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Structure–activity relationships of saponins enhancing the cytotoxicity of ribosome-inactivating proteins type I (RIP-I)

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ABSTRACT

Saponins are amphiphilic secondary plant compounds able to interfere with surfaces and permeabilize membranes. In addition to antimicrobial and anti-inflammatory features, anti-neoplastic activities are described which base on various mechanisms. A very promising anti-cancer strategy is the synergistic enhancement of the cytotoxicity of ribosome-inactivating proteins type I (RIP-I) by suitable saponins. The cytotoxicity of the naturally low cytotoxic activity showing RIP-I can be augmented up to 100,000-fold, if they are applied in combination with appropriate saponins. These saponins have to hold certain structural features.

We analyzed 56 different saponins for their non-synergistic, as well as their synergistic cytotoxicity with the RIP-I saporin from *Saponaria officinalis* L. (Caryophyllaceae). The saponins were assorted into categories, according to their structural features and their cytotoxic behavior, to build up structure–activity relationships. A non-synergistic cytotoxicity as low as possible and a synergistic cytotoxicity as high as possible were desired. The investigations suggest that ideal saponins consist of an oleanane-type scaffold, preferably gypsogenin or quillaic acid, three branched sugars at the C-3–OH-group including glucuronic acid, galactose, xylose and four branched sugars at the C-28–OH-group, including deoxy-sugars and acetyl-residues.

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1. Introduction

Saponins are a very versatile group of secondary plant and marine life-form compounds. A lipophilic backbone (aglycon or sapogenin) of steroidal or triterpenoid derivation is connected with up to three (trisdesmosidic) hydrophilic oligosaccharide chains. In the class of *Magnoliopsida* in the plant kingdom triterpenoid saponins with one (mondesmosidic) or two (bisdesmosidic) sugar moieties are the most prevalent (Vincken et al., 2007). The sugars can be arranged in line or they can be branched. Whilst the oleanane-type triterpen can be seen to be the most common skeleton in the plant orders of *Magnoliopsida*, the sugars apiose (Api), arabinose (Ara) fucose (Fuc), galactose (Gal), glucose (Glc), glucuronic acid (GlcA), quinovose (Qui), rhamnose (Rha) and xylose (Xyl) are widespread in this class (Vincken et al., 2007). Consistent of lipophilic and hydrophilic molecule parts, saponins exhibit surface activity. They are known to lower the surface tension of aqueous solutions and permeabilize cell membranes (Böttger et al., 2012). Besides these physico-chemical properties saponins show pharmacologic effects: anti-viral, anti-fungal, anti-inflammatory, anti-allergic and anti-neoplastic activities are reported (Francis et al., 2002). The latter may base on the inhibition of angiogenesis, the reduction of invasiveness, the arrest of cell cycles or the induction of apoptosis (Gilabert-Oriol et al., 2013). Another promising anti-cancer strategy is provided by the saponins' ability to enhance the cytotoxicity of ribosome-inactivating proteins type I (RIP-I). This effect is reported for agrostin from *Agrostemma*

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githago L. (Caryophyllaceae) and for saporin from *Saponaria officinalis* L. (Caryophyllaceae), two RIP-I (Hebestreit and Melzig, 2003; Hebestreit et al., 2006). The RIP execute a cytotoxic N-glycosidase activity by cleaving adenine nucleotides from the 28S rRNA in the 60S subunit of ribosomes (Endo et al., 1987). While ribosome-inactivating proteins type-II (RIP-II) like ricin from *Ricinus communis* L. (Euphorbiaceae) or viscumin from *Viscum album* L. (Viscaceae) are composed of two protein chains, an A-chain with the enzymatic activity and a B-chain that acts as cell binding domain and mediates cytosolic uptake, RIP-I consist of the A-chain only (Olsnes and Pihl, 1973). The lack of a natural cell binding domain in RIP-I results in insufficient cytosolic liberation and is the reason for their low cytotoxicity. Since certain oleanane-type saponins can trigger the transport of the RIP-I A-chain into the cytosol, they can be seen as a B-chain substitute that enables a high cytotoxicity. Thus the appliance of these saponins at sub-toxic levels along with the naturally low cytotoxic activity showing RIP-I amplifies their cytotoxicity in a synergistic manner up 100,000-fold in cell culture (Hebestreit et al., 2006; Melzig et al., 2005). This makes RIP-I as toxic as naturally highly cytotoxic RIP-II (Weng et al., 2009). If a RIP-I is coupled to a specific ligand (like EGF) that targets cancer-associated antigens (like EGFR), the cytotoxicity can be even increased up to 4,000,000-fold in cell culture, provided that it is combined with appropriate saponins (Weng et al., 2012a). Finding these saponins and investigating which structural features they have to hold was the purpose of our study. We compared the results of cytotoxicity tests with and without combination with a RIP-I for differently structured saponins and tried to develop structure–activity relationships. Ideal saponins would feature a minimal cytotoxicity when applied discretely (non-synergistic cytotoxicity) and a maximal cytotoxicity (synergistic cytotoxicity) in combined application with a RIP-I.

2. Materials and methods

2.1. Saponins

The cytotoxicity of 56 saponins was tested in cell culture. The saponins were also tested for the capability to enhance the cytotoxicity of the RIP-I saporin.

50 saponins were provided by AnalytiCon Discovery GmbH, Potsdam, Germany. The saponins SA1641 and SA1657 were isolated by high performance liquid chromatography (HPLC) from Saponinum album (Merck KGaA, Darmstadt, Germany) a commercial saponin mixture from *Gypsophila arrostii* Guss. and *Gypsophila paniculata* L. (Caryophyllacea) (Weng et al., 2012b) and primulic acid 1 from *Primula vulgaris* Huds. Further saponins are commercially available: beta-escin and glycyrrhizin (Merck KGaA, Darmstadt, Germany), soyasaponin Bb (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

2.2. Cell culture

Human urinary bladder epithelial carcinoma cells (ECV-304, ACC 310) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Leibniz-Institut DSMZ, Braunschweig, Germany). As epithelial cells ECV-304 cells are adapted to function at surfaces and interfaces of tissues. Therefore this cell line seems suitable for experiments with substances that are known to affect the cell membrane integrity. The cells were cultured in Dulbecco's MEM (DMEM) without phenol red (Biochrom AG, Berlin, Germany) with 10% FCS (fetal calf serum), 5% glutamine, 10% HEPES (Biochrom AG, Berlin, Germany) at 37 °C in saturated water vapor atmosphere containing 5% CO_2. Passages from 4 to 33 were used for the experiments. The cultures were split 1:5 twice a week, using trypsin/EDTA (Biochrom AG, Berlin, Germany).

2.3. Cytotoxicity assays

The cells were disseminated at a density of 5000 cells/well and were grown for 24 h in 96-well plates, transparent and black. The culture medium was then replaced by medium containing different concentrations (0.5, 1.0, 2.5, 5.0, 7.5, 10.0 μg/mL) of a saponin with or without 1 nM saporin (Sigma–Aldrich, Steinheim, Germany). The cells were incubated for another 72 h. The cytotoxicity was evaluated using an MTT assay and via DNA quantification.

The colorimetric MTT assay was performed in transparent 96-well plates. The supernatant was removed and replaced by DMEM containing no other additives. The MTT dye (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was dissolved in PBS and added to the cells reaching a concentration of 0.5 mg/mL. After 30 min of incubation at 37 °C living cells transformed the yellowish MTT into a water-insoluble purple formazan. The medium was discarded and the formazan was dissolved in 100 μL/well DMSO (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). After 5 min of careful shaking the absorbance was measured in a Tecan Spectrafluor microplate reader (Tecan Group Ltd., Mainz, Germany) at 580 nm. The absorbance is proportional to the number of viable cells. The cytotoxicity was calculated by comparing saponin-treated wells with untreated control-wells.

The DNA quantification was performed in black 96-well plates. Therefore the supernatant was removed and the cells were washed with isotonic NaCl solution. 100 μL/well ultra-pure water were given into the wells followed by three freeze-thaw-cycles in order to break the cell membranes. After that 100 μL/well double-concentrated DNA buffer pH 7.4 containing 82 mM $Na_2HPO_4 \cdot 2H_2O$, 18 mM $NaH_2PO_4 \cdot H_2O$, 4 mM EDTA and 4 M NaCl were added. The DNA was stained with 10 μL/well of 10 μg/mL Hoechst 33258 dye (Sigma–Aldrich Chemie GmbH, Steinheim, Germany). The fluorescence was measured in a Tecan infinite F200 microplate reader (Tecan Group Ltd., Mainz, Germany) at 360 nm excitation wavelength and 465 nm emission wavelength. The fluorescence is proportional to the amount of dsDNA. The cytotoxicity was calculated by comparing saponin-treated wells with untreated control-wells.

3. Results

The non-synergistic and the synergistic cytotoxicity were assessed for all 56 saponins. Two test systems were used for each kind of cytotoxicity – the colorimetric MTT assay and fluorimetric DNA quantification. All saponins

were applied in the same range and at the same steps of concentration and incubation took place under constant conditions. As Fig. 1 shows both tests provided comparable results.

To facilitate the comparison the saponins were sorted into 13 categories according to their structural features and IC_{50}-values, which were generated from the toxicity tests (Table 1). The complexity of the saponins, the length of sugar chains, as well as the total number of sugars increases from top to bottom. Linear sugar chains are followed by branched sugar moieties. The listing starts with the C-3-O-residues followed by the C-28-O-residues. The saponins' aglyca in the categories are not sorted since they are of subordinate meaning.

The categories 1, 3, 4 and 6 hold saponins that did not show cytotoxicity in this test system, neither non-synergistic nor synergistic. Cat. 2 holds a compound. Although this compound's structure was very similar to those of Cat. 1, 3, 4 and 6, it exhibited a considerable cytotoxicity. Cat. 5 bears two saponins who increased the cytotoxicity of saporin and who can be designated as slightly synergistically toxic. The categories 7–13 contain saponins that enhance the cytotoxicity of saporin, in some cases not without revealing a non-synergistic cytotoxicity.

The highest synergistic cytotoxicity, represented by lowest IC_{50}(syn.)-values, was found for saponins in Cat. 7–9. These saponins also revealed only low non-synergistic cytotoxicity, indicated by high IC_{50}-values (>10 μg/mL). A high difference between IC_{50} and IC_{50}(syn.) is desirable, having a potential therapeutic use in mind. This difference can also be expressed as ratio of IC_{50}(syn.) to IC_{50} wherein the IC_{50}(syn.) is considered 1. A high ratio is favored for the same reason.

4. Discussion

In this study we assessed the cytotoxicity of 56 oleanane-type saponins via two different tests, the MTT assay and the DNA quantification. Both are based on different targets that are measured. The MTT assay detects viable cells by indicating their reductive enzymatic activity of mitochondria and other cell compartments (Berridge et al., 2005). Although widely used for the measurement of metabolic activity in healthy living cells, this assay bears some sources of error that may lead to misinterpretation and false negative results. Cellular repair mechanisms involve a general up-regulation of enzymatic activity which might overlap a decreased reductive activity due to the death of other cells. As Gilabert-Oriol et al. (2013) observed in studies about the cell membrane-perturbing and -permeabilizing effects of oleane-type saponins, cells react with repair mechanisms upon cell membrane damage. Those mechanisms assure the cell's survival unless a critical state of membrane damage is not exceeded. Thus low concentrations of membrane perturbing substances like saponins can be tolerated by the cell. In the case of Gilabert-Oriol et al. (2013) any lytic effects were observed until 6 μM for a choice of oleane-type saponins. This concentration is comparable to 10 μg/mL of the saponins from Cat. 7–11 and 13, and 6 μg/mL of the saponins from Cat. 1–6 and 12. Indeed most of the saponins in our study revealed no or only low non-synergistic cytotoxicity expressed in IC_{50}-values higher than 10 μg/mL. Due to the limited amount of substance, most of the 59 saponins were only tested up to a concentration of 10 μg/mL. To exclude the described overlapping of cytotoxic effects by enzymatic up-regulation, the DNA quantification to assess the cytotoxicity was performed simultaneously. Since severely damaged cells tend to apoptosis and lysis whereas viable (adherent growing) cells remain attached to surfaces, the removal of the culture medium and a washing step allowed a selective detection of double-strained DNA from living cells. Both cytotoxicity test methods gave comparable results for the non-synergistic as well as for the synergistic cytotoxicity expressed in similar IC_{50}- and IC_{50}(syn.)-values for both methods.

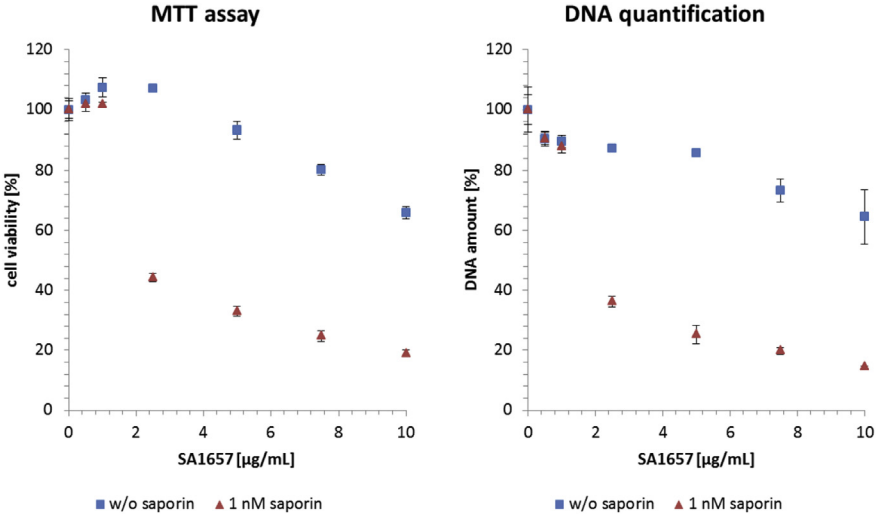

Fig. 1. Comparison of the results from MTT assay and DNA quantification exemplarily for SA1657. The non-synergistic (without saporin; square) and synergistic (with 1 nM saporin, triangle) cytotoxicity of SA1657 were tested via MTT assay and DNA quantification. 1 nM Saporin without any Saponin shows a cell viability of 100% in both assays. Mean ± CV, $n = 6$.

Table 1
Categories of tested saponins. All 56 saponins were assorted into 13 categories according to their chemical structure and cytotoxic behavior. The IC_{50} represents the concentration of a certain saponin with half-maximal cell viability or DNA amount compared to the untreated control. The IC_{50}(syn.) represents the concentration of a certain saponin in combination with 1 nM saporin with half-maximal cell viability or DNA amount compared to the untreated control. The column "No. of saponins" shows the number tested saponins with very similar structural features. The column "Mean ratio of IC_{50} to IC_{50}(syn.)" represents the difference between the non-synergistic and the synergistic cytotoxicity expressed as ratio. Sugars in brackets symbolize a branching of the moiety. MCA = methoxycinnamic acid, FA = fatty acid, OAc = acetyl-residue, + = the sugar moieties bear additionally non-sugar conjugates, * = any position of hydroxylation, *italic* = by estimation, Glc/Gal = glucose or galaktose, Rha/Fuc = Rhamnose or Fucose.

Cat.-No.	Aglycon	C-3-O-residue	C-3-O-residue comment	C-28-O-residue	C-28-O-residue comment	Total No. of sugars attached to the aglycon	IC_{50} [MTT-Assay] in µg/mL (µM)	IC_{50} [DNA-Quant.] in µg/mL (µM)	IC_{50}(syn.) [MTT-Assay] in µg/mL (µM)	IC_{50}(syn.) [DNA-Quant.] in µg/mL (µM)	No. of similar Saponins tested	Mean ratio of IC_{50} to IC_{50}(syn.)
1	Oleanolic acid, Hydroxyoleanolic acid*, Dihydroxyoleanolic acid*, Serjanic acid, Phytolaccagenic acid, Hydroxygypsogenin, Quillaic acid	GlcA-		Glc-		2	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	9	–
				Glc/Gal-			>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	2	–
		OAc-(→6)-GlcA-	Acetylated				>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	1	–
2	Oleanolic acid	GalA-		Glc-		2	2.3 (2.9)	3.3 (4.2)	2.5 (3.1)	3.5 (4.4)	1	–
3	Trihydroxyoleanolic acid*, Medicagenic acid	GlcA-		Rha-(1→2)-Ara-	Short chain (2), linear	3–5	>10 (>10.5)	>10 (>10.5)	>10 (10.5)	>10 (>10.5)	1	–
				Xyl-(1→4)-Rha-(1→2)-Ara-	Medium chain (3), linear		>10 (>9.2)	>10 (>9.2)	>10 (>9.2)	>10 (>9.2)	2	–
				Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-Ara-	Medium chain (4), linear		>10 (>8.2)	>10 (>8.2)	>10 (>8.2)	>10 (>8.2)	1	–
4	Glycyrrhetinic acid, Hydroxyoleanolic acid*, Serjanic acid, Oleanolic acid, Dihydroxyoleanan-12-en	GlcA-(1→2)-GlcA-	2 Sugars, linear	-	(Monodesmosidic)	2–4	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	>10 (>12.2)	1	–
		Xyl-(1→4)-GlcA-		Glc-			>10 (>10.0)	>10 (>10.0)	>10 (>10.0)	>10 (>10.0)	2	–
		Glc/Gal-(1→2/3)-GlcA-		Glc/Gal-			>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	1	–
				Glc-			>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	4	–
		Ara-(1→4)-GlcA-					>10 (>10.8)	>10 (>10.8)	>10 (>10.8)	>10 (>10.8)	1	–
		Rha-(1→2)-Gal-(1→2)-GlcA-	3 Sugars, linear	-	(Monodesmosidic)		>10 (>10.6)	>10 (>10.6)	>10 (>10.6)	>10 (>10.6)	1	–
		Rha/Fuc-(1→2)-Glc/Gal-(1→2)-GlcA-		Glc/Gal-			>10 (>9.1)	>10 (>9.1)	>10 (>9.1)	>10 (>9.1)	2	–
5	Protoescigenin, Quillaic acid	Glc-(1→2)-[Glc-(1→4)]-GlcA-	3 Sugars, branched	-	(Monodesmosidic)	3	> 50 (>44.2)	*62.6 (55.3)*	7.7 (6.8)	7.6 (6.7)	1	7.4
		Gal-(1→2)-[Xyl-(1→3)]-GlcA-		-			>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	>10 (>10.5)	1	–
6	Oleanolic acid, Hydroxyoleanolic acid*	Glc-(1→2)-[Glc-(1→4)]-GlcA-	3 Sugars, branched	Glc-		4–5	>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	1	–
		Gal-(1→2)-[Glc-(1→4)]-GlcA-					>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	1	–
		Glc/Gal-(1→3)-[Glc/Gal-(1→4)]-GlcA-		Glc/Gal-			>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	>10 (>8.9)	1	–
		Api-(1→3)-[Xyl-(1→4)]-GlcA-		Glc-			>10 (>9.3)	>10 (>9.3)	>10 (>9.3)	>10 (>9.3)	1	–
		Rha-(1→3)-[Xyl-(1→4)]-GlcA-		Api-(1→6)-Glc-	2 Sugars, linear		>10 (>8.3)	>10 (>8.3)	>10 (>8.3)	>10 (>8.3)	1	–
7	Gypsogenin	Gal-(1→2)-[Xyl-(1→3)]-GlcA-	3 Sugars, branched	Xyl-(1→4)-Rha-(1→2)-[MCA-(→4)]-Fuc-	3 Sugars, branched, MCA	6+	>10 (>6.6)	>10 (>6.6)	0.5 (0.3)	0.6 (0.4)	2	>20.0
8	Gypsogenin, Quillaic acid	Gal-(1→2)-[Xyl-(1→3)]-GlcA-	3 Sugars, branched	Glc-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[OAc-(→4)]-Fuc-	4 Sugars, linear, acetylated	7+	>10 (>6.4)	*11.3 (7.2)*	1.4 (0.9)	1.5 (1.0)	1	7.3
				Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[FA-(→4)-Fuc-	4 Sugars, linear, FA		>10 (>5.1)	6.7 (3.4)	0.7 (0.3)	0.6 (0.3)	1	12.8
9	Gypsogenin, Quillaic acid	Gal-(1→2)-[Xyl-(1→3)]-GlcA-	3 Sugars, branched	Ara/Xyl-(1→4)-Rha/Fuc-(1→4)-[Glc/Gal-(1→2)]-Fuc-	4 Sugars, branched	7	>10 (>6.5)	*10.4 (6.7)*	3.4 (2.2)	4.2 (2.7)	1	2.7
				Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-Fuc-			>10 (>6.4)	*10.3 (6.7)*	3.3 (2.1)	3.7 (2.4)	1	2.9
				Xyl-(1→4)-[Gal-(1→3)]-Rha-(1→2)-[OAc-(→4)]-Fuc-	4 Sugars, branched, acetylated	7+	>10 (>6.4)	>10 (>6.4)	0.6 (0.4)	0.7 (0.4)	1	>15.4
10	Gypsogenin, Quillaic acid	Gal-(1→2)-[Xyl-(1→3)]-GlcA-	3 Sugars, branched	Xyl-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Qui-(1→4)]-Fuc-	5 Sugars, branched(S1)	8	20.7 (12.6)	14.1 (8.6)	2.0 (1.2)	2.1 (1.3)	2	8.5
				Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-Fuc-	5 Sugars, branched(S2)		>10 (>6)	>10 (>6)	5.4 (3.2)	5.9 (3.5)	1	>1.8
				Api-(1→3)-Xyl-(1→4)-[Qui-(1→3)]-Rha-(1→2)-Fuc-			>10 (>6)	>10 (>6)	>10 (>6)	>10 (>6)	1	–
				Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[OAc-(→4)]-Fuc-	5 Sugars, branched, acetylated	8+	7.3 (4.2)	3.6 (2.1)	1.8 (1.0)	1.8 (1.0)	1	3.0
				Ara/Xyl-(1→3)-Ara/Xyl-(1→4)-Rha/Fuc-(1→2)-[OAc-(→4)-Rha/Fuc-(1→4)]-Rha/Fuc-			*10.9 (6.5)*	*10.5 (6.2)*	1.2 (0.7)	1.5 (0.9)	1	7.9
				Api-(1→3)-Xyl-(1→4)-Rha-(1→2)-[Rha-(1→3)],[OAc-(→4)]-Fuc-			3.5 (2.1)	3.6 (2.1)	0.6 (0.4)	0.7 (0.4)	1	5.5
				Glc-(1→3)-Xyl-(1→4)-Rha-(1→2)-[OAc-(→3)-Rha-(1→3)]-Fuc-			*11.0 (6.4)*	6.3 (3.6)	1.8 (1.0)	1.9 (1.1)	1	4.7
				Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[FA-(→4)-Fuc-	5 Sugars, branched, FA	8+	3.0 (1.4)	3.9 (1.8)	0.6 (0.3)	0.7 (0.3)	1	5.3
11	Gypsogenin, Quillaic acid	Gal-(1→2)-[Xyl-(1→3)]-GlcA-	3 Sugars, branched	Api-(1→3)-Xyl-(1→4)-[Glc-(1→3)]-Rha-(1→2)-[Rha-(1→3)],[OAc-(→4)]-Fuc-	6 Sugars, branched, acetylated	9+	5.6 (3.0)	4.8 (2.6)	1.2 (0.6)	1.2 (0.6)	1	4.3
12	Trihydroxyoleanan-12-en	Rha-(1→2)-Gal-(1→3)-[Glc-(1→2)]-GlcA-	4 Sugars, branched	-	(Monodesmosidic)	4	16.0 (14.5)	14.0 (12.7)	3.5 (3.2)	2.5 (2.3)	1	5.0
13	Dihydroxyoleanolic acid*	Ara/Xyl-(1→4)-Rha/Fuc-(1→2)-Glc/Gal-(1→2)-Rha/Fuc-(1→2)-GlcA-	5 Sugars, linear	Xyl/Ara-		5	>10 (>7.2)	>10 (>7.2)	5 (3.6)	5.7 (4.1)	1	>1.9

The synergistic cytotoxicity between saponins and RIP-I appears to be a conserved defense mechanism of some plants (Weng et al., 2012a). Especially the Caryophyllaceae plant family is known for its saponins and for RIP-I in some genera (Barbieri et al., 1993; Böttger and Melzig, 2011). In the past the cytotoxicity of *A. githago* L. (Caryophyllaceae) gained notoriety when poisoning people who consumed grain contaminated with the plant's seeds. Today the plant is nearly eradicated in modern agriculture (Hebestreit and Melzig, 2003). It has even become an endangered species that was put under protection by law in several countries [http://www.stiftung-naturschutz-hh.de/blume/2003.htm]. Hebestreit et al. showed that the cytotoxicity of the seeds is the result of the presence of oleane-type saponins and the RIP-I agrostin (Hebestreit and Melzig, 2003; Hebestreit et al., 2006; Siepmann et al., 1998). Both, the *Agrostemma* saponins ($IC_{50} = 10.8$ and 11.2 μg/mL) as well as the RIP-I ($IC_{50} > 50$ μg/mL) little cytotoxicity when applied separately, but revealed an increase in the RIP-I cytotoxicity in a synergistic manner, when applied in combination ($IC_{50} = 5.7$ ng/mL with 3 μg/mL agrostemmasaponin 1). Moreover, Hebestreit and Melzig (2003) found an extraordinary high synergistic cytotoxicity, when agrostin was combined with *Agrostemma* saponins instead of other oleane-type saponins of similar molecular mass.

Since membrane-permeabilizing effects can be excluded in this study for most of the tested saponins, the cytosolic liberation of the RIP-I must underlie a different mechanism. Weng et al. recently reported that saponins mediate the endosomal/lysosomal escape of RIP-I after internalization. While the internalization happens autonomously without saponins, most of the RIP-I then stay trapped in the vesicles and are prone to degradation (Weng et al., 2012b). If applied in combination, certain oleanane-type saponins ($M_r > 1600$ g/mol) can bind to internalized saporin (and possibly other RIP-I) in a pH-dependent manner supported by an acidic pH in endosomes/lysosomes (Weng et al., 2012b). The bound saponins then facilitate the passage of the RIP-I through the vesicle's membrane and the liberation into the cytosol. But the nature of the bond still remains unclear. Hebestreit et al. (2006) supposed hydrogen bonds between sugars and the RIP-I, but also Schiff base formation between the aldehyde function of the aglycon and specific amino acids like lysine of the RIP-I. The saponins of Cat. 7–11 possess an aldehyde function in the aglycon and show the highest synergistic cytotoxicity. But other saponins in Cat. 1–6 bear aldehyde functions too and revealed no synergistic cytotoxicity. However the saponins of Cat. 12 and 13 do not have an aldehyde function, they synergistically enhanced the cytotoxicity of saporin. Hence the formation of Schiff bases between saponins and RIP-I as sole mechanism for the enhancement of toxicity is not convincing.

The length of sugar chains seems to be a more relevant structural feature of a saponin to augment a RIP-I's cytotoxicity. In our study saponins with six or more sugar molecules in their structure turned out to be the most potent with a maximum at seven to eight sugar molecules in total. That means those saponins exhibited a low non-synergistic cytotoxicity ($IC_{50} > 10$ μg/mL) and a high synergistic cytotoxicity ($IC_{50} < 5$ μg/mL) with saporin. Due to their amphiphilic properties saponins can interfere with the cell membrane-cholesterol and phospholipids. Saponins are known to complex the membrane-cholesterol (Schlösser, 1969). This aggregation is strongly dependent on the saponin structure (Gestetner et al., 1972; Hu et al., 1996). According to Wang et al. (2007), Hu et al. (1996) and Gauthier et al. (2009) and their colleagues, sugars like glucuronic acid (GlcA), galactose (Gal) and arabinose (Ara) which is an epimer of xylose (Xyl) support this behavior most. Furthermore, the complexity and the length of the sugar chains are important for the complexion of membrane-cholesterol (Nishikawa et al., 1984). Hydrophobic residues in these parts of the saponin can further enhance this effect (Hu et al., 1996).

Taking these facts together, it becomes obvious that saponins, which enhance the cytotoxicity of RIP-I have to hold a certain number of sugars and whose alignment has to be branched for the most potent ones. Moreover, the frequent occurrence of GlcA, Gal, Xyl and Ara in these saponins might affirm the prediction of Wang et al. (2007), Hu et al. (1996) and Gauthier et al. (2009), that these sugars support the complexion of membrane-cholesterol most. Additionally, acetyl residues, methoxycinnamic acid (MCA) and short fatty acid residues can amplify the hydrophobicity and further increase a saponin's influence on the membrane and the cholesterol therein. In our study the two saponins that enhanced the cytotoxicity of saporin most (see Cat. 7 and 9), expressed in IC_{50}(syn.) < 1 μg/mL and $IC_{50} > 10$ μg/mL, consisted of: gypsogenin; three branched sugars at C-3–OH-group containing GlcA, Gal, Xyl; at the C-28–OH-group three/four branched sugars and a methoxycinnamic acid/acetyl residue.

Although the described structural features are decisive for the development of synergistic cytotoxicity between saponins and RIP-I, their part in the structure over a certain extent will not lead to stronger enhancement of the RIP-I cytotoxicity. An increased non-synergistic cytotoxicity due to the damage of the cell membrane will be the result. This can be observed for saponins from Cat. 10 and 11.

Besides the pH-dependent mechanism that binds appropriate saponins to RIP-I in endosomes/lysosomes, it is thinkable that the differences in the concentrations of saponins, that lead to synergistic as well as non-synergistic cytotoxicity, are based on differences in the extent of membrane perturbation they cause. Differences between endosomal/lysosomal membranes and cytosolic membranes and differences in the repair mechanisms for these membranes might also contribute the development of the synergistic cytotoxicity. It is very likely that the damage that saponins cause to the cytosolic membrane is quickly repaired by the cell unless a certain degree is not exceeded (Gilabert-Oriol et al., 2013). Presumably these repair mechanisms do not, or to a lesser extent, work for endosomal/lysosomal membranes. This might explain the lower required concentrations of certain saponins to liberate the RIP-I from the endosomes/lysosomes. It might also explain why only higher concentrations of these saponins lead to non-synergistic cytotoxicity. This underlines the importance of the saponin structure. Regarding a therapeutic use, the potent saponins in this study had significant effects on the endosomal/lysosomal membrane and a substantial

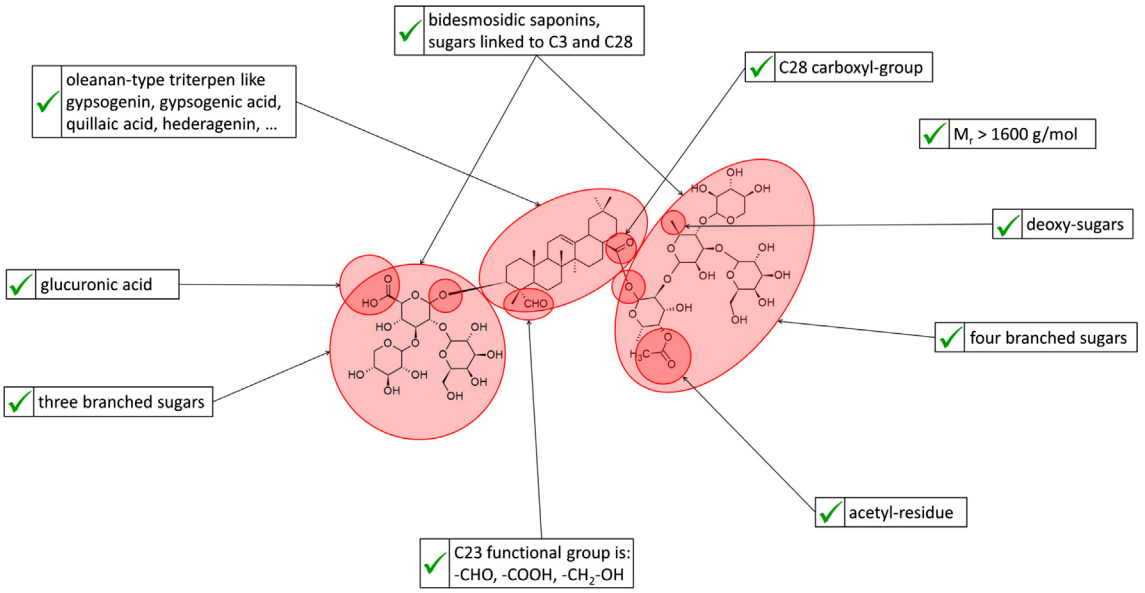

Fig. 2. Structural features of an ideal saponin. An arrangement of structural features that, concluding the results of this investigation, an ideal saponin should hold to exhibit a minimal non-synergistic and a maximal synergistic cytotoxicity in combination with a RIP-I.

binding to saporin resulting in its cytosolic liberation (Weng et al., 2012b). Furthermore, these saponins did not cause severe damage to the cell membrane or at least did not overstress the repair mechanisms. Fig. 2 illustrates which structural features an ideal saponin should hold.

5. Conclusion

56 saponins were analyzed for their non-synergistic cytotoxicity as well as their capability to enhance the toxicity of the RIP-I saporin synergistically. The saponins' structural features were compared with the results of the cytotoxicity assays to get a deeper insight into the structure–activity relationships (SAR). An ideal saponin should exhibit a low cytotoxicity in general, but enables the enhancement of RIP-I toxicity in a highly synergistic manner. According to our findings an ideal saponin is characterized by the following structural features:

- aldehyde at C-4
- a branched trisaccharide at C-3 containing a glucuronic acid
- a preferably branched and partially esterified glycoside at C-28 comprising at least four sugars including desoxy-sugars like fucose and/or rhamnose

Conflict of interest

The authors declare to be in no conflicts of interest.

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