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Liquid-chromatographic profiling of Saponinum album (Merck)

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Saponinum album (Merck) is a complex composite of triterpene saponins. It was shown that Saponinum album (Merck) dramatically enhances the toxicity of the *N*-glycosylase saporin from the seeds of *Saponaria officinalis* L. as well as the toxicity of a saporin based anti-tumor toxin. This study was intended to chromatographically profile the saponins present in Saponinum album (Merck) in order to identify saponins that determine the cytotoxicity enhancing properties of Saponinum album (Merck) on saporin. For this purpose a liquid-chromatographic profiling (HPLC) followed by ESI-TOF-MS analysis and evaluation of cytotoxicity enhancer effects of saponins from Saponinum album (Merck) was performed. This is the first study describing a liquid-chromatographic profiling of saponins from Saponinum album (Merck). Ten different saponins were isolated. There was a lot of variation observed in the cytotoxicity enhancing properties of different isolated saponins, 8 out of 10 isolated saponins showed an enhancer effect on the toxicity of saporin. Based on these results it was concluded that the cytotoxicity enhancer effect of Saponinum album (Merck) is not attributable to a single, activity determining saponin.

1. Introduction

Saponins are plant secondary metabolites, which in general are composed of a hydrophobic backbone (aglycone) and one (at C-3 of the aglycone) or two (at C-3 and C-28 of the aglycone) carbohydrate chains. Monodesmosidic saponins contain one and bisdesmosidic two carbohydrate chains. According to their aglycones saponins can be further classified as steroid (C_{27}) or triterpenoid (C_{30}) saponins (Francis et al. 2002).
Saponinum album (Merck) is a mixture of mono- and bisdesmosidic triterpene saponins with gypsogenin as aglycon. The appellation “Merck” refers to the manufacturer of Saponinum album since the composition may differ when obtained from other sources.
Most of the saponins isolated from Saponinum album are bisdesmosidic and have the carbohydrate moieties attached at the C_3 and C_{28} position of gypsogenin. Saponinum album is isolated by solvent extraction of the roots of the ornamental plant baby’s breath (*Gypsophila paniculata* L.) followed by ether precipitation and concentration in *n*-butanol. In former times Saponinum album was used as an expectorant for bronchial catarrh. In the recent past the commercial production of Saponinum album (Merck) has been halted but the mixture is still available in a large amount in our repository at the department of pharmaceutical biology, Freie Universität Berlin (Berlin, Germany). In some of our previously reported studies Saponinum album (Merck) caused a tremendous toxicity enhancement of the protein toxin saporin (Weng et al. 2008). This was further validated by an *in vivo* assessment in tumor bearing Balb/c mice for a saporin based anti-tumor toxin targeting the epidermal growth factor receptor, known as SA2E (Bachran et al. 2009). Saporin is an *N*-glycosylase that is synthesized by the plant *Saponaria officinalis* L. It inactivates the protein synthesis by depurination the ribosomal RNA, resulting in cell death (Barbieri et al. 1996).
Our long term aim is to develop a combinatorial anti-tumor treatment of saponins and saporin based anti-tumor toxins. For a clinical application of the combinatorial anti-tumor treatment as discussed above it is mandatory to have pure isolated saponins with toxicity enhancing properties. Up to now three triterpene saponins (*Gypsophila* saponins 1, 2, 3) were isolated directly from the roots of *Gypsophila paniculata* L. (Weng et al. 2010). Furthermore, a saponin (saponin-1641) was isolated from Saponinum album (Merck) (Weng et al. 2009). Compared to saponin-1641, *Gypsophila* saponins 1, 2 and 3 showed toxicity enhancing properties only at a higher concentrations (Weng et al. 2009, 2010). Based on these observations we aimed to clarify the question, if there are any activity determining saponins present in Saponinum album (Merck).
For this purpose a liquid-chromatographic profiling of Saponinum album (Merck) was performed in order to isolate single saponins. Each isolated saponin was subjected to ESI-TOF-MS analysis and the individual cytotoxicity of all isolated saponins as well as their toxicity enhancing properties on saporin were investigated.

2. Investigations and results

2.1. *Saponins from Saponinum album (Merck)*

Several triterpene saponins were isolated by HPLC from Saponinum album (Merck). A total of ten saponins were isolated and designated as follows: S-P1.1, S-P1.2, S-P2.1, S-P2.2, S-P3.1, S-P3.2, S-P3.3, S-P3.4, S-P3.5 and S-P4 (“S” refers to saponin and “P” to the HPLC fraction, covering the appropriate saponin; numbers indicate fraction and subfraction). S-P1.1 to S-P4 were used for further cell culture experiments. The Table 1 summarizes the different isolated saponins and the corresponding

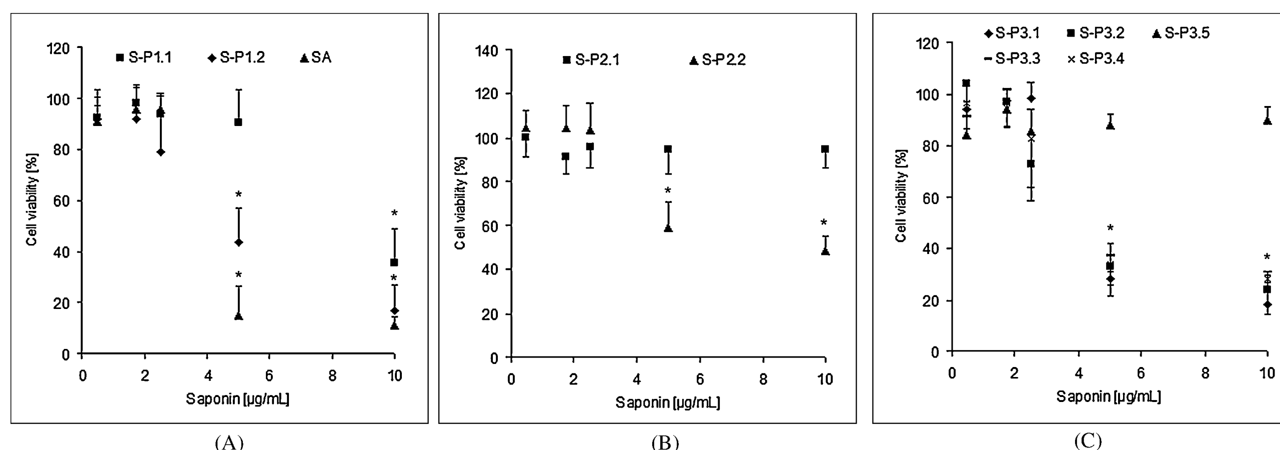

Fig. 1: Cytotoxicity (ECV-304 cells) of saporin in combination with different saponins (S-P1.1 to S-P4.4) isolated from Saponinum album (Merck). ECV-304 cells were incubated with different mixtures of saponins (0.5–10 µg/mL.) and saporin (0.15 nM) for 72 h. Except S-P2.1 (B) and S-P3.5 (C) all saponins showed toxicity enhancing properties on saporin. The cytotoxicity was determined by XTT-assay. Each value represents the mean of at least 5 individual measurements ± SD. * $p < 0.01$, Mann-Whitney-U-Test

masses (*m/z*). According to the mass spectra the saponins were obtained in high purity (mass spectra are shown in supplementary data). S-P3.5 contained two saponins covering the masses *m/z* 939,47 and *m/z* 1053,46 (Table 1). To date we were not able to further separate these saponins from each other.

2.2. *Inherent cytotoxicity of saponins*

The Table 1 (right column) shows the GI_{50} (50% growth inhibitory concentration) of Saponinum album (Merck) and different isolated saponins. The GI_{50} of the saponins increased in the following order: S-P1.2 < S-P3.2 < S-P2.1 = S-P3.3 < S-P3.4 < S-P1.1 < S-P2.2 < S-P3.1= S-P4 < S-P3.5. The inherent cytotoxicity of S-P1.2 was more than 5-fold compared to S-P3.5.

2.3. *Cytotoxicity enhancement effect on saporin*

Figure 1 shows the cytotoxicity enhancing properties of the isolated saponins on saporin at a fixed saporin concentration (0.15 nM). At 0.15 nM saporin exhibited no individual cytotoxicity and the cells were 100% viable as in case of media control (data not shown). In the concentration range from 0.5 to 10 µg/mL S-P2.1 (Fig. 1B) and S-P3.5 (Fig. 1C) showed no cytotoxicity enhancement. S-P1.1 exhibited at 10 µg/mL a significant cytotoxicity enhancement on saporin (Fig. 1A). In contrast, S-P1.2 (Fig. 1A), S-P2.2 (Fig. 1B), S-P3.1, S-P3.2, S-P3.3 and S-P3.4 (Fig. 1C) exhibited a substantial toxicity enhancement effect on saporin at 5 µg/mL.

Table 1: Masses of the isolated saponins detected in the ESI-TOF-MS and their intrinsic GI_{50} values (50% growth inhibitory concentration)

Saponin	*m/z*	GI_{50} [µg/mL]
S-P1.1	1641,72	24
S-P1.2	1699,73	9
S-P2.1	1669,72	21
S-P2.2	1567,69	25
S-P3.1	1641,72	29
S-P3.2	1537,67	17
S-P3.3	1683,73	21
S-P3.4	1683,73	22
S-P3.5	939,47/1053,46	> 50
S-P4	1609,69	29
Saponinum album (Merck)		18

GI_{50} values were deduced from the appropriate dose-response curves (ECV-304 cells) of the saponins.

3. Discussion

In previous studies we characterized the overall impact of Saponinum album (Merck) in terms of the cytotoxicity enhancement effect on the *N*-glycosylase saporin (Weng et al. 2008). This principle could be adapted for a saporin based anti-tumor toxin (SA2E), targeting the epidermal growth factor receptor (Bachran et al. 2009). The benefit of a combinatorial anti-tumor treatment of SA2E and Saponinum album (Merck) is the attenuation of adverse side effects, which is based on a reduction of the SA2E-dosage. For tumor therapy pure saponins are mandatory. However as mentioned, Saponinum album (Merck) is a complex mixture of triterpene saponins (see also MS-spectrum in the supplementary data). Up to now a total of four triterpene saponins have been isolated directly from the roots of *Gypsophila paniculata* L. and Saponinum album (Merck). (Weng et al. 2009, 2010). All these saponins are bisdesmosidic with mainly gypsogenin as aglycon and an acidic trisaccharide at the C_3 of the aglycon (Fig. 2). However, up to now nothing was known about activity determining triterpene saponins from Saponinum album (Merck) in terms of the cytotoxicity enhancing effect on saporin. In order to isolate these potential saponins we performed a liquid-chromatographic profiling of Saponinum album (Merck), followed by ESI-TOF-MS analysis. Thereafter we determined the cytotoxicity enhancement on saporin at a fixed concentration of 0.15 nM. At this concentration saporin alone showed no cytotoxicity. The GI_{50} of saporin in ECV-304 cells was determined to be greater than 2000 nM (Hebestreit et al. 2006) which corresponds to a very low cytotoxicity.

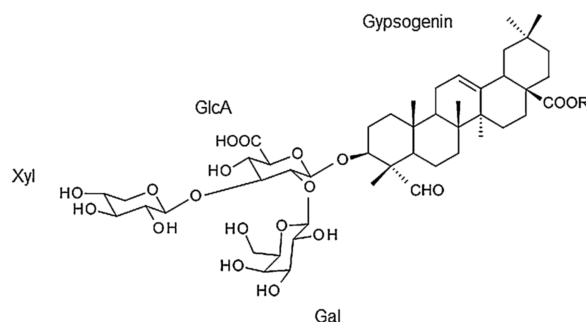

Fig. 2: Common structural element (Mr = 940) of triterpene saponins from Saponinum album (Merck), consisting of gypsogenin as aglycon and an acidic trisaccharide at the C_3 of gypsogenin with glucuronic acid (GlyA), galactose (Gal) and xylose (Xyl)

Ten saponins were isolated with masses ranging from *m/z* 939.47 (S-P3.5) to 1699.73 (S-P2.2). While a mass of 939.47 (S-P3.5) most probably refers to a monodesmosidic saponin (see also Fig. 2), refers the mass represented by S-P2.2 to a bisdesmosidic triterpene saponin. All the isolated saponins were tested for their toxicity enhancing properties in the concentration range 0.5 to 10 μg/mL. In this range S-P3.5 exhibited no cytotoxicity enhancement on saporin (Fig. 1C), which emphasizes the importance of a second carbohydrate chain at the C_{28} of the aglycone. In some other studies we observed, that monodesmosidic saponins show no cytotoxicity enhancement effect on toxins (unpublished results).

However, no enhancement effect were observed for S-P2.1 (*m/z* 1669.72) as well (Fig. 1B), this showed that a second carbohydrate chain at the C_{28} is not the sole criteria for the cytotoxicity enhancement. We hypothesised, that the kind of branching of the carbohydrate chains attached to the aglycone along with some further structural features like presence of glucuronic acid as the starting sugar at C-3 position may have an essential influence on the ability of individual saponins to enhance the cytotoxicity of saporin. This may be the reason why S-P1.1 (*m/z* 1641.72) showed a substantial cytotoxicity enhancement at 10 μg/mL (Fig. 1A) while the mass identical S-P3.1 that was collected as a separate fraction (*m/z* 1641.72) was effective at 5 μg/mL (Fig. 1C). Except S-P2.1 and S-P3.5 all saponins showed a cytotoxicity enhancement on saporin compared to crude Saponinum album (Merck) (Fig. 1A). It can be therefore stated, that the cytotoxicity enhancement of saporin caused by saponins is a general feature of Saponinum album (Merck) and is not attributed to single, activity determining saponins. It seems to be a general feature of bisdesmosidic *Gypsophila* saponins with certain structural attributes, which can lead to the enhancement effect.

4. Experimental

4.1. Materials

Saporin, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate (PMS) were purchased from Sigma Aldrich (Steinheim, Germany). Saponinum album (Merck) was obtained from Merck (Darmstadt, Germany). ECV-304 cells (ACC 304) were purchased form the German Cell Culture Collection (Braunschweig, Germany). Cells were cultured in modified Eagle's medium (MEM) (Biochrom KG, Berlin, Germany) in phenol red-free MEM with 15% fetal bovine serum. All other chemicals were received from Merck (Darmstadt, Germany). Spectra/Por® dialysis membrane (MWCO 1000) was purchased from Carl Roth (Wiesbaden, Germany).

4.2. General experimental procedures

Semi-preparative HPLC was performed using a Wellchrom HPLC pump K-1001 equipped with a Wellchrom solvent organizer K-1500 and a Wellchrom DAD K-2700 (Knauer, Berlin, Germany). An UltraSep ES PHARM RP18E (7 μm, 250 × 8 mm) column from SepServ (Berlin, Germany) was used for the chromatographic profiling and an Agilent 6210 ESI-TOF-MS for the MS-analysis of the saponins.

4.3. Isolation of saponins

Saponinum album (Merck) (2 g) was dissolved in 20 mL distilled water and dialyzed (MWCO 1000) for 72 h against distilled water. The water was changed thrice during the whole course of dialysis. The dialyzed solution was freeze dried and 1.03 g of a pre-purified saponin fraction was obtained. Dialyzed Saponinum album (Merck) (50 mg) was dissolved in 1 mL of 65% methanol, 0.5 mL of dissolved saponins was analyzed by HPLC. A methanol/distilled water (0.01% trifluoroacetic acid) gradient starting with 65% to 80% methanol (30 min, flow rate, 1.5 mL; detection at 210 nm) was used for elution. Four peaks with retention times of 17 (P1), 18 (P2), 20 (P3) and 22 (P4) min were collected and analyzed by ESI-TOF-MS as described elsewhere (Weng et al. 2010). P1 to P3 were subjected to HPLC using the same setup as describe above with an acetonitrile (A)/distilled water (B) gradient (0.01% trifluoroacetic acid) starting with 40% A to 60% A over 30 min. P1 was split into two main peaks with retention time 12.5 (P1.1) and 16.5 min (P1.2). Two peaks with retention time 14 (P2.1) and 15.5 (P2.2) min were collected after injection of P2 into the HPLC. Fraction P3 was split into further 5 peaks with retention time 15 (P3.1), 18 (P3.2), 20.5 (P3.3), 22 (P3.4) and 23.5 (P3.5) min. All peaks were collected and analyzed by ESI-TOF-MS as described elsewhere (Weng et al. 2010). Acetonitrile was evaporated and the remaining water was removed by freeze drying.

4.4. Cytotoxicity assay

ECV-304 cells were seeded in 100 μL MEM in 96-well plates at a density of 2000 cells/well and grown for 24 h. For determining single cytotoxicity of the isolated saponins cells were incubated with S-P1.1, S-P1.2, S-P2.1, S-P2.2, S-P3.1, S-P3.2, S-P3.3, S-P3.4, S-P3.5 or S-P4 (final concentration 1 to 50 μg/mL). For the combination experiments with saporin cells were incubated with saporin (final conc. 0.15 nM) and either one of the isolated saponins S-P1.1 to S-P4 (final conc. 0.5–10 μg/mL). Control cells were only incubated with saporin or phosphate buffered saline (pH 7.4). Cells were incubated for further 72 h and cytotoxicity was determined by XTT-assay as described elsewhere (Weng et al. 2008).

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