The Saponin-Mediated Enhanced Uptake of Targeted Saporin-Based Drugs Is Strongly Dependent on the Saponin Structure

CHRISTOPHER BACHRAN,* MARK SUTHERLAND,*[1] IRING HEISLER,* PHILIPP HEBESTREIT,†[2] MATTHIAS F. MELZIG,† AND HENDRIK FUCHS*[3]

*Institut für Klinische Chemie und Pathobiochemie, Charité–Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany; and †Institut für Pharmazie–Pharmazeutische Biologie, Freie Universität Berlin, Königin-Luise-Str. 2+4, D-14195 Berlin, Germany

Saponins are a group of plant glycosides consisting of a steroid or triterpenoid aglycone to which one or more sugar chains are attached. They exhibit cell membrane–permeabilizing properties and, thus, have been investigated for their therapeutic potential. Recently, at a nonpermeabilizing concentration saponinum album from *Gypsophila paniculata* L. has been described to enhance the cytotoxicity of a chimeric toxin in a cell culture model. To elucidate whether this enhancing effect is also mediated by other saponins, we analyzed the ability of seven different saponins to enhance the cytotoxicity of a targeted chimeric toxin. The chimeric toxin is composed of saporin, a plant ribosome-inactivating toxin, a cleavable adapter, and human epidermal growth factor (EGF). Cytotoxicity on EGF receptor (EGFR)-bearing cells was analyzed both alone and after combined application of saponin and chimeric toxin. Only two of the tested saponins, quillajasaponin and saponinum album, enhanced cytotoxicity by more than 1000-fold, whereas the enhancement factors of the other saponins were only approximately 10-fold. In contrast to saponinum album, quillajasaponin enhanced the cytotoxicity both on control cells lacking EGFR and on target cells, indicating that, in this case, the enhancement is not target cell receptor specific. This is also the case for some of the saponins with low enhancement factors. Saponinum album resulted in a more than 13,600-fold receptor-specific enhancement, decreasing the 50% inhibitory concentration (IC_{50}) from 2.4 n*M* to 0.18 p*M*, which renders it the best option to promote saporin-3–based drug uptake while retaining specificity for the EGFR. Exp Biol Med 231:412–420, 2006

Key words: immunotoxin; chimeric toxin; saporin; saponin; triterpenoids; adjuvants

Introduction

Saponins are a highly diverse group of glycosides of plant origin. They contain either a steroidal or a triterpenoid aglycone to which one or more sugar chains are attached. The sugars are usually glucose, galactose, glucuronic acid, xylose, or rhamnose. The diversity of saponins is a result of the variability in the aglycone structure, the sugar side chains, and the composition of these side chains. Although saponins have been examined in many applications, especially in medicine, their natural role in plants is still a matter of discussion. The main biologic activity ascribed to saponins is their membrane permeabilizing property (1, 2). The main actions are considered changes in membrane permeability and pore formation (3, 4). Saponins are also hemolytic, probably as a result of their interactions with steroids, especially cholesterol (5). The amount of cholesterol in the membrane has been shown to be important for this interaction (6). However, in contrast, Segal *et al.* (7) demonstrated that the absence of cholesterol in membranes does not inhibit pore formation by some saponins. The sugar side chains of saponins also affect activity. The number of side chains influences both hemolytic activity and membrane permeability. Woldemichael *et al.* (8) reported that saponins possessing two side chains induce less activity than saponins containing one sugar. Contrary to this observation, Yamasaki *et al.* (9) showed that increasing the amount of sugar side chains increased the membrane permeability for calcium ions. Taking all information into consideration, the permeabilizing effect may be caused by the combination of target membrane composition, the type of the saponin side chain(s) and the nature of the aglycone (10).

Saponins have additionally been reported to exhibit adjuvant-active properties (11; reviewed in Ref. 12). Based on these observations, an open cage-like immunostimulating complex (ISCOM) of cholesterol, lipid, immunogen, and saponins from the bark of *Quillaja saponaria* Mol. (soap bark tree) has found successful application as an active

[1] Current address: University of Bradford, The Institute of Cancer Therapeutics, BD7 1DP Bradford, West Yorkshire, United Kingdom.
[2] Current address: BASF Aktiengesellschaft, 67056 Ludwigshafen, Germany.
[3] To whom correspondence should be addressed at Institut für Klinische Chemie und Pathobiochemie, Charité–Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany. E-mail: hendrik.fuchs@charite.de

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adjuvant for vaccination (13). Furthermore, formosanin-C, a diosgenin saponin from *Paris formosana*, increases the natural immune defense against malignant cells by activating natural killer cells (14). Saponins have also been shown to inhibit tumor cell growth in mouse models (15, 16). Interestingly, Nakata *et al.* (17) showed that a ginsenoside alone had virtually no effect on tumor growth, but when combined with *cis*-diaminedichloroplatinum(II), significantly inhibited growth. The combined application of saponins with other antitumor drugs, thus, offers an interesting development in cancer treatment. In nature, the occurrence of a synergistic mechanism between a saponin and a cytotoxic compound was recently described. The toxicity of the seeds from *Agrostemma githago* L. was always thought to be caused by the saponin content, however, this is rather caused by the combined action of saponin and agrostin, a Type I ribosome-inactivating protein (18).

With these thoughts in mind, we recently examined the combination of saponin and a targeted chimeric toxin (19) composed of human epidermal growth factor (EGF) and saporin linked *via* a cleavable adapter (19). The combination of saponinum album from *Gypsophila paniculata* L. at a nonpermeabilizing concentration and chimeric toxin enhanced cytotoxicity in an EGF receptor (EGFR) tumor model (20). Furthermore, the highest increase in cytotoxicity was observed on EGFR-expressing cells. This synergistic enhancement of cytotoxicity is both receptor- and ligand-specific. Based on these observations, we analyzed six more saponins from diverse plants and with different structures for their ability to increase the cytotoxicity of chimeric toxins.

The selection of these six saponins should allow, based on their distinct structural properties, conclusions to be drawn regarding the minimal structural elements required for an enhanced uptake of saporin-based drugs. Hederasaponin C and glycyrrhizic acid do not contain a formyl group on C4 nor an acidic branched trisaccharide on C3. Helianthoside 2 shows a branched trisaccharide on C3 but lacks the formyl group on C4. In addition to the branched trisaccharide on C3, β-aescin contains a hydroxymethyl group on C4. The main saponins from saponinum album (gypsoside A) and quillajasaponin (QS-21 and QS-18) show both important structural elements, namely the formyl group at C4 and the branched sugar chains on C3. They differ in the monosaccharide composition of the sugar chain on C3 and C28, and the aglycone of quillajasaponin possesses an additional hydroxyl group in position C16.

The objectives of this investigation were to clarify whether saponins, in general, are able to enhance the receptor- and ligand-specific cytotoxicity of targeted saporin-based drugs, and, based on these results, to determine the basic structural requirements needed for a saponin to enhance uptake.

Materials and Methods

Plasmid Construction and Chimeric Toxin Expression. All primers and oligonucleotides were purchased from Metabion (Martinsried, Germany). The DNA of the chimeric toxin was constructed in pLitmus28 (NEB, Frankfurt am Main, Germany) and transcribed from a pET11d expression vector (Novagen, Madison, WI), as previously described in detail (20). The chimeric saporin-adapter-EGF (SA2E) used in this study is the same as SapAd*EGF in Heisler *et al.* (19) and SA2E in Heisler *et al.* (20), except for the introduction of an N-terminal 10× His-tag. Saporin-3 (Sap-3) complementary DNA (cDNA) was generously provided by Serena Fabbrini, San Raffaele Scientific Institute, Milano, Italy. The basic structure of SA2E is His–Sap-3–adapter–human EGF. The adapter is composed of a cytosolic cleavable peptide (YVH-DEVDRGP) containing caspase cleavage sites and a yeast recognition sequence for cytosolic cleavage; a protein transduction domain (PLSSIFSRIGDP), derived from the PreS2-domain of hepatitis B virus surface antigen (21); and an endosomal cleavable peptide (RHRQPRGNRVGRS) containing the *Pseudomonas* exotoxin and a modified diphtheria toxin cleavage site. As a control for ligand-mediated specificity, His-tagged Sap-3 without adapter and ligand was used.

For expression, plasmid DNA was transformed into the *Escherichia coli* strain, Rosetta DE3 pLysS (Novagen). Transformed cells were grown overnight in LB medium supplemented with 50 μg/ml ampicillin. The overnight culture was used to inoculate 1 liter of LB medium containing 50 μg/ml ampicillin and grown to $A_{600} = 0.5–0.6$. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 m*M* and the culture further incubated for 3 hrs. Cells were harvested by centrifugation (10 mins, 4°C, 4000 *g*) and the culture pellets were resuspended in phosphate-buffered saline (PBS), pH 8.0, supplemented with 0.2% Triton X-100 and stored at –20°C.

Purification of the Chimeric Toxin and Testing of the Enzymatic Activity. Sap-3 and SA2E were purified by nickel-nitrilotriacetic acid chromatography according to the manufacturers' recommendations (Qiagen, Hilden, Germany). Briefly, the resuspended cells were thawed, sonicated on ice five times for 20 secs (Branson Sonopuls, microtip SH213G, duty cycle 20%) with 20-sec breaks, and centrifuged at 16,100 *g* for 30 mins at 4°C. The supernatant was subjected to affinity chromatography on a pre-equilibrated nickel-nitrilotriacetic acid column. The toxins were eluted using 250 m*M* imidazole and the corresponding fractions were dialyzed against PBS. The concentration of purified protein was estimated using Advanced Protein Assay Kit (Cytoskeleton Inc., Denver, CO). The quantity of the full-length toxins was corrected after analyses by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE; 12% [w/v] gel) under reducing conditions, using protein standards. The final

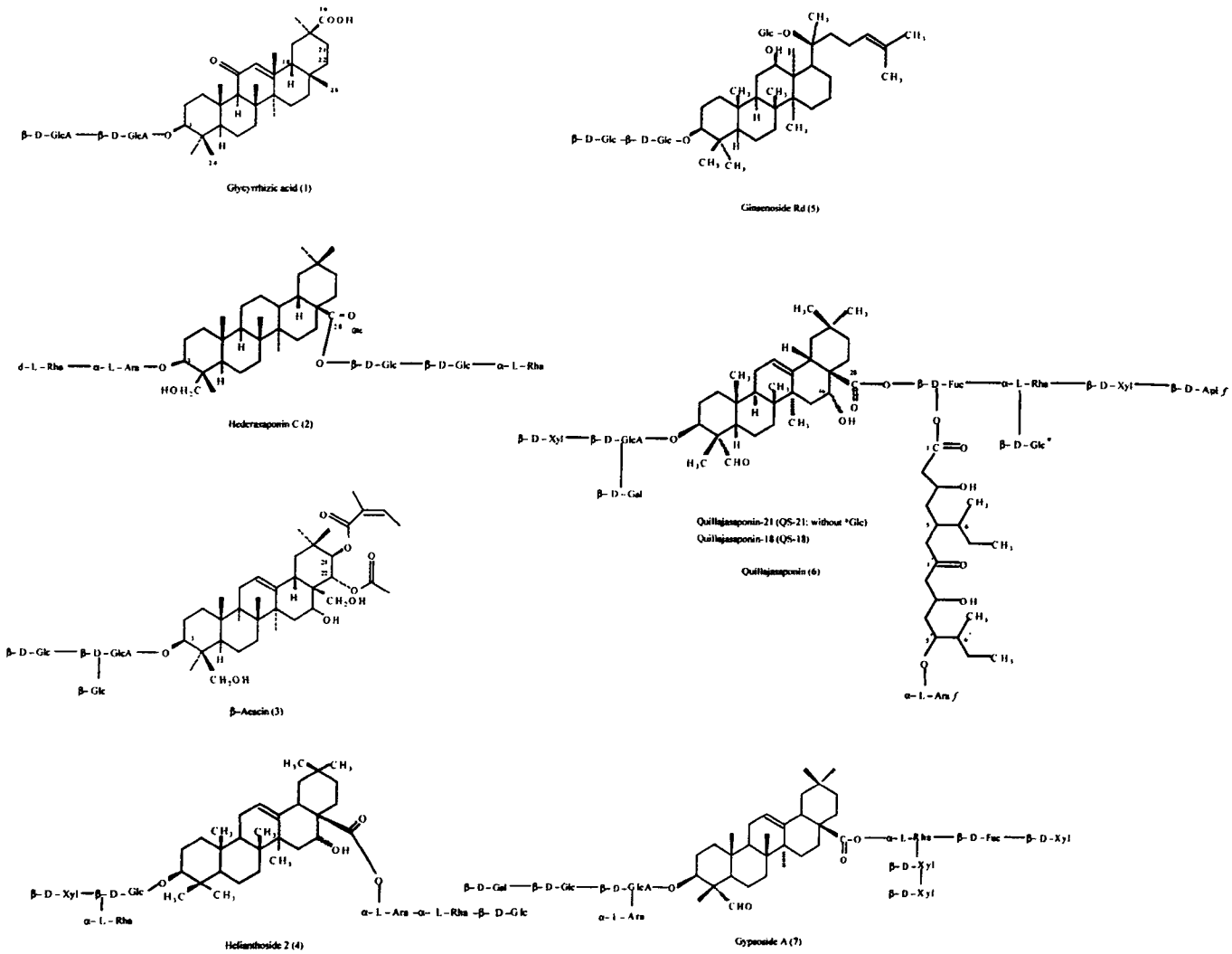

Figure 1. Structures of the saponins (according to Table 1).

materials were estimated to be 95% pure, as evaluated by Coomassie staining after SDS-PAGE.

Saponins. Saponinum album (*Gypsophila paniculata* L.) and β-aescin (*Aesculus hippocastanum* L.) were obtained from Merck (Darmstadt, Germany), quillajasaponin (*Quillaja saponaria* Mol.), hederasaponin C (*Hedera helix* L.), ginsenoside Rd (*Panax ginseng* C.A. Mey.), and glycyrrhizic acid (*Glycyrrhiza glabra* L.) were purchased from Roth (Karlsruhe, Germany). Helianthoside 2 was isolated from *Helianthus annuus* L. and characterized by Dr. G. Bader (Berlin, Germany), according to Hiegemann *et al.* (22; Fig. 1 and Table 1).

Cell Culture Experiments. Cell culture experiments were performed with untransfected Swiss mouse embryo NIH-3T3 cells (obtained from DSMZ, the German Collection of Microorganisms and Cell Cultures) as the control, and NIH-3T3 cells transfected with human EGFR, referred to as HER14 (a kind gift from Professor E. J. van Zoelen, Department of Cell Biology, University of Nijmegen, The Netherlands). The HER14 cells express approximately 4×10^5 EGFR molecules per cell. Cells were maintained in Dulbecco's modified Eagle's medium with Glutamax I (Invitrogen/Gibco, Karlsruhe, Germany) supplemented with 10% fetal calf serum (BioChrom KG, Berlin, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were cultivated at 37°C, 5% CO_2, and 95% humidity.

Cytotoxicity Assay. For the determination of the dose-response curves, the cells were trypsinized and seeded

Table 1. Plant Sources of the Saponins Used

Saponin	Plant Source	Formula no. in Figure 1
Glycyrrhizic acid	*Glycyrrhiza glabra* L.	1
Hederasaponin C	*Hedera helix* L.	2
β-Aescin	*Aesculus hippocastanum* L.	3
Helianthoside 2	*Helianthus annuus* L.	4
Ginsenoside Rd	*Panax ginseng* C.A. Mey.	5
Quillajasaponin	*Quillaja saponaria* Mol.	6
Saponinum album	*Gypsophila paniculata* L.	7

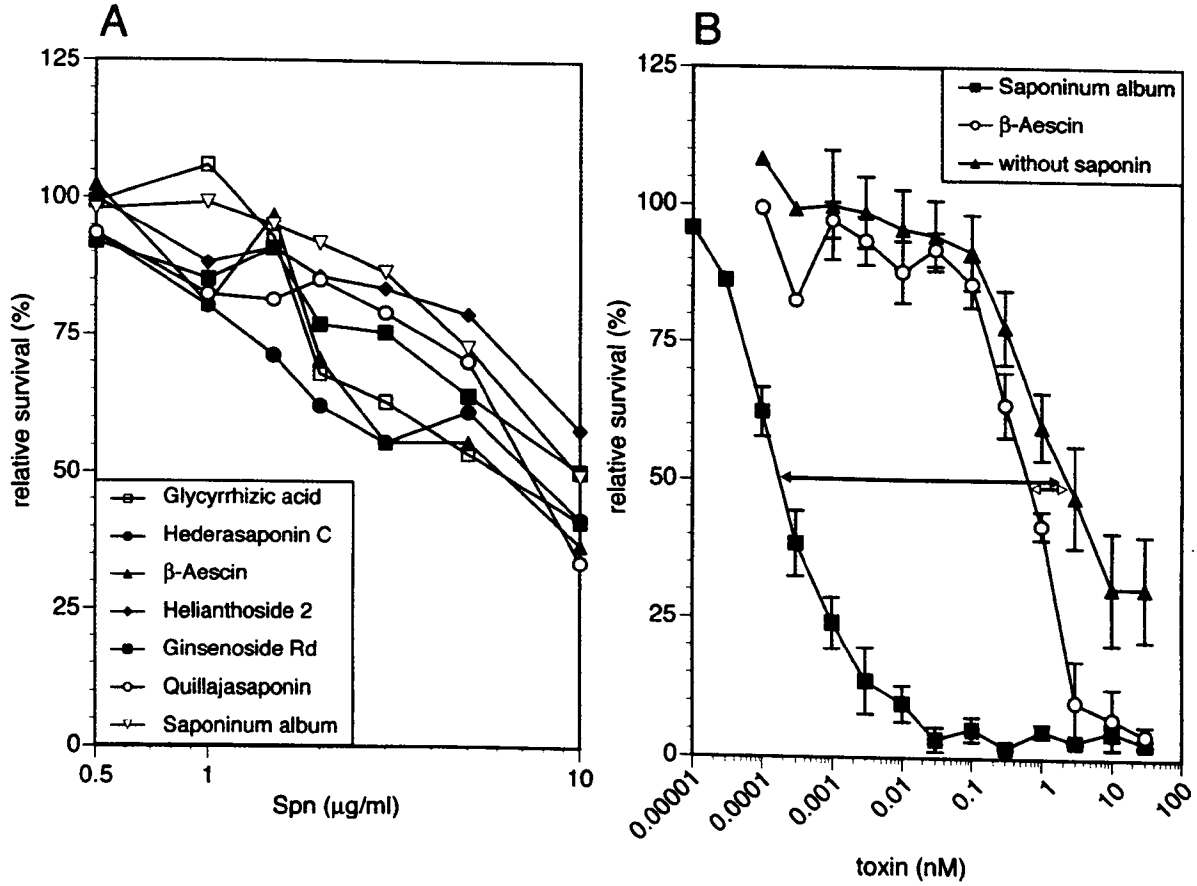

Figure 2. (A) Cytotoxicity of saponins alone. HER14 cells were incubated for 48 hrs in the presence of varying concentrations of the different saponins. Living cells were quantitated by their ability to cleave fluorescein diacetate. Relative survival was calculated as the number of living cells after treatment in relation to untreated cells. Error bars have been omitted for clarity ($n = 4$; average SEM, 6.29%). (B) Receptor-dependent cytotoxicity of the saponins. Relative survival of HER14 cells, expressing human EGFR, after a 48-hr incubation with varying concentrations of SA2E without saponin (triangle), β-aescin (circles), and saponinum album (squares). Data are the mean ± SEM of four independent experiments.

in 96-well plates (pretreated with 0.1% gelatin in water) at a concentration of 2000 cells per well and left untreated for 16 hrs. To determine the toxicity of the saponins (glycyrrhizic acid, hederasaponin C, β-aescin, helianthoside 2, ginsenoside Rd, quillajasaponin, and saponinum album) alone, the cells were washed once with PBS, and 180 µl medium was added. After a 5-min incubation, 20 µl of saponin at different final concentrations (0.5–10 µg/ml) in PBS was added. After 48 hrs, the cytotoxicity was determined.

To determine the synergistic cytotoxic effect, 180 µl medium containing 1.5 µg/ml saponin was added to PBS-washed cells and incubated for 5 mins. Thereafter, 20 µl Sap-3 or SA2E was added and the cells cultivated for a further 48 hrs. The defined final concentrations of the toxins were generally in the range of 10^{-14} to 10^{-7} *M*.

The cytotoxicity assay is based on the cleavage of fluorescein diacetate by living cells and was performed as described by Nygren *et al.* (23). After incubation of the cells with either saponin alone or together with the toxins, cells were washed twice with PBS and incubated with fluorescein diacetate (10 µg/ml; Sigma, St. Louis, MO) for 1 hr. Fluorescence was measured using a microplate reader (Spectra Max Gemini, Molecular Devices, Ismaning, Germany) with an excitation and emission λ of 485 nm and 538 nm, respectively. The survival index was calculated as the percentage of living cells in treated wells in relation to untreated cells (for saponin toxicity alone, cells with medium served as untreated cells; for the synergistic effect, cells without toxin but with one of the saponins served as untreated cells).

Results

Determination of Saponin Cytotoxicity on Cells. The cytotoxicity of the seven saponins was analyzed in the concentration range from 0.5 to 10 µg/ml on HER14 cells. The resulting relative survival of the cells was approximately 100% at 0.5 µg/ml and ranged from 34% (quillajasaponin) to 57% (helianthoside 2) at the highest saponin concentration (10 µg/ml) applied (Fig. 2A). At the intermediate concentration of 1.5 µg/ml, the relative survival of the cells was greater than 90%, with the exceptions of hederasaponin C and quillajasaponin, in

which only 71% and 82% of cells survived, respectively. Analyses on NIH-3T3 cells at 1.5 μg/ml saponin revealed similar values to HER14 cells, with more than 90% relative survival (except for hederasaponin C and quillajasaponin, which each had 87% survival). The cytotoxic effect of the saponins at higher concentrations, however, was lower than with HER14 cells. At 10 μg/ml, a relative survival greater than 70% was measured for all saponins (data not shown). Because treatment with 1.5 μg/ml saponin resulted only in minor influences on cell survival, this concentration was used for all further experiments to determine the effect of combined application of saponin and chimeric toxins. Quillajasaponin and hederasaponin C exhibited slightly reduced cell survival, but for reasons of comparability, the same concentrations of these saponins were used for further experiments. These results correlate with our recent publication regarding the combined application of saponinum album and chimeric toxins, wherein a concentration of 1.5 μg/ml saponin was optimal to enhance the specific toxicity of SA2E (20).

Analysis of the Synergistic Effect of Saponins and Chimeric Toxins on Cytotoxicity. The purification, testing of the enzymatic activity, and determination of dose-response curves is well established for the chimeric toxins (19, 24). To induce enhanced cytotoxicity, HER14 and NIH-3T3 cells were preincubated with the different saponins before adding Sap-3 or SA2E. All tested saponins enhanced the cytotoxicity of Sap-3 and SA2E on human EGFR-expressing HER14 cells as well as on the control NIH-3T3 cells, however, the factor of enhancement and the ligand and receptor specificity differed greatly between different saponins. Examples of the toxin concentration-dependent relative survival for HER14 cells treated with SA2E are shown in Figure 2B. The control without saponin preincubation and the curves resulting from preincubation with β-aescin and saponinum album are presented, corresponding to the saponins with the weakest and strongest enhancement of cytotoxicity, respectively. Whereas the 50% inhibitory concentration (IC_{50}) of the control without saponin was 2.4 n*M*, the values after preincubation with saponins ranged from 0.6 n*M* to 0.18 p*M*. Saponinum album and quillajasaponin enhanced the cytotoxicity of SA2E on HER14 cells by 13,647-fold and 1,434-fold, respectively; in contrast, the other tested saponins, including β-aescin, only increased the IC_{50} up to 11.3-fold (Table 2). These results clearly show that saponins, in general, enhance the uptake of saporin-based drugs, although the effects differ greatly. The next question is whether this effect is ligand and receptor specific.

The EGFR-Specific Enhancement of Cytotoxicity by Saponins and Chimeric Toxins. To determine to what extent the observed enhancement factors depend on specific ligand-receptor interactions, the combination was performed on the control NIH-3T3 cells lacking human EGFR. The enhancement factors on NIH-3T3 cells for glycyrrhizic acid, hederasaponin C, β-aescin, and quillajasaponin were similar to those observed for HER14 cells. Helianthoside 2, ginsenoside Rd, and saponinum album exhibited lower enhancement factors for SA2E cytotoxicity (Table 3), indicating ligand-receptor specificity.

The enhancement factors determined in Tables 2 and 3 allow an estimation of the EGFR-dependent influence on the enhanced cytotoxicity mediated by the saponins. The ratios of the enhancement factors (for each saponin on HER14 cells divided by the corresponding enhancement factor on NIH-3T3 cells) for SA2E cytotoxicity allow for better comparability; the ratios are presented in Figure 3A. A ratio of greater than 1 represents an enhancement of receptor-specific cytotoxicity, because the enhancement factor on EGFR-expressing HER14 cells was higher than that on control NIH-3T3 cells. Saponinum album enhanced the cytotoxicity of SA2E on HER14 cells more than 13,600-fold and was, thus, approximately 7-fold more potent compared with NIH-3T3 (1,980-fold), which was the highest ratio observed. Although quillajasaponin exhibited an enhancement factor of approximately 2000-fold, this effect was not receptor-specific, as revealed by the ratio of 0.7. Helianthoside 2 and ginsenoside Rd possessed receptor-specific enhancement ratios of 2.7 and 3, respectively; however, the overall effect was low. The three remaining saponins, glycyrrhizic acid, hederasaponin C, and β-aescin, had ratios lower than 1.6, and the observed effects were,

Table 2. IC_{50} Values and Saponin-Mediated Factors of Enhancement for Sap-3 and SA2E on HER14 Cells in the Absence and Presence of Different Saponins

	Sap-3		SA2E	
Saponin	IC_{50} (n*M*)	Factor of enhancement	IC_{50} (n*M*)	Factor of enhancement
Without saponin	175	—	2.4	—
Glycyrrhizic acid	50.9	3.4	0.4	6.0
Hederasaponin C	24.4	7.2	0.5	4.9
β-Aescin	80.5	2.2	0.6	3.7
Helianthoside 2	55.8	3.1	0.2	10.9
Ginsenoside Rd	70.5	2.5	0.2	11.3
Quillajasaponin	0.17	1009	0.0017	1434
Saponinum album	0.093	1881	0.00018	13,647

Table 3. IC_{50} Values and Saponin-Mediated Factors of Enhancement for Sap-3 and SA2E on NIH-3T3 Cells in the Absence and Presence of Different Saponins

Saponin	Sap-3		SA2E	
	IC_{50} (n*M*)	Factor of enhancement	IC_{50} (n*M*)	Factor of enhancement
Without saponin	83	—	27.2	—
Glycyrrhizic acid	71.7	1.2	4.6	6.0
Hederasaponin C	153.9	0.5	5.8	4.7
β-Aescin	110.4	0.8	11.6	2.4
Helianthoside 2	25.7	3.2	6.6	4.1
Ginsenoside Rd	73.8	1.1	7.3	3.7
Quillajasaponin	1.3	62.4	0.013	2113
Saponinum album	0.5	174.9	0.014	1977

therefore, not explicitly receptor specific. This suggests that the enhanced uptake by saponins is independent of ligand-receptor specificity for these saponins. The presence of EGF as the ligand may also play an important role for the amplifying effects of the saponins.

The Ligand Specificity for Enhancement of Cytotoxicity by Saponins. To analyze the ability of the saponins to enhance ligand-specific cytotoxicity in more detail, the same experiments were carried out with Sap-3 on HER14 and NIH-3T3 cells. Because Sap-3 lacks EGF as the ligand for the EGFR, it was expected, in combination with the saponins, that similar cytotoxicities would be observed on HER14 cells and NIH-3T3 cells. All saponins enhanced the cytotoxicity of Sap-3, nevertheless, the resulting enhancement factors differed slightly between HER14 cells and NIH-3T3 cells (Tables 2 and 3). Again, quillajasaponin and saponinum album had the highest enhancement factors. Although the enhancement factors on HER14 cells were greater than 1000-fold, the corresponding factors on NIH-3T3 cells were only 62.4-fold and 174.9-fold for quillajasaponin and saponinum album, respectively. All other saponins increased Sap-3 cytotoxicity less than 10-fold. Thus, similar to the receptor specificity ratio, the ligand specificity was calculated from the enhancement factors of the saponins for SA2E and Sap-3 on HER14 cells (Fig. 3B). A ratio greater than 1 represents an enhancement of cytotoxicity caused by the presence of EGF as ligand. The calculated ratios mirrored those for the receptor specificity. Saponinum album had the highest ratio, of 7.3, and helianthoside 2 and ginsenoside Rd again had high specificities, with ratios of 3.5 and 4.6, respectively. The other saponins had ratios less than 2, and hederasaponin C

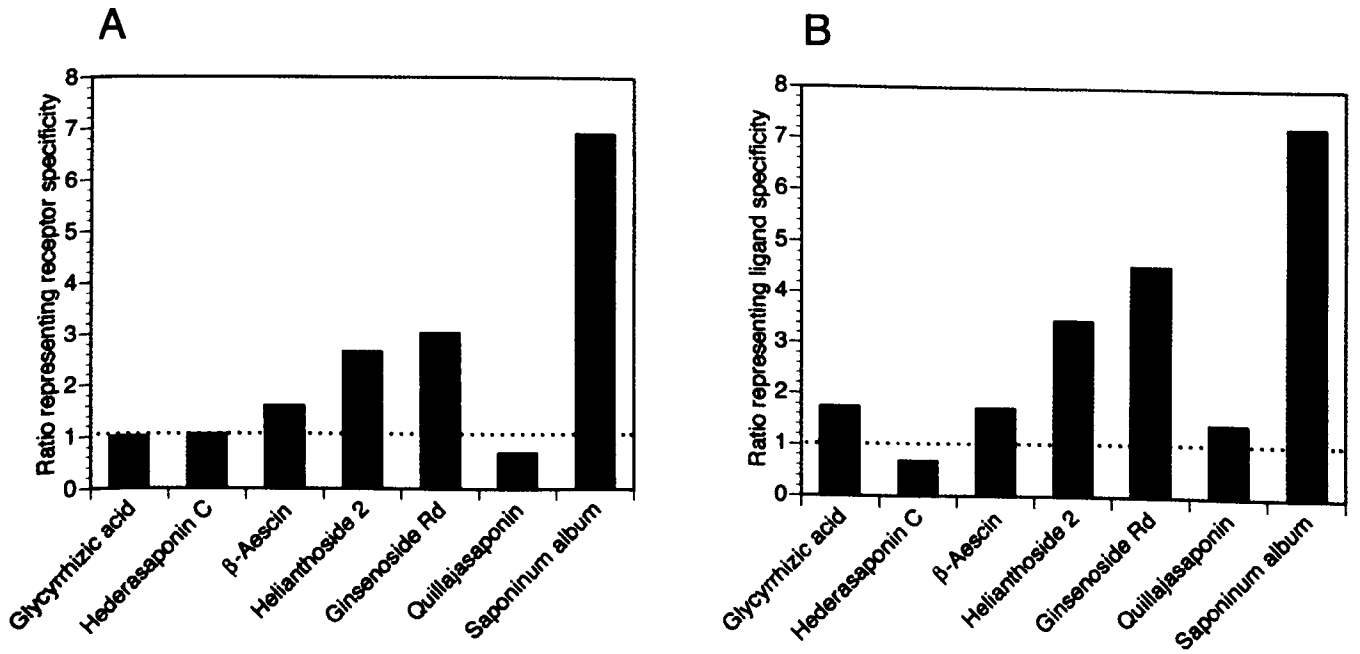

Figure 3. Ratio of cytotoxicity enhancement. (A) Ratio of receptor-dependent enhancement of SA2E cytotoxicity by saponins (saponin enhancement factor of SA2E on HER14 cells divided by saponin enhancement factor of SA2E on NIH-3T3 cells). A ratio greater than 1 (dotted line) indicates higher enhancement factors on HER14 cells compared with NIH-3T3 cells. (B) Ratio of ligand-dependent enhancement of cytotoxicity by saponins on HER14 cells (saponin enhancement factor of SA2E on HER14 cells divided by saponin enhancement factor of Sap-3 on HER14 cells). A ratio greater than 1 (dotted line) means higher enhancement factors for SA2E compared with Sap-3.

had a ratio less than 1, implying a stronger enhancement of the nonspecific Sap-3 cytotoxicity. Because the cell lines and chimeric toxins used were identical, this suggests that other factors, such as saponin structure, are critical in determining the action mediated.

Discussion

The ability of saponins to influence membranes and enhance permeability has been widely used for a variety of applications. The established use of ISCOMs for enhancing the effect of vaccination is one example for their successful application. QuilA from *Quillaja saponaria* Mol. acts as an adjuvant for a variety of viruses (13). The triterpenoid saponin avicin, from *Acacia victoriae*, induces cell cycle arrest in malignant cells (25), whereas other saponins have been shown to induce caspases and concomitant apoptosis (26, 27). Recently, saponins were shown to enhance the membrane penetration of toxins into cells (18).

Saponins are widely examined as possible tumor therapeutics because they result in anticarcinogenic effects (28, 29). However, these effects are only based on the cytotoxicity of the saponins alone. Sim *et al.* (30) described a saponin from the root bark of *Aralia elata* that acts as an efficient adsorption enhancer for chondroitin sulfates. In an EGFR-based tumor model, we clearly showed that Saponinum album exerts an immense enhancing effect on the cytotoxicity of Sap-3–based drugs targeted against the EGFR (20). In contrast to other studies in which the cytotoxic activity of saponins alone was described, saponinum album is able to enhance the cytotoxicity of chimeric toxins at otherwise ineffective concentrations of either the saponin or the toxin alone. Furthermore, this enhancement is higher on cells expressing the target receptor for the chimeric toxin than on cells without the relevant receptor. This broadens the therapeutic window of application for the chimeric toxin. Additionally, because the concentration of the toxin is reduced, this may result in fewer side effects in the context of a desired tumor therapy.

Based on these results, further examinations into this enhancing effect were necessary to better understand the consequences. Because the basic parameters of application were previously optimized (20), these parameters were used for the following experiments for improved comparability. Under these conditions (1.5 μg/ml), the cytotoxicity of the saponins without chimeric toxin on HER14 cells produced no effect for most of the saponins. The increase of cell survival after application of 1.0–1.5 μg/ml β-aescin may be an artifact of the measurements because HER14 cells are very sensitive to the combination therapy. Hederasaponin C and quillajasaponin resulted in 20%–30% cytotoxicity, indicating that these saponins are not ideal candidates for such a combination therapy. This may be because of reversible rearrangements of the membrane, as recently described for quillajasaponin (31), or because of pore formation.

Consecutive intravenous administrations of ginseng saponins, up to 500 μg/mouse/day, did not generate severe side effects and revealed an inhibitory effect on tumor angiogenesis and metastasis (32, 33). Because saponinum album is the most promising candidate for a combination therapy, we have already tested that saponin for possible side effects in mice (unpublished data). For enhanced cytotoxic activity, a dose of approximately 30 μg/20 g mouse, corresponding to an optimal saponin concentration (1.5 μg/ml), should be sufficient and is far below the dose of 500 μg administered by Sato *et al.* (32). Subcutaneous application of 30 and 100 μg saponin did not result in any notable side effects. The dosage needed to induce the desired enhancement of cytotoxicity is not known hitherto, but the presented cell culture experiments indicate that it is clearly lower than 100 μg, corroborating the potential use of saponinum album as an enhancer in targeted tumor therapies.

A closer evaluation of the IC_{50} values of the combination of saponin and toxin clearly reveals the synergistic effect (Fig. 2B). Saponinum album is the only saponin tested in this study that was able to decrease the IC_{50} to less than 1 p*M*. When comparing the nonspecific enhancement on the chimeric toxin in the target receptor-free control cells, again, saponinum album and quillajasaponin reveal the highest factors. However, it is important to note that although both induce similar nonspecific enhancements in NIH-3T3 cells, the effect of saponinum album on target receptor-bearing HER14 cells is approximately 7-fold higher. The saponins helianthoside 2 and ginsenoside Rd also lead to an increase in the receptor-mediated cytotoxicity of SA2E. However, it has to be considered that these saponins only result in a total enhancement factor of 10–12, and are, therefore, not promising candidates for further studies on enhanced drug uptake. A comparison of the saponin-mediated enhancement factors of untargeted Sap-3 with the targeted SA2E reveals the effect of the ligand. Regarding receptor specificity, only saponinum album exerts a ligand-specific amplification on cytotoxicity and, at the same time, producing a relevant enhancement factor. Saponins lacking specificity, such as quillajasaponin, may be considered for other *in vitro* applications, including cell transfection or transduction.

In this study, the effect of the saponins is independent of cell type and composition of the drug; structural components, such as the number of sugar moieties, the composition of the sugar side chains, and the functional groups of the aglycone, seem to be more relevant. Quillajasaponin and saponinum album, which are the most efficient in increasing cytotoxicity, with enhancement factors greater than 1000 on HER14 cells, possess a structurally similar triterpenoid aglycone (Fig. 1). However, the other saponins, except ginsenoside Rd, also possess a triterpenoid aglycone; hence, the overall structure of the triterpenoid aglycone cannot be solely responsible for the enhancement. The role of the sugar side chains may also be

important for the enhancement of cytotoxicity, as has been shown by the loss of permeabilizing properties of a saponin after sugar side-chain hydrolysis (34). Although the saponins used in these experiments exhibit differences in the composition and number of sugar side chains, as well as in the branching, some conclusions can be drawn. The highly active saponins possess branched acidic sugar chains in position C-3 and are bisdesmosidic. These results are in accordance with investigations regarding the enhanced cytotoxicity of agrostin by a variety of saponins, with either gypsogenin or quillajic acid as agylcone, differing only in the sugar side chain at position C-3 (35). Besides the triterpenoid aglycone, gypsoside A from saponinum album and QS-21/QS-18 from quillajasaponin share another common feature, a formyl group in the C-4 position of the aglycone. Helianthoside 2, which possesses the same aglycone as quillajasaponin, except for a methyl group that replaces the formyl group at position 4, fails to induce the enhancing effects. Because this aldehyde has been connected to the adjuvant function of quillajasaponin for vaccination (36) and none of the other triterpenoid saponins in this study possess this group, it supports an important role of the formyl group on the enhancement effects observed. It could be speculated that the formyl group is crucial for interactions with the cell membrane to support the uptake of the toxin into the cell. An interaction with the membrane is likely, because the formyl group is a requirement for the effect of quillajasaponin on effective vaccination. However, it must also be noted that the effect may also be caused by a combination of structural features, including the sugars, and that not only the formyl group plays a critical role. Further investigations and a better understanding of the structural requirements could open the possibility of using saponins in combined tumor therapy.

Analyses with the combination of different saponins and a chimeric toxin were performed to determine the effect of the saponins on enhancement of cytotoxicity. Saponinum album was the only saponin that exerted a strong enhancement and retained the ligand-receptor specificity. Further studies are essential to better understand the role of the different groups within the saponins on their efficacy to enhance uptake.

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