molecular pharmaceutics

ARTICLE

pubs.acs.org/molecularpharmaceutics

The Endocytic Uptake Pathways of Targeted Toxins Are Influenced by Synergistically Acting *Gypsophila* Saponins

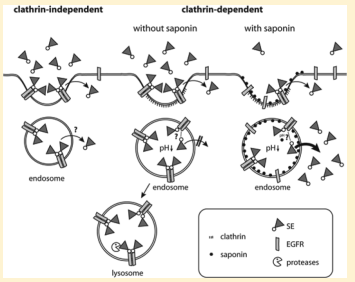
Diana Bachran,[†] Stefanie Schneider,[†] Christopher Bachran,[†,‡] Alexander Weng,[†] Matthias F. Melzig,[§] and Hendrik Fuchs*[,†]

[†]Institut für Laboratoriumsmedizin, Klinische Chemie und Pathobiochemie, Charité — Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany

[§]Institut für Pharmazie-Pharmazeutische Biologie, Freie Universität Berlin, Königin-Luise-Strasse 2+4, D-14195 Berlin, Germany

Supporting Information

ABSTRACT: The expression of the epidermal growth factor (EGF) receptor is upregulated in many human tumors. We developed the targeted toxin SE, consisting of the plant toxin saporin-3 and human EGF. The cytotoxic effect of SE drastically increases in a synergistic manner by a combined treatment with Saponinum album (Spn), a saponin composite from *Gypsophila paniculata* L. Here we analyzed which endocytic pathways are involved in the uptake of SE and which are mandatory for the Spn-mediated enhancement. We treated HER14 cells (NIH-3T3 cells transfected with human EGF receptor) with either chlorpromazine, dynasore, latrunculin A, chloroquine, bafilomycin A1 or filipin and analyzed the effect on the cytotoxicity of SE alone or in combination with Spn. We demonstrated that SE in combination with Spn enters cells via clathrin- and actin-dependent pathways and the acidification of the endosomes after endocytosis is relevant for the cytotoxicity of SE. Notably, our data suggest that SE without Spn follows a different endocytic uptake pathway. SE cytotoxicity is independent of blocking of clathrin or actin, and the decrease in endosomal pH is irrelevant for SE cytotoxicity. Furthermore, Spn has no influence on the retrograde transport. This work is important for the better understanding of the underlying mechanism of Spn-enhanced cytotoxicity and helps to describe the role of Spn better.



KEYWORDS: targeted toxins, endocytosis, dynamin-2, intracellular trafficking, retrograde transport, endosomes, EGFR, saporin, saponins

INTRODUCTION

Endocytosis is a very important feature of eukaryotic cells. It is required for many cell functions, including uptake of nutrients, transcellular transport, control of the density of surface receptors and immune defense. To date many endocytic pathways are identified. They are classified according to the transported compounds as well as to the proteins and structures involved in the endocytic process. Two intensely studied endocytic pathways are the clathrin-dependent[1] and the caveolin-dependent[2] pathway. As shown for the transferrin (Tf) receptor (TfR)[3,4] and the epidermal growth factor (EGF) receptor (EGFR),[5,6] these receptors are ordinarily endocytosed via clathrin-coated vesicles. A number of different endocytic pathways are described in the literature, which can be dependent on or independent of clathrin, caveolin or dynamin-2,[2] and several others not yet identified may exist that do not fit into the known classifications.

Plant and bacterial toxins that target an intracellular process need to enter their target cells. Some toxins, such as the pertussis adenylate cyclase toxin from *Bordetella pertussis*,[7] are able to directly cross the cell membrane;[7−9] others are endocytosed and translocate into the cytosol via endosomal membranes,[10−12] for example diphtheria toxin.[10] A completely different mechanism to enter cells is used by *Pseudomonas* exotoxin A (PE),[13] ricin,[14] or cholera toxin.[15] After binding to their receptors on the cell surface they are retrogradely transported to the endoplasmic reticulum before finally entering the cytosol.[16,17] PE binds to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein,[18] ADP-ribosylates the eukaryotic elongation factor 2 after endocytosis and thus inhibits protein synthesis.

Targeted toxins consist of a cell-targeting moiety and a cytotoxic compound, chemically coupled or expressed as recombinant proteins. Radioactive substances, cytotoxic small molecules, or natural toxins are used as cytotoxic compound and delivered to the target cells by antibodies, antibody fragments or natural ligands of tumor-associated antigens. A couple of targeted toxins were already approved for the treatment of cancer (reviewed in ref 19) including Ontak, Zevalin, Bexxar and Mylotarg; however, Mylotarg was withdrawn from the market in 2010 due to missing improvement in clinical benefit and a greater number of deaths compared to chemotherapy.[20] This demonstrates the great importance of knowing the detailed molecular

Received: March 15, 2011
Accepted: October 9, 2011
Revised: August 25, 2011
Published: October 10, 2011

mechanisms of a drug for the development of new treatment strategies and better understanding of targeted toxins including those currently under investigation in clinical trials.[21] Many of these toxins brought promising results for the treatment of cancer, but in some cases the uptake into the target cells is ineffective and nonspecific cytotoxicity often results in side effects, such as vascular leak syndrome and hypersensitivity reactions.[22,23]

A recently published approach to decrease side effects demonstrates that a saponin composite from *Gypsophila paniculata* L. (designated as Saponinum album, Spn) synergistically enhances the cytotoxicity of different targeted toxins up to more than 100,000-fold in cell culture experiments.[24−26] In BALB/c mice bearing a solid tumor, this highly synergistic effect resulted in 94% tumor volume reduction at a 50-fold lower concentration of the targeted toxin compared to treatment without Spn.[27] Notably, both single substances, the targeted toxins and Spn, were applied at nontoxic and nonpermeabilizing concentrations. Saponins are mostly plant-derived glycosides and contain either a steroidal or triterpenoidal aglycon to which one or more sugar chains are attached. Saponins exhibit many different functions: they permeabilize cell membranes, and they have hemolytic, anticancer and antimicrobial effects (reviewed in ref 28).

In this study, we used the targeted toxin SE, consisting of the plant toxin saporin isoform 3 (Sap-3) as cytotoxic moiety and human EGF to target tumor cells. Sap-3 is a type I ribosome-inactivating protein with an N-glycosidase activity. SE binds to the EGFR and is internalized into the cells, where Sap-3 inhibits protein synthesis. However, the delivery pathway for SE, especially in combination with Spn to enhance its cytotoxicity, is not known. Here, we reveal which processes are necessary for the impact of Spn.

EXPERIMENTAL SECTION

Expression and Purification of the Targeted Toxin. The construction of the plasmid for the expression of the targeted toxin SE was described previously[29] (SE was referred to as SapEGF in this publication). SE consists of an N-terminal 6 × His-tag, the plant toxin Sap-3 (as defined in ref 30) and human EGF as tumor-specific ligand. The expression was performed for 3 h at 37 °C in an isopropyl β-D-thiogalactopyranoside-inducible *Escherichia coli* system as described before.[31] Afterward cells were harvested by centrifugation (10 min, 4 °C, 5000*g*), and culture pellets were resuspended in PBS (150 mM NaCl, 8.3 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4) supplemented with 20 mM imidazole and stored at −20 °C. SE was purified under native conditions by nickel−nitrilotriacetic acid agarose affinity chromatography as described in detail in ref 31. Fractions were dialyzed against PBS. The final materials were estimated to be >90% pure as evaluated by Coomassie staining after SDS−PAGE. The concentration of purified proteins was first estimated using the Advanced Protein Assay Kit (Cytoskeleton, Denver, CO, USA). The quantity of full-length toxins was then corrected after analyses by SDS−PAGE (12% gel) under reducing conditions by comparison to protein standards.

Cell Culture. Cell culture experiments were performed with the human cervical carcinoma cell line HeLa (obtained from ATCC, The American Type Culture Collection, Manassas, VA, USA) and HER14 cells (Swiss mouse embryo cells transfected with human EGFR, obtained from Professor E. J. van Zoelen, Department of Cell Biology, University of Nijmegen, The Netherlands). Both cell lines were maintained in Dulbecco's modified Eagle's medium with Glutamax-1 (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin. For HER14 cells, the cell culture dishes were pretreated with 0.1% gelatin in water. All cells were cultivated at 37 °C, 5% CO_2 and 95% humidity.

Cytotoxicity Assay. To generate dose response curves with or without inhibitors influencing the uptake of SE, cells were seeded in 96-well plates at concentrations of 10,000 (HeLa) or 2000 (HER14, NIH-3T3) cells per well in 100 μL of culture medium. After 24 h the conditioned medium was replaced with 180 μL of fresh medium and 20 μL of SE or Sap-3 in PBS was added to adjust the desired toxin concentrations on the cells. To investigate synergistic effects between SE or Sap-3 and Spn, 180 μL of medium containing 1.5 μg/mL Spn (Merck, Darmstadt, Germany, and further characterized in ref 32) was preincubated for 5 min before the toxin was added accordingly. Cells were cultivated for 48 h as described above, and relative survival was measured by a cytotoxicity assay based on the cleavage of fluorescein diacetate by living cells.[31] The cells were washed twice with PBS and incubated for 1 h with 200 μL of fluorescein diacetate (10 μg/mL; Sigma Aldrich, Munich, Germany) in PBS. Developing fluorescence was measured in a microplate reader (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA, USA) at excitation and emission wavelengths of 485 and 538 nm, respectively. The relative cell survival, designated as survival index, was calculated after blank subtraction (wells without cells) as the percentage of living cells in treated wells in relation to untreated cells (cells without toxin). To inhibit endocytosis, HER14 or NIH-3T3 cells were preincubated for 1 h with 10 μM chlorpromazine (Sigma Aldrich, Munich, Germany), 100 μM dynasore (Sigma Aldrich, Munich, Germany), 60 nM latrunculin A (Biomol, Hamburg, Germany), 3 μM chloroquine (Sigma Aldrich, Munich, Germany), 3 nM bafilomycin A1 (Alexis, Lörrach, Germany) or 3 μM filipin complex (Sigma Aldrich, Munich, Germany) before SE was added. In the case of chlorpromazine and dynasore the medium with the inhibitors was replaced by 180 μL of fresh medium before starting the incubation with SE or Spn/SE. All inhibitory agents were used at nontoxic concentrations, as determined by cytotoxicity studies. In addition to the use of dynasore the influence of dynamin-2 on endocytosis of SE was investigated by a dominant negative mutant: HeLa cells were stably transfected with the K44A dynamin-2 mutant (a gift of Sandra L. Schmid; Scripps Research Institute, La Jolla, CA), designated as HeLaΔ-Dyn, and cultivated in the presence of 1 mg/mL Geneticin. To investigate whether the enhancing ability of Spn is restricted to clathrin-dependent uptake mechanisms, HER14 cells were incubated with Spn/SE in the presence of free human EGF (2.5 nM or 15 ng/mL), which induces clathrin-independent endocytosis of EGF in HeLa cells.[33] For the analysis of retrograde transport, HER14 cells were incubated with PE (Sigma Aldrich, Munich, Germany) instead of SE with or without Spn as described. The effect of different incubation times of either SE or Spn/SE was analyzed on HER14 cells. Cells were incubated for 0.5, 6, 24, and 48 h with SE or Spn/SE. In the first three samples, the medium was replaced with 200 μL of fresh medium and the cells were incubated for the remaining time to complete the period of 48 h; cytotoxicity was then measured as above.

Flow Cytometry. The different inhibitors were validated by flow cytometry. HeLa cells were removed from cell culture plates with PBS supplemented with 2.3 mM EDTA, blocked with PBS containing 0.2% fetal calf serum and incubated for 30 min at 4 °C

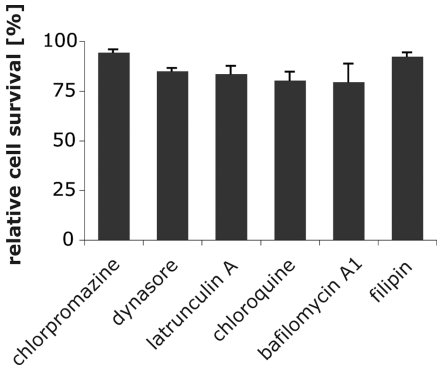


Figure 1. Cytotoxicity of the inhibiting substances applied in subsequent experiments. HER14 cells were on one side incubated with 10 μM chlorpromazine or 100 μM dynasore for 1 h before the medium was replaced with fresh medium and cells were further incubated for 48 h, or otherwise with 60 nM latrunculin A, 3 μM chloroquine, 3 nM bafilomycin A1 or 3 μM filipin for 48 h without replacement of the medium. Living cells were quantitated by their ability to cleave fluorescein diacetate. Relative survival was calculated as the percentage of living cells after treatment in relation to untreated cells. Error bars indicate SEM of up to 8 experiments ($N_{chlorpromazine}$ = 3, $N_{dynasore}$ = 2, $N_{latrunculinA}$ = 5, $N_{chloroquine}$ = 5, $N_{bafilomycinA1}$ = 8, $N_{filipin}$ = 5) performed in triplicate.

with either blocking solution or fluorescein isothiocyanate-labeled transferrin (Tf-FITC, 4 ng/μL) in blocking solution. After a subsequent incubation at 37 °C for 15 min, the cells were washed twice with ice-cold PBS and fluorescence was analyzed by flow cytometry (EPICS-XL from Coulter, Krefeld, Germany). The data were evaluated using the Cyflogic software 1.2.1. Cells were stained with propidium iodide to identify cell debris and dead cells and excluded from unstained cells for analyses by gating. To solely include internalized dye and exclude Tf-FITC located on the cell surface, cells were incubated for two minutes at 37 °C with a trypan blue quench solution according to Pearson et al.[34] (20 mM Na_2HPO_4, 150 mM NaCl, 1.5 mM KCl, 0.04% trypan blue, pH 5.6) before we measured the fluorescence of 10,000 cells using a filter at 525 nm. To block endocytosis, cells were incubated for 30 min at 37 °C with 10 μM chlorpromazine, 100 μM dynasore, 60 nM latrunculin A, 3 nM bafilomycin A1 or 3 μM chloroquine and washed prior to the incubation with Tf-FITC. To determine the enduring effect of dynasore, the medium containing the inhibitor was replaced with fresh medium for a further 30 min. After two washing steps the cells were incubated with Tf-FITC and then treated as described. For the investigations with filipin, HeLa cells were incubated with 3 μM filipin as described for the other inhibitory substances. Instead of Tf-FITC the cells were incubated for 30 min at 4 °C with fluorescein isothiocyanate-labeled EGF (EGF-FITC, 1 μM) and thereafter treated as described above.

Western Blot. EGFR and TfR expression was analyzed for NIH-3T3, HER14, HeLa and HeLaΔDyn (HeLa cells transfected with mutant dynamin-2 DNA) cells in a Western blot. Shortly, the cells were lysed and 50 μg of total protein was separated on a 10% SDS−PAGE and transferred to a nitrocellulose membrane. Immunodetection was performed using an anti-EGFR (ab231, Abcam, Cambridge, U.K.) and anti-TfR (H68.4, Zymed Laboratories, Bad Homburg, Germany) antibody.

Statistical Analyses. The significance of results was determined by the nonparametric Mann−Whitney U-test using SPSS 17.0 on a Mac OS 10.4 operating system. A two-tailed asymptotic significance of $p \leq 0.05$ was interpreted as being statistically significant.

RESULTS

Cytotoxicity of SE on HER14 and NIH-3T3 Cells. The cytotoxicity of SE alone and in combination with Spn on NIH-3T3 cells (Swiss mouse embryo fibroblast cells) and related HER14 cells (NIH-3T3 cells stably transfected with human EGFR) was recently described:[35] On HER14 cells, SE alone was highly cytotoxic (SI_{50} = 2.5 nM) whereas NIH-3T3 cells were, as expected, more resistant (SI_{50} = 30 nM). The combination with Spn enhanced the cytotoxicity of SE 2800-fold on HER14 cells (SI_{50} = 0.9 pM) and only 50-fold on NIH-3T3 cells (SI_{50} = 600 pM).[35] Additionally, the toxin Sap-3 alone shows a comparable toxicity on HER14 and NIH-3T3 cells with a SI_{50} of 53 nM and 30 nM, respectively (Figure S-1 in the Supporting Information). This clearly demonstrates that the cytotoxicity of SE depends on cell surface expression of the target receptor, which is more prominent in the presence of Spn. Thus, Spn improves not only the sensitivity but also the specificity of SE.

Inhibition of Clathrin-Mediated Endocytosis. Inhibitor experiments were performed on HER14 cells since these cells express a high level of EGFR.[35] Cells preincubated with chlorpromazine, a specific inhibitor of clathrin-mediated endocytosis,[36,37] were only slightly affected in their survival rate at the used concentration (Figure 1). After chlorpromazine preincubation, the cells were treated with either SE alone or Spn/SE. The cytotoxicity of SE alone was not altered after chlorpromazine pretreatment (Figure 2a, Table 1), indicating that SE acts independently of clathrin-mediated endocytosis. In contrast, in combination with Spn, the preincubation with the inhibitor dramatically decreased the cytotoxicity of SE (Figure 2a, Table 1). At the tested concentrations no SI_{50} value was achieved.

The effect of chlorpromazine on endocytosis was analyzed in flow cytometry using HeLa cells since these cells express high amounts of TfR. Tf-FITC was used for flow cytometry experiments instead of EGF. TfR is internalized by clathrin-dependent receptor-mediated endocytosis after binding of Tf[3,4] and is commonly used for the analysis of receptor-mediated endocytosis. Furthermore, at high EGF concentrations the endocytosis of EGFR is shifted from a clathrin-dependent to a clathrin-independent process, which would interfere with the investigations of the inhibitory agents.[33] HeLa cells, either pretreated with chlorpromazine or not, were incubated with Tf-FITC. The internalized Tf-FITC was quantitated by flow cytometry after quenching of extracellular dye molecules with trypan blue. Compared to unstained control cells we observed a clear shift of the fluorescence intensity without chlorpromazine pretreatment but only a slight shift after pretreatment (Figure 3a), indicating that chlorpromazine successfully inhibited clathrin-mediated endocytosis.

Inhibition of Actin Polymerization. The effect of actin polymerization on SE-mediated cytotoxicity was analyzed on HER14 cells by use of the inhibitor latrunculin A, which binds actin monomers near the nucleotide binding cleft.[38] The cells were preincubated with the inhibitor at a nontoxic concentration (Figure 1) and afterward treated with SE or Spn/SE. Similar to chlorpromazine, latrunculin A does not affect the cytotoxicity of SE alone (Figure 2c, Table 1) whereas the Spn-mediated cytotoxicity of SE was clearly inhibited. The efficacy of latrunculin A was validated by flow cytometry using HeLa cells. The cells were treated with the inhibitor prior to incubation with Tf-FITC, and

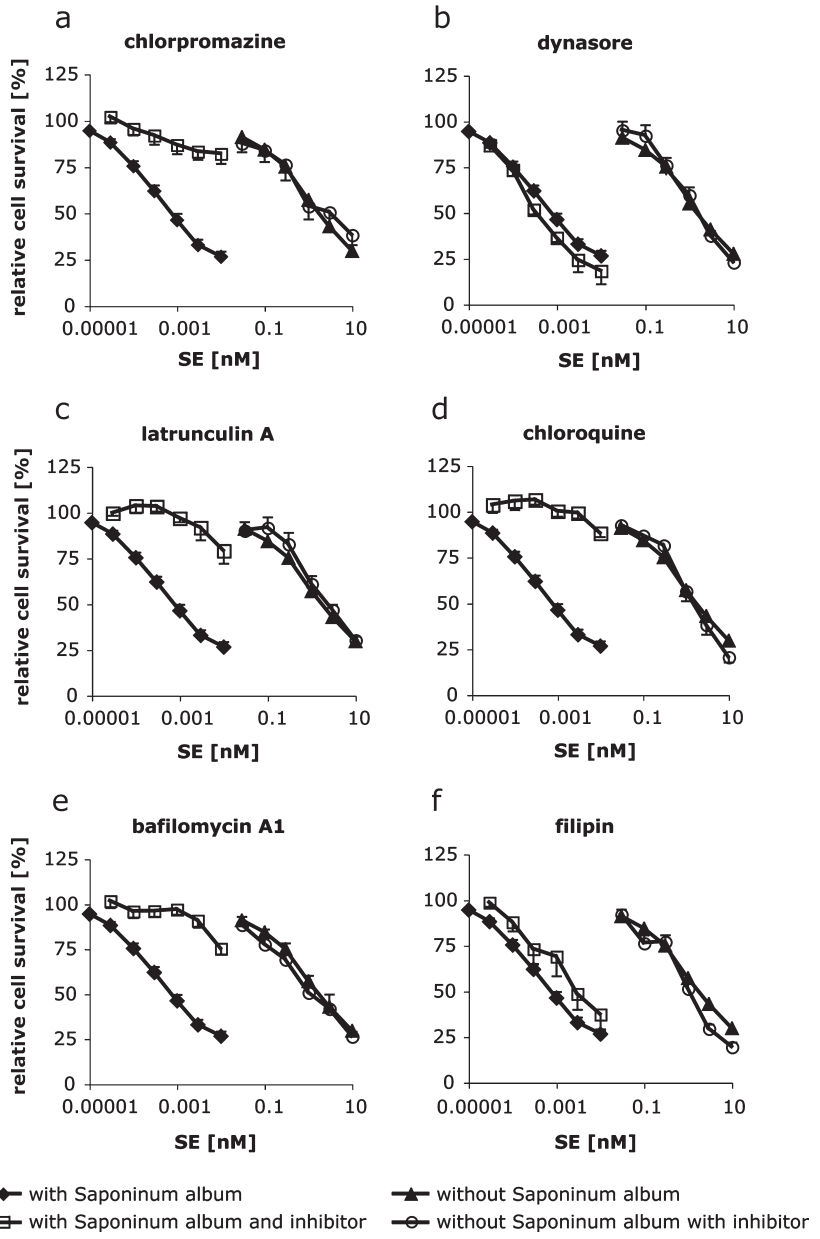


Figure 2. Cytotoxicity of SE and Spn/SE on HER14 cells in the presence and absence of chlorpromazine (a), dynasore (b), latrunculin A (c), chloroquine (d), bafilomycin A1 (e) or filipin (f). Cells were preincubated for 1 h with the inhibitors followed by a 48 h incubation with SE or Spn/SE. In the case of chlorpromazine and dynasore, the inhibitors were replaced by fresh medium before SE or Spn/SE was added. Relative survival was calculated as described. In all panels, the corresponding data points that represent experiments without inhibitor are identical and are shown repeatedly only for better comparison with samples treated with the inhibitor. For experiments with inhibitor, relative survival refers to cells incubated without SE but treated with the inhibitor. Error bars indicate SEM of 4 to 76 experiments ($N_{\mathrm{SEwithoutSpn}}$ = 76, $N_{\mathrm{SEwithSpn}}$ = 40, $N_{\mathrm{chlorpromazinewithoutSpn}}$ = 10, $N_{\mathrm{chlorpromazinewithSpn}}$ = 8, $N_{\mathrm{dynasorewithoutSpn}}$ = 10, $N_{\mathrm{dynasorewithSpn}}$ = 8, $N_{\mathrm{latrunculinAwithoutSpn}}$ = 6, $N_{\mathrm{latrunculinAwithSpn}}$ = 8, $N_{\mathrm{chloroquine}}$ = 6, $N_{\mathrm{bafilomycinA1withoutSpn}}$ = 4, $N_{\mathrm{bafilomycinA1withSpn}}$ = 6, $N_{\mathrm{filipinwithoutSpn}}$ = 4,$N_{\mathrm{filipinwithSpn}}$ = 3) performed in triplicate. The inhibitory effect was significant for chlorpromazine, latrunculin A, chloroquine and bafilomycin A1 when cells were treated with Spn/SE ($p < 0.001$) but not significant when treated with SE alone ($p_{\mathrm{chlorpromazine}} = 0.451$; $p_{\mathrm{latrunculinA}} = 0.700$; $p_{\mathrm{chloroquine}} = 0.326$; $p_{\mathrm{bafilomycinA1}} = 0.151$). For dynasore and filipin significant differences were detectable neither for SE ($p_{\mathrm{dynasore}} = 0.667$; $p_{\mathrm{filipin}} = 0.114$) nor for Spn/SE ($p_{\mathrm{dynasore}} = 0.667$; $p_{\mathrm{filipin}} = 0.1$).

fluorescence of internalized dye was read out as before. Comparable to chlorpromazine, only a small shift in the fluorescence intensity between unstained cells and cells treated with Tf-FITC in the presence of the inhibitor occurred, demonstrating that latrunculin A indeed prevented the uptake of transferrin (Figure 3b).

Inhibition of Endosomal Acidification. In order to analyze whether the acidification of the endosomes is required for the uptake of SE and the enhancing property of Spn, HER14 cells were incubated with either one of two different inhibitors. Bafilomycin A1 is a strong specific inhibitor of the vacuolar H^+-ATPase,[39−41] and chloroquine is a compound that diffuses into the cells and becomes protonated within the acidic interior of the endosome−lysosome system,[42] thus leading to a direct increase in endosomal pH. Both bafilomycin A1 and chloroquine

Table 1. Overview of the Inhibitory Agents and the SI_{50} Values on HER14 Cells after Incubation with SE and Spn/SE in the Absence and Presence of the Inhibitory Agents

		SI_{50} (nM)		
inhibitor	concentration	without Spn	with Spn	inhibition of
no inhibitor		2.5	0.0009	
chlorpromazine	10 μM	3.1	>0.01	clathrin-mediated endocytosis[36,37]
dynasore	100 μM	1.3	0.00051	GTPase activity of dynamin-2[47]
latrunculin A	60 nM	2.5	>0.01	actin-polymerization[38]
chloroquine	3 μM	1.3	>0.01	endosomal acidification[42]
bafilomycin A1	3 nM	1.5	>0.01	endosomal acidification[39−41]
filipin	3 μM	1.1	0.003	caveolae-dependent endocytosis[49]

were used at nontoxic concentrations (Figure 1), and after preincubation with the inhibitors the cells were incubated with SE or Spn/SE. The cytotoxicity of SE alone was affected neither by bafilomycin A1 nor by chloroquine (Figure 2d,e, Table 1) but drastically decreased in combination with Spn, where no SI_{50} was reached within the tested concentrations. The acidification of the endosomes is thus not essential for the delivery of SE to the cytosol; however, it is mandatory for the enhancement of SE delivery by combination with Spn. Both inhibitors were tested for their ability to block the uptake of Tf-FITC. After preincubation with either of the two inhibitors, the fluorescence intensity of internalized transferrin was clearly decreased compared to cells not treated with any of the inhibitors (Figure 3c,d). Although bafilomycin A1 inhibits transport from early to late endosomes in HeLa cells and not endocytosis,[43] the amount of TfR on the cell surface is clearly diminished, since the recycling of TfR is inhibited,[44] resulting in reduced uptake of Tf.

Effects of Inhibitors on Nontarget Cells. The different inhibitors were analyzed on nontarget cells (NIH-3T3) to demonstrate their specificity for the uptake of SE via the EGFR pathway. The cells were preincubated with the inhibitors as described before, and their influence on the cytotoxicity of Spn/SE was tested. Chlorpromazine, latrunculin A and chloroquine had no inhibitory effects (Figure S-2 in the Supporting Information). Only bafilomycin A1 decreased the cytotoxicity of Spn/SE slightly, indicating a certain relevance of endosomal acidification for non-EGFR dependent uptake of SE.

Inhibition of Vesicle Budding. Dynamin-2 mediates the fission of the endocytic vesicle from the cell membrane.[45] After transfection of cells with mutant dynamin-2 DNA (K44A), dynamin-2-dependent endocytosis is blocked.[46] Therefore, we stably transfected HeLa cells with dynamin-2^{K44A} (HeLaΔDyn) and observed a considerable inhibition of both SE and Spn/SE cytotoxicity; however, a detailed investigation showed that EGFR was lost in these cells (Figure 4). Thus, dynamin-2 appears to be essential for the intracellular trafficking of EGFR in HeLa cells. Interestingly, the level of TfR was also reduced but the protein was still present in the functional dynamin-2 knockout (Figure 4), indicating different intracellular trafficking. This observation leads to the conclusion that these experiments cannot provide evidence whether the inhibition of toxin uptake is directly dynamin-2-dependent or simply a consequence of induced target receptor absence. Therefore, we investigated the effect of dynasore, a specific inhibitor of dynamin.[47] Dynasore was used at a nontoxic concentration for the applied incubation period of 1 h (Figure 1) and showed no significant influence on the cytotoxicity of SE and Spn/SE (Figure 2b, Table 1). In the flow cytometry dynasore showed a clear inhibition of the endocytosis of Tf-FITC, which demonstrates the ability of dynasore to inhibit receptor-mediated endocytosis (Figure 5a). Interestingly, this inhibitory effect is already lost 30 min after removal of dynasore (Figure 5b), which might explain that dynasore had no influence on the cytotoxicity of SE and Spn/SE. For the cytotoxicity assay, the medium containing dynasore was removed prior to the incubation with SE or Spn/SE due to the high toxicity of dynasore at longer incubation periods.

Inhibition of Caveolae-Dependent Endocytosis. At high EGF concentrations, the ligand−receptor complex is internalized via clathrin-independent mechanisms. Mineo et al. demonstrated that phosphorylated EGFR (after binding of the ligand) is not located in the caveolae.[48] To demonstrate that SE is internalized in the same manner as EGF, we preincubated HER14 cells with the caveolae inhibitor filipin at an effective concentration compared to the work of Schnitzer et al.[49] Filipin destroys the structural assembly of caveolae and thereby reduces their amount in the cell membrane.[49] As expected, filipin showed no significant inhibition of the cytotoxicity of SE in the presence as well as in the absence of Spn (Figure 2f, Table 1). Similarly, filipin had no inhibitory effect on the uptake of EGF-FITC determined in flow cytometry (Figure 3e). Here, EGF-FITC was used instead of Tf-FITC to evoke a shift from clathrin-dependent to clathrin-independent endocytosis.[33]

Effect of Spn on Clathrin-Independent Endocytosis. For the combined treatment (Spn/SE) SE is applied at a low concentration (<0.01 nM or 0.36 ng/mL), which results in the clathrin-dependent uptake of SE. To further investigate the enhancing mechanism of Spn, HER14 cells were incubated with Spn/SE in the presence of additional EGF at a total concentration of 2.5 nM, which in total mimics the SE concentration that is used without Spn to achieve the SI_{50} value on HER14 cells. This should shift the uptake to a clathrin-independent process. As described by Sigismund et al. a concentration of 10 ng/mL (1.7 nM) EGF is sufficient for this shift.[33] At the same time, this EGF concentration is not sufficient to block SE binding to EGFR, which occurs efficiently only at 100 nM EGF.[35] The results showed no difference between the treatment with Spn/SE and Spn/SE supplemented with EGF in a concentration that is too low for a competitive effect (see Discussion) (Figure 6), demonstrating that the enhancing effect of Spn is independent of the uptake mechanism of EGFR.

Effect of Spn on Retrograde Transport Pathways. The enhancing ability of Spn on the cytotoxicity of PE was analyzed on HER14 cells, which are, as mentioned before, transfected NIH-3T3 cells. The expression of the receptor for PE (α2-macroglobulin

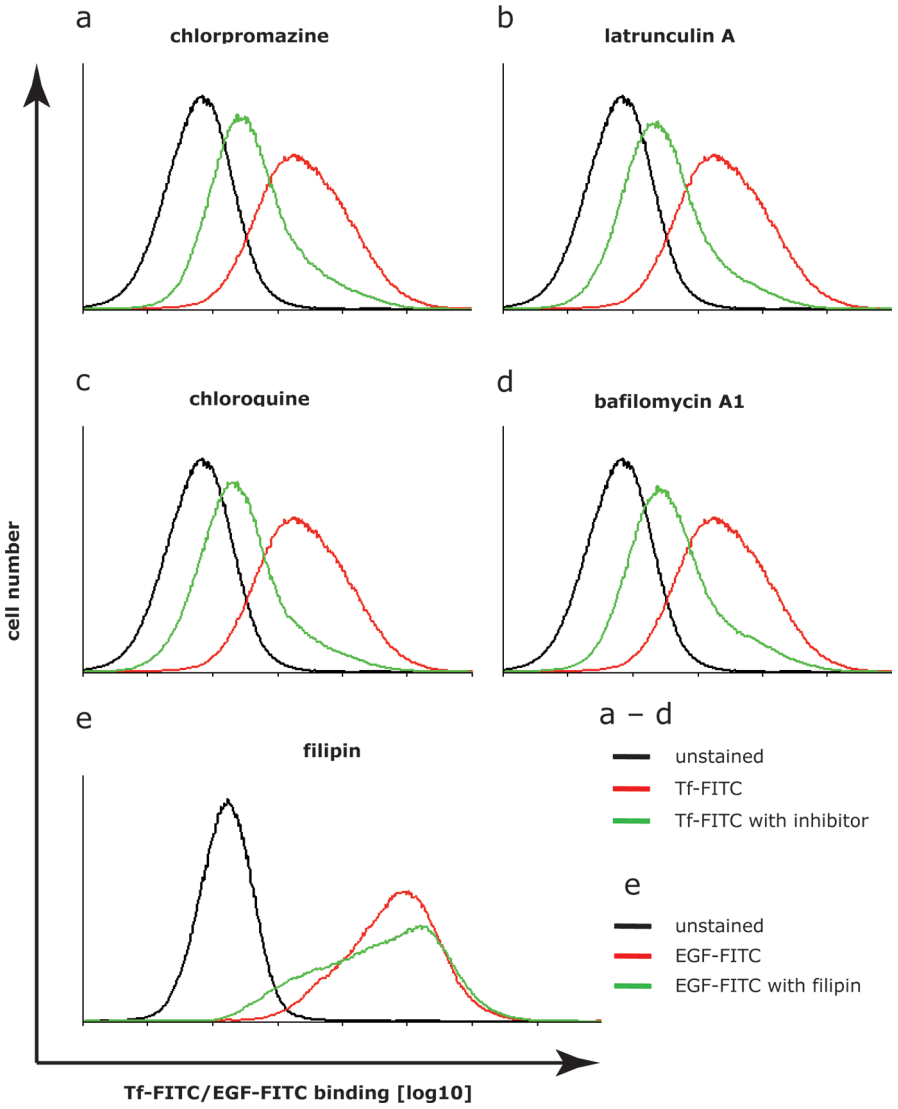


Figure 3. Endocytosis of Tf-FITC and EGF-FITC in flow cytometry. HeLa cells were incubated for 30 min with Tf-FITC or EGF-FITC (red lines) or PBS (black lines) at 4 °C. After a further incubation for 15 min at 37 °C, cells were washed and extracellular fluorescence quenched by a trypan blue solution. Endocytosis was inhibited (green lines) by 10 μM chlorpromazine (a), 60 nM latrunculin A (b), 3 μM chloroquine (c), 3 nM bafilomycin A1 (d) or 3 μM filipin (e). Cells were preincubated with the inhibitors for 30 min at 37 °C before incubating with Tf-FITC or EGF-FITC. The curves representing data without inhibitor are repeatedly shown only for better comparison.

receptor/low density lipoprotein receptor-related protein) was demonstrated for NIH-3T3 cells,[50] and they are often used in studies on the impact of PE.[51,52] PE enters target cells by receptor-mediated endocytosis, is then retrogradely delivered to the endoplasmic reticulum and subsequently enters the cytosol. On HER14 cells, PE showed a high cytotoxicity with a SI_{50} value of 8.6 nM (Figure 7). The combined treatment of the cells with Spn and PE had no enhancing effect on the cytotoxicity of PE. These results evince that Spn does not affect the retrograde transport.

Kinetics of SE and Spn/SE Cytotoxicity. As demonstrated, the uptake of low doses of SE into cells depends on Spn while SE itself can gain access to cells in higher concentrations. As shown further, the enhancing effect is based on an uptake mechanism different from the mechanism for SE in higher concentrations. Therefore, we analyzed whether these differences follow different kinetics. HER14 cells were incubated with SE or Spn/SE for 0.5, 6, 24, or 48 h and further incubated to a total of 48 h without toxin. In samples without Spn, a cytotoxic effect (SI_{50} = 10.6 nM) was already detectable in samples that were incubated with SE for only 30 min (Figure 8a). The cytotoxicity continuously increased with the toxin incubation time (6 h incubation, SI_{50} = 7.7 nM). In the sample exposed 24 h to SE the maximal cytotoxicity comparable to that of 48 h toxin incubation was reached. In contrast, the incubation of Spn/SE revealed no cytotoxicity after 30 min on HER14 cells at the relevant concentration range for the combination treatment (Figure 8b). The sample incubated for 6 h with Spn/SE displayed a significant toxicity with an SI_{50} greater than 0.01 nM. As for SE alone, incubation for 24 h was sufficient to achieve cytotoxicity equal to that after 48 h toxin incubation. Thus, although for both kinetic experiments maximal toxicity is reached after a similar time period, the detectable onset of the enhancer effect is substantially later than the onset of the basic toxin effect of SE alone.

dx.doi.org/10.1021/mp200130j |*Mol. Pharmaceutics* 2011, 8, 2262–2272

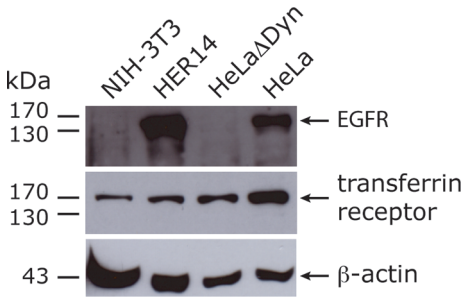


Figure 4. Analysis of the EGFR and TfR expression by Western blotting. 50 μg of total protein of NIH-3T3, HER14, HeLaΔDyn and HeLa was applied to SDS−PAGE and Western blotting. EGFR and TfR were detected by a monoclonal anti-EGFR antibody and a monoclonal anti-TfR antibody, respectively.

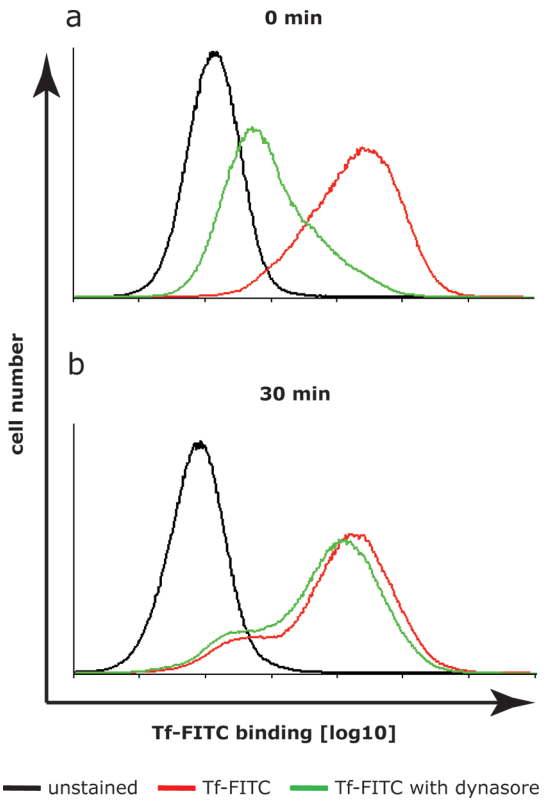


Figure 5. Endocytosis of Tf-FITC in flow cytometry for the determination of long-lasting effects of dynasore. After 30 min of preincubation with 100 μM dynasore (green lines) or without inhibitor (red lines) cells were incubated with (a) Tf-FITC at 4 °C or (b) for additional 30 min with fresh medium at 37 °C prior to the incubation with Tf-FITC at 4 °C. Cells incubated with PBS (black lines) instead of Tf-FITC served as control for fluorescence background. After a further incubation for 15 min at 37 °C, cells were washed and extracellular fluorescence was quenched by a trypan blue solution.

DISCUSSION

The cytotoxicity of the targeted toxin SE[29,53] was drastically enhanced by combination with Spn in a cell culture model[24,31,54,55] and in a mouse model.[27,56] The enhancing properties were also found for other saponins, but were less prominent and often not specific for target cells.[28] The effect is clearly synergistic and resulted in a broader therapeutic window.

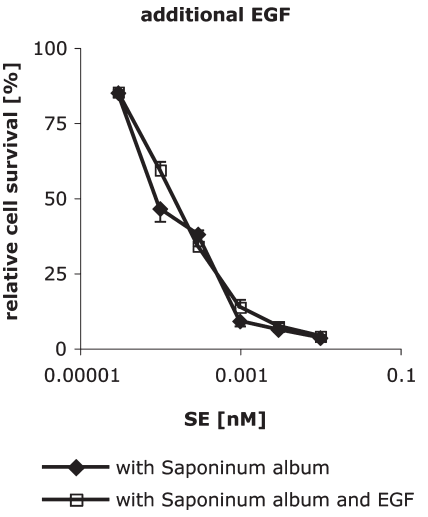


Figure 6. Influence of free human EGF on the enhancing ability of Spn. HER14 cells were incubated for 48 h with Spn/SE in the absence or presence of additional EGF (2.5 nM), and relative survival was then determined as described. Error bars indicate SEM of 6 experiments performed in triplicate. No significant difference was observed ($p = 0.24$).

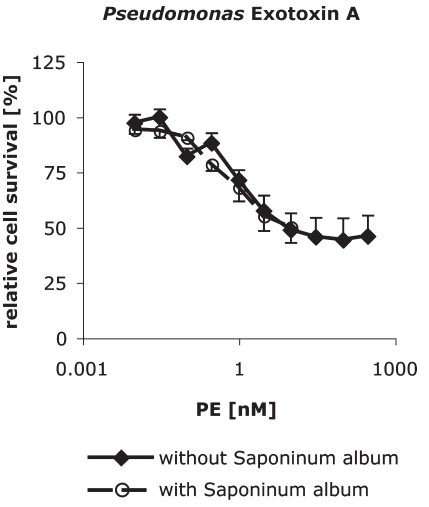


Figure 7. Cytotoxicity of PE on HER14 cells. Cells were incubated for 48 h with PE in the presence and absence of Spn, and relative survival was determined. Error bars indicate SEM of 5 experiments performed in triplicate. No significant difference was observed ($p = 0.602$).

In spite of the promising and clearly described enhancer effect, the molecular mechanism by which Spn augments the cytotoxicity of the targeted toxin SE remains a topic of discussion. Since EGF is ordinarily internalized by clathrin-dependent endocytosis after binding to its receptor,[5] we assumed that Spn affects the EGFR-mediated endocytic uptake of SE or acts on compartments that are located further downstream in the uptake process. In this study we therefore analyzed possible targets for the mode of action of Spn within the endocytic delivery pathway by use of six inhibitory substances (Table 1). All inhibitors despite filipin clearly decreased the uptake of the reference molecules Tf-FITC or EGF-FITC (Figures 3 and 5).

The cytotoxicity of SE was not altered by any of these inhibitors (Figure 2) even though EGFR is mandatory for the

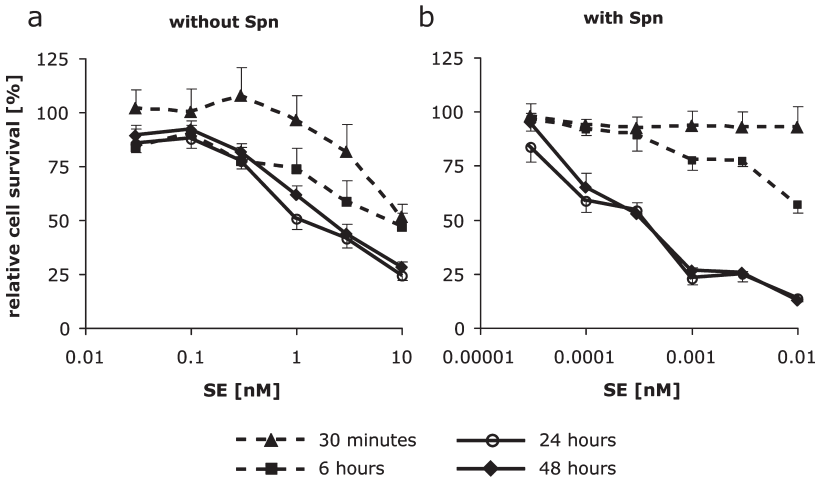


Figure 8. Cytotoxicity of SE (a) and Spn/SE (b) on HER14 cells after different incubation periods. Cells were incubated for 0.5, 6, 24, and 48 h with SE or Spn/SE, and the cells are then further grown with fresh medium to achieve a total of 48 h. Error bars indicate SEM of the relative survival obtained from 3 to 9 experiments ($N_{0.5hwithoutSpn}$ = 4, $N_{0.5hwithSpn}$ = 3, $N_{6hwithoutSpn}$ = 5, $N_{6hwithSpn}$ = 3, $N_{24hwithoutSpn}$ = 6, $N_{24hwithSpn}$ = 3, $N_{48hwithoutSpn}$ = 9, $N_{48hwithSpn}$ = 3) performed in triplicate.

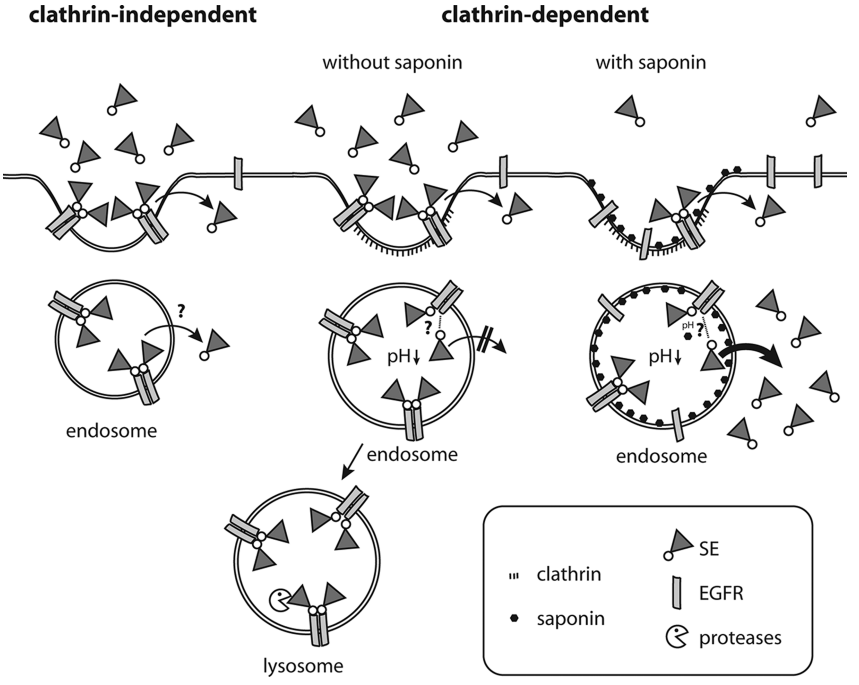


Figure 9. Model for cellular SE uptake in the absence and presence of Spn. After binding to EGFR, SE can enter the cytosol in small amounts via clathrin-independent pathways or from coated pits, but is unable to cross the membrane of acidic endosomes so that most of internalized SE is degraded. Spn accumulates in endosomes[61,62] after clathrin-dependent endocytosis and mediates the membrane transfer of SE. Spn might be also involved in acidic SE dissociation from EGFR.

uptake of SE (Figure S-1 in the Supporting Information) and free human EGF diminishes the cytotoxicity of SE at a concentration of 100 nM.[35] Here, we demonstrated that SE is not internalized via clathrin-dependent endocytosis since the cytotoxicity of SE was not attenuated by chlorpromazine. In accordance with our observations Sigismund et al. described EGF uptake via clathrin-mediated endocytosis at low concentrations (<1 ng/mL) but in a clathrin-independent pathway at high concentrations (>10 ng/mL).[33] Thus, we conclude that SE binds as expected to EGFR and is, similar to EGF in higher concentrations, internalized via clathrin-independent mechanisms.[6,33] Again comparable to EGF, this is a caveolae-independent process as demonstrated by the lack of influence by filipin (Figure 2). It is known that Sap-3 alone can also be internalized; however, it is unlikely that SE uptake follows the same pathway of internalization on EGFR expressing cells. Notably, it was shown that endocytic uptake of Sap-3 is dependent on clathrin.[57]

Robertson et al. have divided the endocytic process into three steps (assembly of the endocytic coat complex, invagination to form a vesicle by actin polymerization, and vesicle fission).[58]

dx.doi.org/10.1021/mp200130j |*Mol. Pharmaceutics* 2011, 8, 2262–2272

Actin appears to play an indirect role in the vesicle fission by ensuring that regulatory proteins are recruited, but is, although not essential, actively involved in the subsequent movement of the vesicle from the cell membrane into the cell.[59] Since latrunculin A had no effect on SE cytotoxicity, the uptake of the targeted toxin seems to be independent of actin. After acidification of the endosomes, the internalized EGF remains bound to the EGFR and both are degraded.[60] Preventing endosomal acidification by chloroquine and bafilomycin A1 did not hamper the cytotoxicity of SE on HER14 cells (Figure 2), indicating that dissociation of SE from internalized EGFR in experiments using nM SE concentrations is independent of pH.

Notably, in contrast to treatment with SE alone, the efficacy of the combination of Spn/SE is strongly dependent on endocytic mechanisms. Chlorpromazine, latrunculin A, chloroquine and bafilomycin A1 induced a significant decrease in cytotoxicity (Figure 2), demonstrating that the inhibited processes are required for the action of Spn. Interestingly, the addition of free human EGF to a total concentration of 2.5 nM (reflecting the equivalent amount of EGF in SE treatment without Spn) had no influence on the cytotoxicity of Spn/SE (Figure 6). This EGF concentration (2.5 nM) is too low to compete for the binding of SE as has been shown for a higher concentration (100 nM)[35] but is sufficient to induce clathrin-independent uptake of EGF and thus most likely of SE.[33] We expected the 2.5 nM EGF concentration to shift the SE uptake into the clathrin-independent pathway, which is not affected by Spn. The results indicated no change in cytotoxicity and demonstrated that SE in pM concentrations is sufficient to induce cell death when combined with Spn. Moreover, we clearly demonstrated that the enhancing ability of Spn is independent of the uptake mechanism observed for SE in nM concentrations. Weng et al. showed that a small portion of Spn molecules remained associated with the cell after washing and that this portion was sufficient to induce a drastic toxicity enhancement of Spn-mediated endosomal escape of the toxin.[61,62] Adapting these results to the present study, we suggest that Spn integrates into endosomal membranes and then mediates the transfer of SE into the cytosol. Endosomal acidification seems to be necessary for either or both of these steps, as shown here by the inhibition by chloroquine and bafilomycin A1. Protonation of the glucuronic acid at C3 of Spn may play an important role. The exact molecular mechanism for endosomal escape of SE is not known and will be the topic of future research. Additionally, we could exclude an effect of Spn on the retrograde transport, as Spn showed no enhancing ability on the protein toxin PE (Figure 7).

Since a functional dynamin-2 knockout prevents EGFR from appearing at the cell surface in HeLa cells (Figure 4) and the specific dynamin-2 inhibitor dynasore induces no long-lasting effects, we were unable to investigate the direct influence of dynamin-2 on SE internalization. The idea that SE is taken up via different pathways in presence or absence of Spn is further supported by the considerably slower kinetics of its cytotoxicity (Figure 8), indicating that the synergistic interaction between Spn and SE requires a certain setup time.

In conclusion, we demonstrated that the uptake of SE follows a completely different pathway upon combination with Spn after binding selectively to EGFR. Assuming that Spn has no direct influence on the intracellular trafficking of EGFR, the receptor together with bound SE is expected to be distributed between cell surface, coated pits, budding vesicles, early and late endosomes in the same manner in the absence or presence of Spn. We suggest that a small number of SE molecules can cross the membrane early after binding to EGFR, for instance directly from the cell membrane, from coated pits or after internalization via pathways not dependent on clathrin (Figure 9). These molecules cause the moderate cytotoxicity of SE at nM concentrations. The majority of the SE molecules remain bound to the receptor and are transported to lysosomes for degradation as described for natural EGF-EGFR.[60] Alternatively, in contrast to EGF, SE dissociates at acidic pH but cannot pass through the membrane. In combination with Spn, Spn may mediate the endosomal escape of dissociated SE and even facilitate the acidic dissociation of SE from EGFR beforehand. The Spn-dependent endosomal escape relies on precedent intracellular trafficking and is thus slower than the early clathrin-independent but less efficient cytosolic uptake of SE shortly after binding to cell surface EGFR. This hypothesis and the results presented here are an important step toward the understanding of the mode of action of Spn. Further studies on this process will help to unravel the precise mechanism by which Spn directs SE delivery to the cytosol.

■ ASSOCIATED CONTENT

Supporting Information. Information about the cytotoxicity of SE, Spn/SE, Sap-3 and Spn/Sap-3 on HER14 and NIH-3T3 cells and the inhibitory effects of chlorpromazine, latrunculin A, bafilomycin A1 and chloroquine on NIH-3T3 cells. The data are presented as Figure S-1 and S-2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Institut für Laboratoriumsmedizin, Klinische Chemie und Pathobiochemie, Charité — Universitätsmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, D-12200 Berlin, Germany. Tel: +4930 8445 2559. Fax: +4930 8445 4152. E-mail: hendrik.fuchs@charite.de.

Present Addresses

‡Laboratory of Bacterial Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 33 North Drive, Bethesda, MD 20892, USA.

■ ACKNOWLEDGMENT

We acknowledge the financial support of the Deutsche Forschungsgemeinschaft (FU 408/3-1), the Sonnenfeld-Stiftung and the Berliner Graduiertenförderung (NaFöG) (grants to D.B.).

■ ABBREVIATIONS USED

EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; SI_{50}, survival index (50% cell survival); Sap-3, saporin isoform 3; SE, targeted toxin consisting of saporin and EGF; Spn, Saponinum album (saponin composite from *Gypsophila paniculata* L.); PE, *Pseudomonas* exotoxin A; Tf, transferrin; TfR, transferrin receptor; Tf-FITC, fluorescein isothiocyanate-labeled transferrin

■ REFERENCES

(1) Royle, S. J. The cellular functions of clathrin. *Cell. Mol. Life Sci.* **2006**, *63* (16), 1823–32.

(2) Mayor, S.; Pagano, R. E. Pathways of clathrin-independent endocytosis. *Nat. Rev. Mol. Cell Biol.* **2007**, *8* (8), 603–12.
(3) Ciechanover, A.; Schwartz, A. L.; Dautry-Varsat, A.; Lodish, H. F. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. *J. Biol. Chem.* **1983**, *258* (16), 9681–9.
(4) Dautry-Varsat, A. Receptor-mediated endocytosis: the intracellular journey of transferrin and its receptor. *Biochimie* **1986**, *68* (3), 375–81.
(5) Pastan, I. H.; Willingham, M. C. Receptor-mediated endocytosis of hormones in cultured cells. *Annu. Rev. Physiol.* **1981**, *43*, 239–50.
(6) Sorkin, A.; Goh, L. K. Endocytosis and intracellular trafficking of ErbBs. *Exp. Cell Res.* **2009**, *315* (4), 683–96.
(7) Osickova, A.; Osicka, R.; Maier, E.; Benz, R.; Sebo, P. An amphipathic alpha-helix including glutamates 509 and 516 is crucial for membrane translocation of adenylate cyclase toxin and modulates formation and cation selectivity of its membrane channels. *J. Biol. Chem.* **1999**, *274* (53), 37644–50.
(8) Bhakdi, S.; Tranum-Jensen, J. Alpha-toxin of Staphylococcus aureus. *Microbiol. Rev.* **1991**, *55* (4), 733–51.
(9) Fivaz, M.; Abrami, L.; Tsitrin, Y.; van der Goot, F. G. Not as simple as just punching a hole. *Toxicon* **2001**, *39* (11), 1637–45.
(10) Pappenheimer, A. M., Jr. Diphtheria toxin. *Annu. Rev. Biochem.* **1977**, *46*, 69–94.
(11) Mock, M.; Fouet, A. Anthrax. *Annu. Rev. Microbiol.* **2001**, *55*, 647–71.
(12) Binz, T.; Rummel, A. Cell entry strategy of clostridial neurotoxins. *J. Neurochem.* **2009**, *109* (6), 1584–95.
(13) Wolf, P.; Elsasser-Beile, U. Pseudomonas exotoxin A: from virulence factor to anti-cancer agent. *Int. J. Med. Microbiol.* **2009**, *299* (3), 161–76.
(14) Balint, G. A. Ricin: the toxic protein of castor oil seeds. *Toxicology* **1974**, *2* (1), 77–102.
(15) Vanden Broeck, D.; Horvath, C.; De Wolf, M. J. Vibrio cholerae: cholera toxin. *Int. J. Biochem. Cell Biol.* **2007**, *39* (10), 1771–5.
(16) Spooner, R. A.; Smith, D. C.; Easton, A. J.; Roberts, L. M.; Lord, J. M. Retrograde transport pathways utilised by viruses and protein toxins. *Virol. J.* **2006**, *3*, 26.
(17) Sandvig, K.; Spilsberg, B.; Lauvrak, S. U.; Torgersen, M. L.; Iversen, T. G.; van Deurs, B. Pathways followed by protein toxins into cells. *Int. J. Med. Microbiol.* **2004**, *293* (7−8), 483–90.
(18) Kounnas, M. Z.; Morris, R. E.; Thompson, M. R.; FitzGerald, D. J.; Strickland, D. K.; Saelinger, C. B. The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds and internalizes Pseudomonas exotoxin A. *J. Biol. Chem.* **1992**, *267* (18), 12420–3.
(19) Yan, L.; Rosen, N.; Arteaga, C. Targeted cancer therapies. *Chin J Cancer* **2011**, *30* (1), 1–4.
(20) U.S. Food and Drug Administration. Mylotarg (gemtuzumab ozogamicin): Market Withdrawal. http://www.fda.gov/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/ucm216458.htm 2010, Accessed 21 June 2010.
(21) Fuchs, H.; Bachran, C. Targeted tumor therapies at a glance. *Curr. Drug Targets* **2009**, *10* (2), 89–93.
(22) Frankel, A. E.; Fleming, D. R.; Hall, P. D.; Powell, B. L.; Black, J. H.; Leftwich, C.; Gartenhaus, R. A phase II study of DT fusion protein denileukin diftitox in patients with fludarabine-refractory chronic lymphocytic leukemia. *Clin. Cancer Res.* **2003**, *9* (10 Pt 1), 3555–61.
(23) Soler-Rodriguez, A. M.; Ghetie, M. A.; Oppenheimer-Marks, N.; Uhr, J. W.; Vitetta, E. S. Ricin A-chain and ricin A-chain immunotoxins rapidly damage human endothelial cells: implications for vascular leak syndrome. *Exp. Cell Res.* **1993**, *206* (2), 227–34.
(24) Heisler, I.; Sutherland, M.; Bachran, C.; Hebestreit, P.; Schnitger, A.; Melzig, M. F.; Fuchs, H. Combined application of saponin and chimeric toxins drastically enhances the targeted cytotoxicity on tumor cells. *J. Controlled Release* **2005**, *106* (1−2), 123–37.
(25) Bachran, C.; Sutherland, M.; Heisler, I.; Hebestreit, P.; Melzig, M. F.; Fuchs, H. The saponin-mediated enhanced uptake of targeted saporin-based drugs is strongly dependent on the saponin structure. *Exp. Biol. Med. (Maywood)* **2006**, *231* (4), 412–20.
(26) Fuchs, H.; Bachran, D.; Panjideh, H.; Schellmann, N.; Weng, A.; Melzig, M. F.; Sutherland, M.; Bachran, C. Saponins as tool for improved targeted tumor therapies. *Curr. Drug Targets* **2009**, *10* (2), 140–51.
(27) Bachran, C.; Durkop, H.; Sutherland, M.; Bachran, D.; Muller, C.; Weng, A.; Melzig, M. F.; Fuchs, H. Inhibition of tumor growth by targeted toxins in mice is dramatically improved by saponinum album in a synergistic way. *J. Immunother.* **2009**, *32* (7), 713–25.
(28) Bachran, C.; Bachran, S.; Sutherland, M.; Bachran, D.; Fuchs, H. Saponins in tumor therapy. *Mini-Rev. Med. Chem.* **2008**, *8* (6), 575–84.
(29) Heisler, I.; Keller, J.; Tauber, R.; Sutherland, M.; Fuchs, H. A cleavable adapter to reduce nonspecific cytotoxicity of recombinant immunotoxins. *Int. J. Cancer* **2003**, *103* (2), 277–82.
(30) Fabbrini, M. S.; Rappocciolo, E.; Carpani, D.; Solinas, M.; Valsasina, B.; Breme, U.; Cavallaro, U.; Nykjaer, A.; Rovida, E.; Legname, G.; Soria, M. R. Characterization of a saporin isoform with lower ribosome-inhibiting activity. *Biochem. J.* **1997**, *322* (Part 3), 719–27.
(31) Bachran, C.; Heisler, I.; Bachran, D.; Dassler, K.; Ervens, J.; Melzig, M. F.; Fuchs, H. Chimeric toxins inhibit growth of primary oral squamous cell carcinoma cells. *Cancer Biol. Ther.* **2008**, *7* (2), 237–42.
(32) Weng, A.; Jenett-Siems, K.; Gorick, C.; Melzig, M. F. Enhancement of cytotoxicity of ribosome-inactivating-protein type I by saponinum album is not based on stimulation of phagocytosis. *J. Pharm. Pharmacol.* **2008**, *60* (7), 925–30.
(33) Sigismund, S.; Woelk, T.; Puri, C.; Maspero, E.; Tacchetti, C.; Transidico, P.; Di Fiore, P. P.; Polo, S. Clathrin-independent endocytosis of ubiquitinated cargos. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (8), 2760–5.
(34) Pearson, A. M.; Baksa, K.; Ramet, M.; Protas, M.; McKee, M.; Brown, D.; Ezekowitz, R. A. Identification of cytoskeletal regulatory proteins required for efficient phagocytosis in Drosophila. *Microbes Infect.* **2003**, *5* (10), 815–24.
(35) Bachran, D.; Schneider, S.; Bachran, C.; Urban, R.; Weng, A.; Melzig, M. F.; Hoffmann, C.; Kaufmann, A. M.; Fuchs, H. Epidermal growth factor receptor expression affects the efficacy of the combined application of saponin and a targeted toxin on human cervical carcinoma cells. *Int. J. Cancer* **2010**, *127* (6), 1453–61.
(36) Wang, L. H.; Rothberg, K. G.; Anderson, R. G. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* **1993**, *123* (5), 1107–17.
(37) Sofer, A.; Futerman, A. H. Cationic amphiphilic drugs inhibit the internalization of cholera toxin to the Golgi apparatus and the subsequent elevation of cyclic AMP. *J. Biol. Chem.* **1995**, *270* (20), 12117–22.
(38) Yarmola, E. G.; Somasundaram, T.; Boring, T. A.; Spector, I.; Bubb, M. R. Actin-latrunculin A structure and function. Differential modulation of actin-binding protein function by latrunculin A. *J. Biol. Chem.* **2000**, *275* (36), 28120–7.
(39) Bowman, E. J.; Siebers, A.; Altendorf, K. Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85* (21), 7972–6.
(40) Yoshimori, T.; Yamamoto, A.; Moriyama, Y.; Futai, M.; Tashiro, Y. Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *J. Biol. Chem.* **1991**, *266* (26), 17707–12.
(41) Clague, M. J.; Urbe, S.; Aniento, F.; Gruenberg, J. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *J. Biol. Chem.* **1994**, *269* (1), 21–4.
(42) de Duve, C.; de Barsy, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. Commentary. Lysosomotropic agents. *Biochem. Pharmacol.* **1974**, *23* (18), 2495–531.
(43) Bayer, N.; Schober, D.; Prchla, E.; Murphy, R. F.; Blaas, D.; Fuchs, R. Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection. *J. Virol.* **1998**, *72* (12), 9645–55.

(44) Johnson, L. S.; Dunn, K. W.; Pytowski, B.; McGraw, T. E. Endosome acidification and receptor trafficking: bafilomycin A1 slows receptor externalization by a mechanism involving the receptor's internalization motif. *Mol. Biol. Cell* **1993**, *4* (12), 1251–66.

(45) Takei, K.; McPherson, P. S.; Schmid, S. L.; De Camilli, P. Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. *Nature* **1995**, *374* (6518), 186–90.

(46) Damke, H.; Baba, T.; Warnock, D. E.; Schmid, S. L. Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **1994**, *127* (4), 915–34.

(47) Macia, E.; Ehrlich, M.; Massol, R.; Boucrot, E.; Brunner, C.; Kirchhausen, T. Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* **2006**, *10* (6), 839–50.

(48) Mineo, C.; Gill, G. N.; Anderson, R. G. Regulated migration of epidermal growth factor receptor from caveolae. *J. Biol. Chem.* **1999**, *274* (43), 30636–43.

(49) Schnitzer, J. E.; Oh, P.; Pinney, E.; Allard, J. Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.* **1994**, *127* (5), 1217–32.

(50) Mucci, D.; Forristal, J.; Strickland, D.; Morris, R.; Fitzgerald, D.; Saelinger, C. B. Level of receptor-associated protein moderates cellular susceptibility to pseudomonas exotoxin A. *Infect. Immun.* **1995**, *63* (8), 2912–8.

(51) Hwang, J.; Fitzgerald, D. J.; Adhya, S.; Pastan, I. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. *Cell* **1987**, *48* (1), 129–36.

(52) Chow, J. T.; Chen, M. S.; Wu, H. C.; Hwang, J. Identification of the carboxyl-terminal amino acids important for the ADP-ribosylation activity of Pseudomonas exotoxin A. *J. Biol. Chem.* **1989**, *264* (31), 18818–23.

(53) Fuchs, H.; Bachran, C.; Li, T.; Heisler, I.; Durkop, H.; Sutherland, M. A cleavable molecular adapter reduces side effects and concomitantly enhances efficacy in tumor treatment by targeted toxins in mice. *J. Controlled Release* **2007**, *117* (3), 342–50.

(54) Hebestreit, P.; Weng, A.; Bachran, C.; Fuchs, H.; Melzig, M. F. Enhancement of cytotoxicity of lectins by Saponinum album. *Toxicon* **2006**, *47* (3), 330–5.

(55) Weng, A.; Melzig, M. F.; Bachran, C.; Fuchs, H. Enhancement of saporin toxicity against U937 cells by Gypsophila saponins. *J. Immunotoxicol.* **2008**, *5* (3), 287–92.

(56) Bachran, C.; Weng, A.; Bachran, D.; Riese, S. B.; Schellmann, N.; Melzig, M. F.; Fuchs, H. The distribution of saponins in vivo affects their synergy with chimeric toxins against tumours expressing human epidermal growth factor receptors in mice. *Br. J. Pharmacol.* **2010**, *159* (2), 345–52.

(57) Weng, A.; Bachran, C.; Fuchs, H.; Melzig, M. F. Soapwort saponins trigger clathrin-mediated endocytosis of saporin, a type I ribosome-inactivating protein. *Chem.-Biol. Interact.* **2008**, *176* (2−3), 204–11.

(58) Robertson, A. S.; Smythe, E.; Ayscough, K. R. Functions of actin in endocytosis. *Cell. Mol. Life Sci.* **2009**, *66* (13), 2049–65.

(59) Fujimoto, L. M.; Roth, R.; Heuser, J. E.; Schmid, S. L. Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic* **2000**, *1* (2), 161–71.

(60) Wells, A. EGF receptor. *Int. J. Biochem. Cell Biol.* **1999**, *31* (6), 637–43.

(61) Weng, A.; Gorick, C.; Melzig, M. F. Enhancement of toxicity of saporin-based toxins by Gypsophila saponins--kinetic of the saponin. *Exp. Biol. Med. (Maywood)* **2009**, *234* (8), 961–6.

(62) Weng, A.; Bachran, C.; Fuchs, H.; Krause, E.; Stephanowitz, H.; Melzig, M. F. Enhancement of saporin cytotoxicity by Gypsophila saponins--more than stimulation of endocytosis. *Chem.-Biol. Interact.* **2009**, *181* (3), 424–9.