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Preclinical Evaluation of the Synthetic Adjuvant SQS-21 and its Constituent Isomeric Saponins

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Abstract

The saponin fraction QS-21 from *Quillaja saponaria* has been demonstrated to be a potent immunological adjuvant when mixed with keyhole limpet hemocyanin conjugate vaccines, as well as with other classes of subunit antigen vaccines. QS-21 adjuvant is composed of two isomers that include the apiose and xylose forms in a ratio of 65:35, respectively. The chemical syntheses of these two isomers in pure form have recently been disclosed. Herein we describe detailed *in vivo* immunological evaluations of these synthetic QS-21 isomeric constituents, employing the GD3-KLH melanoma antigen. With this vaccine construct, high antibody titers against GD3 ganglioside and KLH were elicited when GD3-KLH was co-administered with adjuvant, either as the individual separate synthetic QS-21 isomers (SQS-21-Api or SQS-21-Xyl), or as its reconstituted 65:35 isomeric mixture (SQS-21). These antibody titer levels were comparable to that elicited by vaccinations employing naturally derived QS-21 (PQS-21). Moreover, toxicities of the synthetic saponin adjuvants were also found to be comparable to that of naturally derived PQS-21. These findings demonstrate unequivocally that the adjuvant activity of QS-21 resides in these two principal isomeric forms, and not in trace contaminants within the natural extracts. This lays the foundation for future exploration of structure-function correlations to enable the discovery of novel saponins with increased potency, enhanced stability, and attenuated toxicity.

Keywords

Cancer Vaccines; Synthetic adjuvant; Saponin adjuvant; Conjugate vaccines

1. INTRODUCTION

The most widely expressed cell surface antigens on majority of cancers are carbohydrate-associated molecules such as gangliosides (GM2, GD2, GD3, fucosyl GM1, sialyl Lewis^a), the neutral glycolipid (globo H) and mono and disaccharides epitopes (Tn, sTn and TF) on mucins [1–3]. We have developed conjugate vaccines that are consistently immunogenic in

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mice and patients against each of these carbohydrate cancer antigens (reviewed in [4]). Vaccines against cancer and infectious diseases have employed increasingly homogeneous antigens for vaccine construction; however, a marked decrease in immunogenicity of these antigens is observed as they are purified. In the case of viral and bacterial antigens, this is a consequence of the loss of the surrounding xenoantigens and other components at the surface of these pathogens during purification. This can be offset to a significant degree by conjugating these antigens to immunogenic carrier proteins and/or by the use of a potent immunological adjuvant. We have investigated both approaches [5–7] but focus here on immunological adjuvants.

We have previously tested more than 25 adjuvants in mice with a conjugate melanoma vaccine comprising the ganglioside antigen GD3 and the carrier protein KLH. Adjuvants were evaluated based on their ability to augment antibody responses to GD3 and KLH, as well as the T-cell response to KLH [6,7]. Saponin adjuvants significantly outperformed several other classes of adjuvants including glucan formulations, peptidoglycans, amphiphilic block copolymers, bacterial nucleosides and bacterial lipopolysaccharides. Of these, the purified *Quillaja saponaria* fraction QS-21 exhibited the highest responses. These responses were found to be generally correlated to dose; at the highest doses tolerated in cancer patients, where repeated vaccinations are required, QS-21 was the most effective adjuvant. QS-21 has also demonstrated superior adjuvant potency with other types of vaccines targeting cancer [4], HIV [8,9] and malaria [10,11].

QS-21 comprises of two principal constitutional isomers in 65:35 ratio that bear either a xylose or apiose as the terminal residue in the linear tetrasaccharide fragment of the saponin (see Figure 1) [12,13]. We have previously described the synthesis of QS-21-Api and QS-21-Xyl and demonstrated the potent adjuvant activity of a reconstituted 65:35 mixture of SQS-21-Api and SQS-21-Xyl (SQS-21)[14]. We compare here for the first time the adjuvant potency of each individual synthetic isomer within SQS-21 (SQS-21-Api, SQS-21-Xyl) and the 65:35 SQS-21 mixture against GD3-KLH antigen, with simultaneous comparison of toxicity in preparation for subsequent clinical trials.

2. MATERIALS AND METHODS

2.1 Antigens and Reagents

Keyhole limpet hemocyanin (KLH), human serum albumin (HSA), sodium cyanoborohydride, and human serum complement were purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal antibody R24 recognizing GD3 was provided by Dr. Chapman, at MSKCC. Goat anti-mouse IgG and IgM conjugated with alkaline phosphatase and goat anti-mouse IgG and IgM fluorescence-isothiocyanate (FITC) were obtained from Southern Biotechnology Associated (Birmingham, AL). The PQS-21 was obtained from *Quillaja saponaria*, according to the literature procedure, and served as the positive control[15]. SQS-21-Api, SQS-21-Xyl, SQS-21 (mix of 65% SQS-21-Api and 35%SQS-21-Xyl) were synthesized by the laboratory of Dr. Gin at MSKCC. The syntheses of SQS-21-Api and SQS-21-Xyl are summarized in Figure 1.

2.2 Preparation of the SQS-Adjuvants

SQS-21-Api and SQS-21-Xyl were synthesized as described previously.[14,16,17] Briefly (Figure 1), construction of the branched trisaccharide and the isomeric linear tetrasaccharide portions of QS-21-Api/Xyl isomers involved the synthesis of the corresponding selectively protected monosaccharides, which were assembled with a combination of sulfoxide-mediated dehydrative[18] and oxidative[19] glycosylation reactions, TESOTf-catalyzed glycosylation with anomeric acetates[20], and trichloroacetimidate anomeric coupling reactions[21].

Enantioselective synthesis of the fatty acyl chain within QS-21 employed the convergent steps of diastereoselective Brown crotylation[22], diastereoselective aldol reaction[23], sulfoxide-mediated dehydrative glycosylation[18], and Yamaguchi esterification[24]. Finally, quillaic acid was obtained in gram quantities from strong acid hydrolysis of the tree bark extracts. These efforts successfully yielded all four structural quadrants of QS-21-Api/Xyl in suitably protected form, allowing for their subsequent late-stage coupling, careful global deprotection, and RP-HPLC purification to provide synthetic QS-21-Api and QS-21-Xyl for immunological evaluation.

2.3 Preparation of GD3-KLH conjugate

GD3-KLH conjugate was prepared as described previously [5,25] (see Figure 2). The GD3-KLH conjugation procedure was initiated by ozonolytic cleavage of the GD3 ceramide alkene, and the resulting aldehyde was reacted with native KLH in the presence of sodium cyanoborohydride to effect reductive amination with the ϵ -amino acid groups of lysine residues on the carrier protein. GD3 concentration and GD3/KLH epitope ratio were determined using resorcinol to measure sialic acid content of GD3. The GD3/KLH epitope ratio for the combined preparation was found to be 748. The dose of GD3-KLH refers exclusively to the quantity of GD3 in the conjugate.

2.4 Animals and Immunization

Female C57BL/6J mice, six weeks of age, were obtained from Jackson Laboratory (Bar Harbor, ME). Groups of five mice were immunized subcutaneously 3 times at one-week intervals with 10 μ g of GD3-KLH conjugate mixed with 10, 20 or 50 μ g of SQS-21, SQS-21-Api or SQS-21-Xyl (see Table 1). A positive control group was vaccinated with 10 μ g GD3-KLH plus 10 μ g of PQS-21. A negative control group was vaccinated with 10 μ g of GD3-KLH without adjuvant. Mice were bled 7 days after third immunization and tested for the presence of antibody against GD3 and KLH by ELISA. A booster was administered 5 weeks after the third injection and mice were bled 7 days after booster and tested for GD3 by ELISA and against a tumor cell-line expressing GD3 by FACS. As a measure of toxicity, loss of weight was monitored at 0 h, 24 h, 48 h and 72 h after each injection for the first three immunizations.

2.5 ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) was performed as previously described [5, 25]. GD3 or KLH titers were measured by coating ELISA plates with 0.1 μ g GD3 or KLH per well. Serially diluted antisera or monoclonal antibody R24 as positive control were added to the wells and incubated for 1 h at room temperature. Goat anti-mouse IgM or IgG conjugated with alkaline phosphatase were used as secondary antibodies. Absorbances were measured at 405 nm. The antibody titer was defined as the highest serum dilution showing an absorbance of ≥ 0.1 over that of pre-vaccination sera.

2.6 Flow Cytometry

Cells from the GD3-positive melanoma cell line SK-Mel-28 served as targets. Cells were incubated on ice with 20 μ l of 1/20 diluted pre-vaccination sera, post vaccination sera or murine mAb R24 (5 μ g/mL) for 30 min. After washing, 20 μ l of 1/15 goat anti-mouse IgM or IgG labeled with FITC (Southern Biotechnology Associates, Inc., Birmingham, AL) was added, mixed and incubated for 30 minutes on ice. After washing, the positive population and mean fluorescence intensity (MFI) of the stained cells were analyzed by flow cytometry (FACScan, Becton and Dickinson, San Jose, CA). Pre- and post-vaccination sera were analyzed together and the pre-treatment percent positive cells gaied at 10%. Results were considered positive when percent positive cells were 3-fold greater than the negative control and the MFI was 150% of the negative control.

3. RESULTS

3.1 Synthetic (SQS) Adjuvants

The SQS-saponin adjuvants were obtained through chemical synthesis via de novo construction of the oligosaccharide and acyl chain portions of the natural product, followed by modular assembly with its triterpene core, Quillaic acid. The products of each synthesis were purified to >95% purity by reverse phase HPLC as judged by ¹H NMR and analytical RP-HPLC. SQS-21 was prepared by combining the two purified synthetic isomers SQS-21-Api and SQS-21-Xyl in a 65:35 ratio respectively, in order to mimic the isomeric ratio reported to be isolated from the natural source[12]. This reconstituted isomeric mixture (SQS-21) can be considered as a highly purified synthetic surrogate for naturally derived QS-21.

3.2 Toxicity

The median weight change for groups of five mice receiving SQS-21, SQS-21-Api or SQS-21-Xyl at the three different doses is illustrated in Figures 3, 4 and 5. Weight loss was proportional to the dose of synthetic adjuvants. The saponin SQS-21-Xyl was marginally more toxic than SQS-21-Api or the SQS-21 mixture. One mouse in the SQS-21-Api 50 µg dose group and one mouse in the SQS-21-Xyl 50 µg dose group died over the course of immunizations. The mice in both 50 µg dose groups, especially in the SQS-21-Xyl group, looked ill with a hunched posture and scruffy coat in addition to low weight, though all recovered and eventually gained weight normally. Overall weight loss was greatest in the SQS-21-Xyl groups.

3.3 Antibody Response

The antibody response after vaccination with GD3-KLH conjugate with or without adjuvant was determined with an ELISA assay using either GD3 ganglioside or KLH protein as target. The results are summarized in Table 1. Comparison of the different adjuvants at the same doses (10, 20 and 50 µg) of SQS-21, SQS-21-Api and SQS-21-Xyl were all equally effective at inducing an IgM antibody response against GD3 after 3 weekly vaccinations, with antibody titers. In each case, significantly higher titers were observed compared to the GD3-KLH alone group, with the exception of the SQS-21-Api 50 µg dose group (see toxicity description above). After the third vaccination, no IgG antibodies against GD3 were detected; however, after the fourth immunization, IgM and IgG antibody titers were induced in most mice. Again, there were no significant differences between the various groups with the exception of the 50 µg SQS-21-Api group, which had lower IgG antibody titers. The IgG antibody response against KLH was also strikingly elevated in all groups with no group demonstrating significantly higher or lower titers than the others. All were at least 20 fold higher than the GD3-KLH alone group. The results against GD3 (IgM) after the fourth vaccination in Figure 6 and against KLH after the third vaccination are illustrated in Figure 7.

The high immunogenicity of KLH combined with the potency of SQS-21 components at doses of 10µg and higher, as well as our 3 weekly immunization schedule, result in maximum titers that may have precluded legitimate comparison of the adjuvant potency of the Api and Xyl components. Consequently mice were immunized with a 1µg dose of KLH mixed with either 3 or 20 µg of SQS-21-Api, SQS-21-Xyl or SQS-21 (mix) on days 1 and 28, and sera tested at intervals before and after the second immunization. The results are demonstrated in Table 2. The Xyl and Api components have comparable activity both at the low 3 µg dose and the high 20 µg dose. They also generate comparable antibody titers against KLH both after the initial immunization and after the second immunization. However, 3µg of SQS-21-Xyl induced statistically significant ($p<0.004$) anti-KLH titer on day 42. Overall the SQS-21 (mix) induced high titer antibody at all time points when compared with SQS-21 isomer (Table 2). These data, arising from a less intense immunization schedule and lower administered doses of both KLH and SQS congeners, confirm the results in Table 1.

3.4 Cell Surface Reactivity by Flow Cytometry

Sera from 7 days after the 4th vaccination were tested for cell surface reactivity by flow cytometry using SK-Mel-28 (GD3 positive) cell line. The median FACS results are summarized in Table 1. Presera obtained from mice before immunization showed less than 10% positive cells, and sera from mice vaccinated with GD3-KLH plus adjuvant (all three synthetic SQS-21 adjuvants or the PQS-21) showed comparable significant positive reactivity with SK-Mel-28.

4. DISCUSSION

Recent advances in the target-oriented synthesis of saponin immunostimulating agents have enabled the completion of the first syntheses of the adjuvant QS-21-Api and QS-21-Xyl. These efforts have resulted in a highly modular synthesis via assembly of the four principle substructures of the molecules. The successful results from these synthetic efforts provides samples of QS-saponins with a heretofore unparalleled degree of purity. With these SQS-21-Api/Xyl products, there is no chance of trace saponin contaminants derived from purification protocols from the natural source, thereby eliminating uncertainties associated with uncontrolled heterogeneity in QS-adjuvants derived from traditional isolation protocols. Notably, the synthetic process has also been successfully adapted to produce GMP-grade SQS-adjuvant for upcoming clinical evaluation.

Herein, we describe in detail the adjuvanticity and toxicity of the synthetic apiose and xylose isomers that comprise QS-21, and compare this to the synthetic 65:35 mixture that simulates QS-21 obtained from the South American soap bark tree *Quillaja saponaria*. Our findings are that 10, 20 or 50 µg of either isomer or the 65:35 mixtures is equally potent as immunological adjuvants in mice. The antibody responses induced against the ganglioside GD3 and the highly immunogenic carrier protein KLH after immunization with GD3-KLH conjugates plus synthetic adjuvant (SQS-21-Api or SQS-21-Xyl) or the mixture were comparable to each other and to PQS-21, which is QS-21 fractionated from *Quillaja saponaria* [15]. The SQS-21-Xyl isomer, however, appeared to be slightly more toxic than either the corresponding apiose isomer, or the reconstituted SQS-21 mixture. The results described here, both adjuvanticity and toxicity, are quite similar to those that we have previously described with the same GD3-KLH conjugate and QS-21 isolated from *Quillaja saponaria*[5].

These data demonstrate that the two synthetic isomers, SQS-21-Api and SQS-21-Xyl, are equally potent whether administered individually or as a mixture comparable to that of the QS-21 isomeric ratio reported to be obtained from natural sources [28]. This leaves no doubt that the highly purified synthetically derived isomer constituents of the QS-21 fraction are competent adjuvants in ganglioside-KLH conjugate vaccines. These findings lay the groundwork for investigation and imminent establishment of a thorough structure-activity profile for this potent adjuvant, for which concrete mechanism-of-action studies are sorely lacking. For example, Marciani has described three components of QS-21 that are required for optimal immunogenicity [26]. These include: (1) the hydrophobic chain, postulated to facilitate binding to the cell surface lipid bilayer; (2) the oligosaccharide moieties, postulated to mediate the interaction of saponin (or associated antigen complexes) to cell-surface lectins on antigen presenting cells; and (3) the saponin aldehyde, postulated to engage in covalent (Schiff-base) conjugation with certain T-cell surface-NH₂ groups and evoking a co-stimulatory second signal leading to Th1 responses. It is this proposed combination of immunological effects on the macrophage, dendritic cell and T-cell arms of the immune system that may be important for the potency of saponins as immunological adjuvants. While SQS-21 has the academic advantage of unambiguously defining the structures of the active components in QS-21, it does not compete on a cost basis with QS-21 obtained by extraction. However, the advantages of the synthetic SQS products are evident when considering the high level of purity of these

synthetic entities relative to naturally isolated QS-saponins, often incorporating variable quantities of trace natural impurities. Moreover, having demonstrated the potency and relative safety of the SQS-21 isomers, we are now uniquely poised to determine the contribution of these and additional QS-saponin molecular components to adjuvant potency and toxicity as we systematically probe structure/function correlations. This indeed highlights the true value of the SQS-chemical synthesis technologies – the ability to engage in unfettered systematic design and synthesis of novel SQS-analogues. The need for these advances arises as a result of some drawbacks associated with the use of naturally derived QS-21, including local and systemic toxicity [27], as well as the rapid loss of adjuvant activity when stored at room temperature as a consequence of spontaneous chemical degradation arising from acyl chain hydrolysis[12]. Using the immunological evaluation protocols described herein in combination with the chemistry innovations in SQS-saponin synthesis, the design and preparation of novel saponin adjuvants with increased potency, enhanced stability, and attenuated toxicity are currently underway, and will be reported in due course.

Based on these results, a clinical trial with SQS-21 containing a 65:35 ratio mix of the two synthetic isomers has recently been initiated with a GD3-KLH and GD2-KLH bivalent vaccine to confirm the safety and potency of SQS-21 in melanoma patients.

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The abbreviations used are

BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
HSA	human serum albumin
KLH	keyhole limpet hemocyanin
MFI	mean fluorescence intensity
PBS	Phosphate buffered saline
PQS	Purified QS-21
QS	<i>Quillaja saponaria</i>

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Figure 1.
Scheme of QS-21-Api and QS-21-Xyl synthesis

Figure 2.
Scheme for preparation of the GD3-KLH conjugate

Figure 3.

Toxicity study in C57BL/6J female mice injected with GD3-KLH conjugate plus SQS-21 (65% SQS-21-Api + 35% SQS-21-Xyl) or PQS-21 adjuvant

Figure 4.
Toxicity study in C57BL/6J female mice injected with GD3-KLH conjugate plus SQS-21-Api or PQS-21 adjuvant

Figure 5.
Toxicity study in C57BL/6J female mice injected with GD3-KLH conjugate plus SQS-21-Xyl or PQS-21 adjuvant

Figure 6.

Median serological response against GD3 for mice vaccinated with 10 μg dose of GD3-KLH plus 10, 20, or 50 μg of SQS-21 adjuvant. Serum collected 7 days after 4th vaccination.

Figure 7.

Median serological response against KLH for mice vaccinated with 10 μ g dose of GD3-KLH plus 10, 20, or 50 μ g of SQS-21 adjuvants. Serum collected 7 days after 3rd vaccination.

Anti-GD3 and Anti-KLH antibody titers by ELISA and cell surface reactivity against SK-Mel-28 by FACS after vaccination with GD3-KLH conjugate with SQS-21, SQS-21-Api or SQS-21-Xyl. Each value represents median value of sera tested from 5 mice. Sera were drawn 7 days after the third vaccination (KLH) or fourth vaccination (GD3, FACS).

Table 1

Group#	Vaccine	Adjuvant	Dose	KLH	IgG	GD3	IgM	GD3	IgG	SK-MEL-28
										% Pos IgM (MFI)
1	GD3-KLH	No Adjuvant	NA	12800	0	0	0	0	0	36% (15)
2	GD3-KLH	SQS-21	10µg	819200	320	320	320	320	320	71% (137)
3	GD3-KLH	SQS-21	20µg	819200	640	320	320	320	320	92% (220)
4	GD3-KLH	SQS-21	50µg	819200	240	320	320	320	320	71% (132)
5	GD3-KLH	SQS-21-Api	10µg	204800	320	320	320	320	320	95% (62)
6	GD3-KLH	SQS-21-Api	20µg	819200	320	320	320	320	320	80% (174)
7	GD3-KLH	SQS-21-Api	50µg	266240	80	160	160	160	160	90% (297)
8	GD3-KLH	SQS-21-Xyl	10µg	819200	320	240	240	240	240	77% (152)
9	GD3-KLH	SQS-21-Xyl	20µg	819200	640	240	240	240	240	92% (271)
10	GD3-KLH	SQS-21-Xyl	50µg	819200	640	480	480	480	480	71% (216)
11	GD3-KLH	PQS-21	20µg	819200	320	320	320	320	320	82% (163)

Table 2

Anti-KLH antibody titers by ELISA after mice vaccination with 1µg KLH alone or with 3 and 20µg of SQS-21, SQS-21-Api or SQS-21-Xyl. Each value represents median value of sera tested from 5 mice vaccinated on days 1 and 28, and sera were obtained from blood drawn on day 21 and 42 from the first vaccination.

Group#	Vaccine	Adjuvant	Dose	Median anti-KLH IgG Titer	
				IgG	IgG
1	1µg KLH	No Adjuvant	None	0	0
2	1µg KLH	SQS-21	3µg	1600	819200
3	1µg KLH	SQS-21	20µg	3200	819200
4	1µg KLH	SQS-21-Api	3µg	800*	51200‡
5	1µg KLH	SQS-21-Api	20µg	3200	819200
6	1µg KLH	SQS-21-Xyl	3µg	1600*	204800‡
7	1µg KLH	SQS-21-Xyl	20µg	3200	512000

* Statistical analysis showed no significant antibody titers obtained from day 21 sera between mice immunized with 3µg of SQS-21- Api and SQS-21-Xyl. The p value is 0.4.

‡ Statistical analysis showed a significant antibody titers obtained from day 42 sera between mice immunized with 3µg of SQS-21- Api and SQS-21-Xyl. The p value is 0.004.