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Effect of betulinic acid and its ionic derivatives on M-MuLV replication

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ABSTRACT

Murine leukemia virus (MuLV) is a retrovirus known causing leukemia and neurological disorders in mice, and its viral life cycle and pathogenesis have been investigated extensively over the past decades. As a natural antiviral agent, betulinic acid is a pentacyclic triterpenoid that can be found in the bark of several species of plants (particularly the white birch). One of the hurdles for betulinic acid to release its antiviral potency is its poor water solubility. In this study, we synthesized more water-soluble ionic derivatives of betulinic acid, and examined their activities against Moloney MuLV (M-MuLV). The mouse fibroblast cells stably infected with M-MuLV, 43D cells, were treated with various doses of betulinic acids and its derivatives, and the viral structural protein Gag in cells and media were detected by western blots. Two ionic derivatives containing the benzalkonium cation were found to inhibit the virus production into media and decreased Gag in cells. However, a cell proliferation assay showed that the benzalkonium cation inhibited the growth of 43D cells, suggesting that our ionic derivatives limited virus production through the inhibition of metabolism in 43D cells. Interestingly, all of these betulinic acid compounds exhibited a minimum impact on the processing and release of Gag from 43D cells, which outlines the differences of viral maturation between MuLV and human immunodeficiency virus.

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1. Introduction

Retroviruses comprise a large and diverse family of enveloped RNA viruses defined by common taxonomic denominators including the structural, compositional, and replicative properties [1]. Murine leukemia viruses (MuLVs) are simple retroviruses and encode only three polyproteins that are used in the assembly of progeny virus particles; Gag (structural protein), Pol (three retroviral enzymes, i.e. protease, reverse transcriptase, and integrase), and Env [2]. MuLVs are composed of diverse strains in both endogamous and exogenous viruses, and some of them are causative agents of leukemia and neurological disorders in mice [3]. Moloney murine leukemia virus (M-MuLV) is a well-studied replication-competent oncogenic retrovirus of gammaretrovirus genus which has enabled the understandings of general phenomenon of leukemogenesis; therefore, M-MuLV has been considered as a model retrovirus [4]. The viruses bind to the host cell via interactions between the envelope protein and its receptor on target cells. Subsequently, the penetrated viral cores are endocytosed and the uncoating occurs. The single stranded viral RNA genome is reverse transcribed and cDNA is integrated into the host genome by integrase. In the late phase, viral transcripts make the viral proteins. At the same time, the unspliced viral RNA works as the viral genome. These viral materials are trafficked to plasma membranes and assembled, and new viruses bud from the membranes at the end.

Betulinic acid (3β-hydroxyl-lup-20(29)-en-28-oic acid) is a natural pentacyclic lupine type triterpene that can be extracted from plants (e.g. birch tree bark) [5]. This natural compound has a number of medically relevant biological properties such as anti-inflammatory, anti-cancer, and anti-viral activities [5–8]. A major obstacle with the releasing of the antiviral potency of betulinic acid is due to its poor solubility in aqueous solutions and common organic solutions (such as esters, alcohols, and ethers). As a matter of fact, the solubility of betulinic acid in water is only approximately

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0.02 μg/ml at room temperature [9]. In common organic solvents, the solubility of betulinic acid is also fairly low, e.g. 1% (w/v) in ethanol and 5% (w/v) in DMSO at 25 °C [10]. Some derivatives of betulinic acid had shown improved water solubility, as well as enhanced biological activities when compared with betulinic acid itself [6,11,12]. Recently, we developed new ionic derivatives of betulinic acid with a higher water solubility and thus a stronger antiviral activity [10,13,14]. In the present study, we further examined the effect of betulinic acid and its derivatives (see Fig. 1) on the retroviral replication of M-MuLV.

2. Materials and methods

Synthesis of ionic derivatives of betulinic acid. Betulinic acid, glycine methyl ester hydrochloride, *N*, *N*′-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)pyridine (DMAP), choline chloride, benzalkonium chloride, and Amberlyst A26 hydroxide form (Sigma-Aldrich, St. Louis, MO) were used to prepare ionic salts of betulinic acid conjugated with glycine (Fig. 1). The procedures for preparing ionic derivatives of betulinic acid are described previously [15,16]. In brief, betulinic acid (**BA1**, 204.4 mg), glycine methyl ester (104.0 mg), and triethylamine (8 mg) were dissolved in 50 ml of anhydrous tetrahydrofuran (THF) at room temperature. Into the reaction mixture, DCC (107.9 mg) and DMAP (30.3 mg) were added and the mixture was stirred at room temperature under argon for a duration of 48 h. After the reaction completed, the precipitate (dicyclohexylurea as byproduct) was filtered off and the filtrate was further evaporated under vacuum to remove THF. The crude product was then dissolved in 100 ml of ether/ethyl acetate (2:1, v/v) and extracted with 100 ml of water to remove the excess water-soluble carbodiimide reagent and any remaining dicyclohexylurea. The organic layer was further extracted with 1.0 N HCl (2 × 100 ml) to remove DMAP [17], saturated $NaHCO_3$ solution (1 × 100 ml), and lastly with water (1 × 100 ml) [18,19]. The purified organic layer was then dried over Na_2SO_4. After filtration, ethyl acetate was evaporated under vacuum to yield the glycine methyl ester conjugate of betulinic acid. The methyl ester (298 mg) was dissolved in 100 ml of THF/H_2O (4:1, v/v) solution, followed by the addition of LiOH (67.5 mg). The reaction mixture was then stirred at room temperature for 4 h under argon. Once the reaction completed, THF was evaporated under vacuum and the product obtained was dissolved in 200 ml of ethyl acetate, followed by sequential washing with water, 0.1 M HCl, and water once again. The organic layer was then dried with Na_2SO_4, and after filtration the solvent was removed under vacuum to yield glycine conjugate of betulinic acid (231.4 mg). The choline hydroxide was prepared from choline chloride following an anion-exchange column approach used previously [15]. The glycinylated betulinic acid, **BA4**, was dissolved in 100 ml of THF/H_2O (4:1, v/v), followed by the addition of a five-fold molar excess of choline hydroxide (0.55 g). The reaction mixture

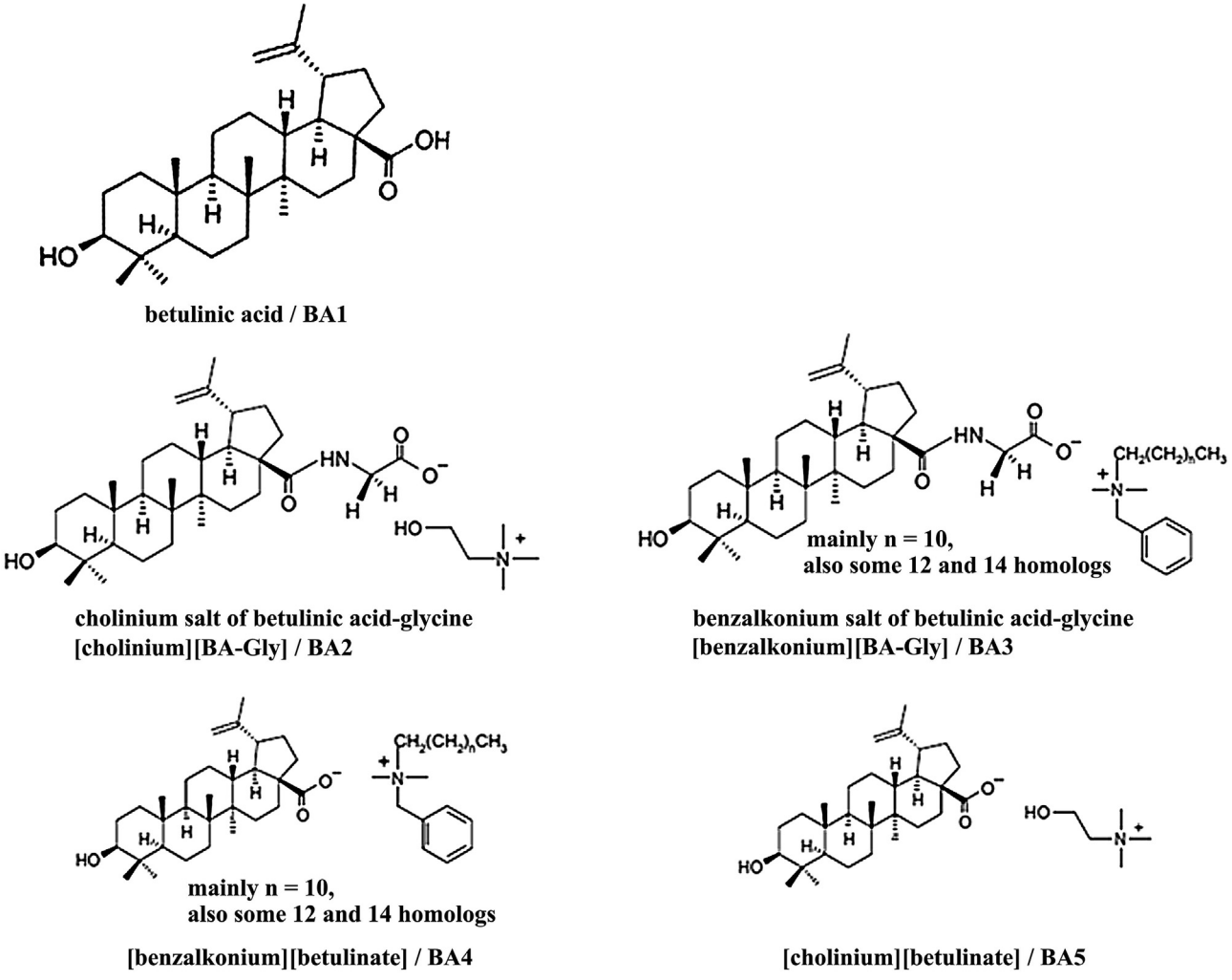

Fig. 1. Structures of betulinic acid (BA**1**) and ionic derivatives (BA**2**–BA**5**).

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