{7.68 \pm 0.39}$ $TCID_{50}$/ml.

The cytotoxicity and cytopathic effect (CPE) reduction assay

The cytotoxicity of betulin, betulinic acid and oleanolic acid was examined by measuring their effects on the growth and viability of HSF cells. HSF cells were seeded at a density of 5×10^4 cells/ml onto 96-well plates (Nunc, Roskilde, Denmark) and grown at 37° for 24 h. The culture medium was replaced with a fresh medium containing compounds at various concentrations, and the cells were allowed to grow for the next two days. The cells (in three wells for each concentration) were treated with MTT (5 mg/ml, Cell Proliferation Kit I, Boehringer, Mannheim, Germany) and incubated for 4 h at 37°. Formazan crystals were solubilized overnight in SDS buffer (10% SDS in 0.01N HCl) and the product was quantified spectrophotometrically by measuring the absorbance at 570 nm using E-max Microplate Reader (Molecular Devices Corp., Menlo Park, CA, USA). The results were expressed as CC_{50} (cytotoxic concentration for 50% of the cells).

The compounds were examined for their antiviral activity in the CPE reduction assay. HSF cells were seeded at a density of 5×10^4 cells/ml onto 96-well plates (Nunc, Roskilde, Denmark) and grown at 37° for 24 h. The cells were infected with viruses at a multiplicity of infection (MOI) of 0.01, incubated for 1 h at 37°, washed and covered with fresh MEM supplemented with 2% of FCS containing different concentrations of the examined compounds. After two days of incubation at 37°C under 5% CO_2 in humidified atmosphere, cell viability was quantified by the MTT assay, as described above. All the assays were done in triplicate. The 50% effective concentration (EC_{50}) was then calculated from cell viability for each concentration of the compound.

The virucidal activity

The virucidal activity of the compounds was estimated by incubation of undiluted stock virus samples with equal volumes of the compounds at different concentrations. After 1 h of incubation at 37°, the titer of the viruses was estimated in HSF cells. The virucidal concentrations of the compounds for 50% reduction of infectious virus particles were determined from the curve relating the number of TCID50 units to the concentration of the sample.

RESULTS

THE VIRUCIDAL EFFECT OF TRITERPENE COMPOUNDS

Betulin and betulinic acid at concentrations higher than 26 μg/ml exerted direct virucidal effect on VSV after incubation of the viral suspension with the compound for 1 h at 37° (Table 1). EMCV was more resistant, and concentrations higher than 39 μg/ml were needed to inactivate half of the viral particles in the suspension. Oleanolic acid at concentrations above 30 μg/ml inactivated both VSV and EMCV.

Table 1. Virucidal activity of betulin (B), betulinic acid (Ba) and oleanolic acid (Oa) against *vesicular stomatitis virus* (VSV) and *encephalomyocarditis virus* (EMCV)

Compound	EC_{50} μg/ml	
	VSV	EMCV
B	26.8	39.5
Ba	26.8	87.4
Oa	31.7	32.4

The virus suspension was incubated with different concentrations of the compounds at 37° for 1 h. The concentration of the compound which caused the decrease of the virus titer in 50% was estimated as EC_{50}.

THE ANTIVIRAL ACTIVITY OF TRITERPENE COMPOUNDS

Anti-VSV and anti-EMCV activity of triterpene compounds was examined in a CPE reduction assay. The EC_{50} of all the compounds was about 1 μg/ml (Table 2). Their CC_{50} differed, and from among the three compounds examined, betulin was less toxic (30 μg/ml) than oleanolic acid (25 μg/ml) and betulinic acid (20 μg/ml). When therapeutic index (TI) was calculated (CC_{50}/ED_{50}), the most potent substance was betulin (TI 23–25) (Table 2), then oleanolic acid (TI 19–25) and betulinic acid (TI 19–20); however, the differences in TI values were very small.

Table 2. Cytotoxic and anti-viral activities of betulin (B), betulinic acid (Ba) and oleanolic acid (Oa)

Compound	CC_{50}(μg/ml)[a]	VSV		EMCV	
		EC_{50} (μg/ml)[b]	TI[c]	EC_{50} (μg/ml)	TI
B	30	1.2	25	1.26	23.8
Ba	20	1.0	20	1.1	18.1
Oa	25	1.0	25	1.26	19.8

[a] Concentration which is toxic to 50% of HSF cells.
[b] Concentration which inhibits viral replication by 50%.
[c] Therapeutic index which is defined by CC_{50}/ EC_{50}.

DISCUSSION

All the tested compounds expressed modest virucidal activity. We suppose that because of poor solubility of the compounds in water, the virucidal activity

was a result of coating of viral particles by a film formed from precipitating triterpenes.

To study the effect of triterpenes on VSV and EMC replication in human skin fibroblast (HSF), a virus CPE reduction assay was applied. All the triterpenes examined in our study were active and inhibited the replication of RNA viruses independently of the presence of an envelope (VSV) or its absence (EMCV); however, TI values were also rather low.

Several plant-derived triterpenoids such as betulonic acid and moronic acid extracted from *Rhus javanica,* were potent anti-herpes simplex 1 (HSV-1) agents *in vitro* and in mice (14). Betulinic acid, a triterpenoid isolated from *Syzygium claviflorum*, exhibited inhibitory activity against HIV-1 replication *in vitro* (6, 8) with EC_{50} of 1.4 μM and TI 9.3. Several derivatives of betulinic acid characterized by better solubility in water than the original compound, were obtained, and one of them, 3-0-(3′,3′-dimethylsuccinyl) betulinic acid, was found to possess extremely high anti-HIV activity. It affected virion assembly and/or budding of HIV virions and was also described as an inhibitor of HIV-induced cell membrane fusion. However, this derivative was not active in the inhibition of influenza virus or *Herpes simplex 1* replication *in vitro* (8, 10, 11). Further experiments exhibited that the interaction of several betulinic acid derivatives with the glycoproteins of the HIV envelope is most important in anti-HIV activity (9, 20). Moreover, betulin and its derivatives such as 3-0-3′,3′-(dimethylsuccinyl)-betulin were also potent anti-HIV agents *in vitro* (11) and it was shown that betulin diacetate may act as an inhibitor of purified HIV-1 reversed transcriptase (1). Except for betulin or betulinic acid derivatives also ursolic and oleanolic acid derivatives possess anti-HIV activity (12).

Triterpenoid saponins, which are naturally occurring sugar conjugates of triterpenes, possess various biological activities, including antiviral effects. Among them, oleanane-type and ursane-type triterpenoidal saponins were shown to inhibit HSV-1 replication *in vitro* (13, 19).

Triterpene compounds of saponin group such as glycyrrhizinic acid, carbenoxolone, and cicloxolone were demonstrated to possess the activity against both RNA and DNA viruses. Cicloxolone sodium was virucidal and inhibited VSV replication *in vitro* (4), while glycyrrhizinic acid exhibited virucidal activity against varicella-zoster virus as well as inhibited varicella-zoster virus and hepatitis A (HAV) virus (*Picornaviridae* family) replication *in vitro* (2, 3, 5). Studies on the mechanism of triterpene action have not identified any specific targets in viral synthesis, but rather these compounds have been found to affect one or more steps in the cellular processes that control viral replication. It has been proved that at least two compounds, namely glycyrrhizinic acid and car-

benoxolone, had a synergistic effect with prostaglandin A1 in the inhibition of vaccinia virus replication *in vitro* (15).

Despite the fact that several papers concerning antiviral activity of plant--derived triterpenoids have been written, there are still no in-depth investigations concerning the structure-function relationships of triterpenoids in the activity against different viral families. Also the mechanisms of their antiviral action should be clarified. Such experiments should be done in the future.

REFERENCES

1. Akihisa T., Ogihara J., Kato J., Yasukawa K., Ukiya M., Yamanouchi S., Oishi K. 2001. Inhibitory effects of triterpenoids and sterols on human immunodeficiency virus-1 reverse transcriptase. Lipids 36: 507–512.
2. Baba M., Shigeta S. 1987. Antiviral activity of glycyrrhizin against varicella-zoster virus *in vitro*. Antiviral Res. 7: 99–107.
3. Crance J. M., Leveque F., Biziagos E., van Cuyck-Gandre H., Jouan A., Deloince R. 1994. Studies on mechanism of action of glycyrrhizin against hepatitis A virus replication *in vitro*. Antiviral Res. 23: 63–76.
4. Dargan D. J., Galt C. B., Subak-Sharpe J. H. 1992. The effect of cicloxolone sodium on the replication of vesicular stomatitis virus in BSC-1 cells. J. Gen. Virol. 73: 397–406.
5. Dargan D. J., Galt C. B., Subak-Sharpe J. H. 1992. The effect of cicloxolone sodium on the replication in cultured cells of adenovirus type 5, reovirus type 3, poliovirus type 1, two bunyaviruses and Semliki Forest Virus. J. Gen. Virol. 73: 407–411.
6. De Clercq E. 2001. New developments in anti-HIV therapy Curr. Med. Chem. 8: 1543–1572.
7. Fulda S., Debatin K. M. 2000. Betulinic acid induces apoptosis through a direct effect on mitochondria in neuroectodermal tumors. Med. Pediatr. Oncol. 35: 616–618.
8. Hashimoto F., Kashiwada Y., Cosentino L. M., Chen C. H., Garrett P. E., Lee K. H. 1997. Anti-AIDS agents-27. Synthesis and anti-HIV activity of betulinic acid and dihydrobetulinic acid derivatives. Bioorg. Med. Chem. 5: 2133–2143.
9. Holz-Smith S. L., Sun IC., Jin L., Matthews T. J., Lee K. H., Chen C. H. 2001. Role of human immunodeficiency virus (HIV) type 1 envelope in the anti-HIV activity of the betulinic acid derivative IC9564. Antimicrob. Agents Chemother. 45: 60–66.
10. Kanamoto T., Kashiwada Y., Kanbara K., Goth K., Yoshimori M., Goto T., Sano K., Nakashima H. 2001. Anti-human immunodeficiency virus activity of YK-FH312 (a betulinic acid derivative), a novel compound blocking viral maturation. Antimicrob. Agents Chemother. 45: 1225–1230.
11. Kashiwada Y., Chiyo J., Ikeshiro Y., Nagao T., Okabe H., Cosentino L. M., Fowke K., Lee K. H. 2001. 3,28-di-0-(dimethylsuccinyl)-betulin isomers as anti-HIV agents. Bioorg. Med. Chem. Lett. 22: 183–185.
12. Kashiwada Y., Nagao T., Hashimoto A., Ikeshiro Y., Okabe H., Cosentino L. M., Lee K. H. 2000. Anti-AIDS agents. 38. Anti-HIV activity of 3-0-acyl ursolic derivatives. J. Nat. Prod. 63: 1619–1622.
13. Kinjo J., Yokozimo K., Hirakawa T., Shii Y., Nohara T., Uyeda M. 2000. Anti-herpes virus activity of fabaceous triterpenoidal saponins. Biol. Pharm. Bull. 23: 887–889.

14. Kurokawa M., Basnet P., Ohsugi M., Hozumi T., Kadota S., Namba T., Kawana T., Shiraki K. 1999. Anti-herpes simplex virus activity of moronic acid purified from *Rhus javanica in vitro* and *in vivo.* J. Pharmacol Exp. Ther. 289: 72–78.
15. Lampis G., Ingianni A., Pompei R. 1997. Synergistic effects of triterpenic compounds with prostaglandin A1 on vaccinia virus infected L929 cells. Antiviral Res. 36: 191–195.
16. Mahato S. B., Sarkar S. K., Poddar G. 1988. Triterpenoid saponins. Phytochemistry 27: 3037–3067.
17. Recio M. C., Giner R. M., Manez S., Gueho J., Julien H. R., Hostettmann K., Rios J. L. 1995. Investigations on the steroidal anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. Planta Med. 61: 9–12.
18. Ryu S. Y., Oak M. H., Yoon S. K., Cho D. I., Yoo G. S., Kim T. S., Kim K. M. 2000. Anti-alergic and anti-inflammatory triterpenes from herb of *Prunella vulgaris*. Planta Med. 66: 358–360.
19. Simoes C. M., Amoros M., Girre L. 1999. Mechanism of antiviral activity of triterpenoid saponins. Phytother. Res. 13: 323–328.
20. Vlietinck A. J., De Bruyne T., Apers S., Pieters L. A. 1998. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Med. 64: 97–109.
21. Zuco V., Supino R., Righetti S. C., Cleris L., Marchesi E., Gambacorti-Paserini C., Formelli F. 2002. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Lett. 175: 17–25.