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Natural and synthetic saponin adjuvant QS-21 for vaccines against cancer

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Abstract

One of the most widely used and potent immunological adjuvants is a mixture of soluble triterpene glycosides purified from the soap bark tree (*Quillaja saponaria*). Despite challenges in production, quality control, stability and toxicity, the QS-21 fraction from this extract has exhibited exceptional adjuvant properties for a range of antigens. It possesses an ability to augment clinically significant antibody and T-cell responses to vaccine antigens against a variety of infectious diseases, degenerative disorders and cancers. The recent synthesis of active molecules of QS-21 has provided a robust method to produce this leading vaccine adjuvant in high purity as well as to produce novel synthetic QS-21 congeners designed to induce increased immune responsiveness and decreased toxicity.

Keywords

adjuvants; saponins; synthetic adjuvants; vaccines

Molecular identification of human tumor antigens found to be immunogenic in humans after natural exposure or treatment with cancer vaccines has resulted in a wide array of increasingly focused vaccines designed to further augment their immunogenicity. However, as vaccines against cancers (and infectious diseases) have employed increasingly focused and homogeneous antigens for vaccine construction, a marked decrease in the immunogenicity of these antigens has been observed. For viral or bacterial antigens this resulted from the loss of the surrounding pathogen-associated molecular patterns (PAMPs) in and at the surface of these pathogens during purification [1,2]. This can be offset to a significant degree by conjugating these homogeneous antigens to immunogenic carrier proteins and by the use of potent immunological adjuvants. In the case of viral or bacterial vaccines, either one of these approaches has proven sufficient. However, in the case of vaccines against cancer antigens, which are inevitably autoantigens or minimally modified autoantigens, the use of both carrier protein conjugation and a potent adjuvant has resulted in the highest immune responses. In both infectious disease and cancer settings the

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immunological adjuvant selected is critically important and in both settings saponin adjuvants, such as QS-21, and adjuvants that contain QS-21, have proven to be among the most potent.

Selection of QS-21 as a uniquely potent immunological adjuvant

The adjuvant effect of saponins was first mentioned in 1925 (alongside the adjuvant effect of bread crumbs, tapioca and soy starch), when it was found to greatly augment the antibody response against diphtheria or tetanus [3,4]. Beginning in 1964, saponins extracted from the bark of the South American tree, *Quillaja saponaria* Molina, became the major focus for saponin research focused on adjuvant activity [5]. From the start saponins were known to augment not only antibody responses but also helper and cytotoxic T-cell responses. Since that time, Quillaja saponins have been extensively used alone and also mixed with aluminum salts [6], liposomes and oil-in-water emulsions [7] or with amphipathic proteins and lipids forming detergent/lipid/saponin complexes termed immune-stimulating complexes (ISCOMs) [8]. Kensil *et al.* purified a series of fractions from *Q. saponaria* Molina bark by reverse-phase chromatography (RP-HPLC), with fractions QS-7, QS-17, Q-18 and QS-21 noted to be particularly potent adjuvants when mixed with a series of xenoantigens [9]. The main saponin component, QS-18, was found to be highly toxic in mice but saponins QS-7 and QS-21 were far less toxic. QS-21, being more abundant than QS-7, was selected and has been the most widely studied saponin adjuvant for more than 15 years.

Because QS-21 was originally designated as a particular fraction on a complex RP-HPLC trace, it is not surprising that it comprises several distinct saponin molecules. There are two principal isomeric molecular constituents of the QS-21 fraction (Figure 1). Both of these saponins incorporate a central triterpene core, to which a branched trisaccharide is attached at the terpene C3 oxygen functionality, and a linear tetrasaccharide is linked to the triterpene C28 carboxylate group. A fourth component within the saponin structure is a glycosylated pseudo-dimeric acyl chain attached to the fucose moiety via a hydrolytically labile ester linkage. The isomeric components differ in the constitution of the terminal sugar residue of the tetrasaccharide, in which the major and minor compounds incorporate either an apiose (65%) or a xylose (35%) carbohydrate, respectively.

Adjuvant active saponins from a variety of sources other than *Q. saponaria* Molina have been identified and their structure determined. Based on this empiric, but nevertheless relevant, survey it is possible to draw some tentative conclusions concerning the contributions of different portions of the QS-21 molecule to toxicity on the one hand and adjuvant activity on the other, as recently reviewed by Sun *et al.* [10]. The sugar side chains of QS-21 [11,12], the presence of both hydrophilic and hydrophobic functional groups, and the aldehyde groups all contribute to QS-21 adjuvant activity. The mechanism underlying the aldehyde importance may relate to formation of a Schiff base with free amino groups on the surface of immune cell targets [13]. Also, the property of Quillaja saponins to stimulate cytotoxic T-cell proliferation appears to depend on the lipophilic acyl side chain [14]. The amphipathic nature of QS-21 makes it an ideal adjuvant for mixing with most large protein antigens or conjugate vaccines where adjuvant and antigen are likely to stay in close proximity where injected (depot effect) until incorporated into antigen-presenting cells (APCs). Unconjugated carbohydrate or peptide antigens on the other hand may require administration with aluminum salts, liposomes, ISCOMs, ISCOMATRIX® (CSL Behring) or other adjuvant formulations conferring a depot effect or facilitating uptake of antigen and adjuvant by APCs.

We have previously tested 19 different adjuvants in mice with conjugate vaccines containing ganglioside antigen GD3 and mucin peptide MUC1 conjugated to the carrier protein keyhole limpet hemocyanin (KLH) [15]. The differences were evaluated based on their ability to augment antibody responses against GD3, MUC1 and KLH, as well as their ability to augment the T-cell response against KLH. QS-21 significantly outperformed the other classes of adjuvants including glucan formulations, peptidoglycans, amphophilic block copolymers, bacterial nucleosides and bacterial lipopolysaccharides. Purified *Q. saponaria* fraction QS-21 was most potent, with antibody titers induced against the three antigens generally proportional to cytokine response induced against KLH and also proportional to dose. However, when the dose resulted in greater than 10% weight loss, increasing doses were seen to result in decreasing titers. These studies emphasized the two critical and generally proportional variables in adjuvant discovery: immunologic potency and toxicity.

Clinical trials with vaccines targeting the ganglioside GM2 have come to the same conclusion: conjugation to KLH and the use of immunological adjuvant QS-21 is the optimal approach. Initially, GM2 ganglioside was incorporated onto the surface of liposomes containing *Salmonella minnesota* mutant R595, BCG, proteasomes and monophosphoryl lipid A [16]. Of these, the use of BCG was found to be optimal, but subsequent comparison of this GM2 adhered BCG vaccine with GM2–KLH conjugate vaccines containing no adjuvant, or mixed with BCG, detox (BCG cell wall skeletons plus monophosphoryl lipid A) or QS-21 found that GM2–KLH conjugate plus QS-21 was strikingly superior, inducing both IgM and IgG antibody responses [17]. Doses of QS-21 in the 100–200 µg range were optimal and well tolerated in the cancer patient population [18]. The toxicity in this dose range was 2–10 cm of erythema and induration at injection sites in most patients, as well as occasional mild low-grade flu-like symptoms. At this time, over 1000 patients have been vaccinated with QS-21 containing vaccines with a dose of 100 µg and no dose-limiting toxicity has been described. Although reformulations of QS-21 with certain excipients helps to reduce pain and improve acceptability of 50 µg doses [19], the consistent grade I or II local toxicity and occasional grade I flu-like symptoms after vaccination will probably exclude the use of QS-21 for routine immunizations in general pediatric or adult populations at doses above 50 µg. The semisynthetic saponin adjuvant GPI-0100, which had been identified in our initial preclinical studies to exceed QS-21 in potency [15], was also tested in clinical trials. While a GPI-0100 dose of 2 mg was well tolerated in prostate cancer patients, and antibody titers appeared to be superior to those induced by 100 µg of QS-21, four out of six women immunized with vaccines containing GPI-0100 at this dose demonstrated grade II or III hepatic toxicity (transaminitis) lasting 3–6 months prior to normalization. Low (500 µg) doses of GPI-0100 were well tolerated but antibody responses were no longer as high as those obtained with 100 µg of QS-21 [10–14].

Current status of clinical trials utilizing QS-21 as an immunological adjuvant

The majority of clinical experience with QS-21 in therapeutic vaccinology has occurred in cancer patient populations where it has been widely utilized, especially in the adjuvant setting (after surgical resection of all known local or systemic metastases). Phase I trial formulations containing QS-21 have been conducted in patients with melanoma [18,20,21], and patients with cancers of the breast [22–24], prostate [25–27], ovary [28] or lung [29–31]. Additional QS-21 containing studies have been conducted in lymphoma [101] and leukemia [102], neuroblastoma [KUSHNER BH *ET AL.* EVALUATION OF ANTI-GD2 AND ANTI-GD3 IMMUNE RESPONSE OF PATIENTS VACCINATED WITH GD2 LAC-TONE AND GD3 lactone-keyhole limpet hemocyanin conjugate vaccine plus immunological adjuvant OPT-821, MANUSCRIPT IN PREPARATION] and renal cell carcinoma [103], including numerous Phase II studies. All trials demonstrated potent antibody responses against the carbohydrate and peptide antigens in the associated KLH-

conjugate vaccines. The doses utilized in these trials varied between 100 and 200 µg per vaccination and side effects were again restricted to local erythema and induration in most patients as well as occasional flu-like symptoms. The dosing schedule found to be optimal for immunization against these cancer-related autoantigens was three immunizations at 1-week intervals followed by a fourth immunization 4 weeks later and finally booster immunizations at 3-month intervals. No vaccine doses had to be held, delayed or decreased owing to toxicity that was clearly related to the vaccinations.

Two randomized Phase III trials with GM2 ganglioside-KLH conjugate vaccine plus QS-21 have been conducted with more than 1000 patients receiving this vaccine with a QS-21 dose of 100 µg per patient [32,33]. Again, the vaccines were well tolerated even in these early stage melanoma patients, and even though the trials were negative (no benefit in terms of progression rate or survival in patients receiving the vaccine when compared with randomized controls), more than 90% of tested patients produced a potent antibody response against GM2. It may be that there is an insufficient amount of GM2 on most melanomas to be useful as the sole target (less than 20% of melanomas can be killed with immune sera or monoclonal antibodies and human complement).

Based on this experience, there are currently four randomized, double-blind, multicenter Phase II trials with monovalent or polyvalent antigen-KLH conjugate plus immunological adjuvant natural QS-21 vaccines ongoing in cancer patients. A total of 68 unresectable stage III or IV melanoma patients are being randomized to receive one of two adjuvant formulations; SB-AS15 or SB-ASO2B (containing QS-21), plus MAGE3 protein, in a small Phase II trial conducted by GlaxoSmithKline and the European Organization for Research and Treatment of Cancer (EORTC) in Europe. A total of 126 patients with resected Stage IV sarcomas are being randomized to receive either a dose of 150 µg of QS-21 alone or mixed with a trivalent KLH-conjugate vaccine targeting GM2, GD2 and GD3 gangliosides in a trial conducted in the USA by MabVax Therapeutics. A total of 162 ovarian cancer patients in second complete remission will be randomized to receive either 100 µg of QS-21 or QS-21 with a pentavalent vaccine targeting GM2, globo H, MUC1, Thompson-Friedenreich antigen and Tn, in a trial conducted by the National Cancer Institute sponsored Gynecologic Oncology Group. There is also a Phase II trial of an NY-ESO-1 protein plus ISCOMATRIX (containing a saponin mix) vaccine in 110 stage III melanoma patients being conducted by The Ludwig Institute. Accrual for each of these possibly pivotal trials should be completed by the end of 2012 with preliminary results available a year later.

The safety and potency of QS-21 as an immunological adjuvant has also been demonstrated in Phase I trials of vaccines against HIV-1 envelope subunit [34], *Plasmodium falciparum* malaria peptides SPf 66 [35] and RTS,S/AS02D [36] and hepatitis B surface antigen [37]. Based on these trials, and the larger experience with cancer vaccines, there are a variety of Phase II trials currently underway with vaccines containing QS-21 in the noncancer setting. These include a large Phase II/III trial in children up to 35 months of age in Africa of a malarial vaccine versus control, with AS01 (containing QS-21) as an immunological adjuvant. This pivotal trial is projected to enroll 16,000 patients across seven countries. There are also six Phase II trials being conducted by Pfizer and one by Wyeth in over 800 patients targeting Alzheimer's Disease with QS-21-containing vaccines.

Challenges with the use of QS-21 adjuvant

There are several problems with the continued focus on natural QS-21 as an adjuvant in vaccines against cancer and infectious diseases. The first relates to the toxicity/potency issue raised earlier. A dose of 100 or 150 µg of QS-21 is relatively well tolerated in the cancer patient population and it has proven more potent than other immunological adjuvants for

augmenting immune responses against a variety of antigens. However, correlations of QS-21 dose to immune potency indicate that if higher doses could be administered safely, immunogenicity could be further increased. For most patient populations, however, the side effects associated with QS-21 limit doses to about 50 µg, with the exception of cancer. Clearly if toxicity could be reduced, dose, and therefore immunogenicity, in both settings might be significantly increased.

The chemical instability of QS-21 is a second drawback [38]. Hydrolytic removal of the acyl chain fragment of the QS-21 molecule occurs spontaneously in solution, especially at a pH 7.4 and in warmer temperatures. As described above, loss of the acyl chain results in an immunologically inactive product. This precludes the use of QS-21 as a stand-alone adjuvant in many third world settings where storage at 4°C or colder is difficult. Notably, the only current methods to address this liability are to modify the multi-component QS-21 fraction to even more heterogeneous compositions, such as formulations in ISCOMS (e.g., ISCOMATRIX) [39], liposomes (e.g., AS01) and proprietary surfactant emulsions (e.g., AS02) [7].

The third drawback, limited supply, has increasingly become the major constraint on the widespread use of QS-21 [40]. There is high variability in saponin composition between *Q. saponaria* trees, even in the same local environment due to factors that are not completely understood. QS-21 is one of many fractions and represents a small, in some case increasingly small, component of the 22 saponin fractions extracted from this bark [41]. Overexploitation of *Q. saponaria* bark has resulted in ecological damage and shortage of available supplies even under the current demand. While novel methods of extraction from Quillaja greatly improves the availability of saponins from ecologically friendly tree prunings, this model of Quillaja harvesting does not improve the shortage of QS-21 [42]. Although the quality and composition of this crude quillai is certainly enough to accommodate the global needs of food and agriculture, the quality of these extracts does not support large scale extraction of QS-21 for global vaccine efforts. A major hurdle to understanding global QS-21 supply is the obscure reporting of yields and processing of Quillaja extracts. This dearth of reported QS-21 yield is a direct result of the tremendous variability in the content and composition of Quillaja saponins, ultimately determined by weather, soil composition, season and age of the trees, which makes it difficult to have a reliable source of QS-21 from *Q. saponaria*. We believe that a carefully detailed study of the yields of QS-21 from all available starting materials and purification methods would greatly benefit the vaccine industry. In an extremely optimistic calculation, accounting for global supply and consumption of Quillaja bark, competition for the bark with the food and beverage industry as well as the best reported Quil A extraction efficiency and our best yielding conversion of Quil A to QS-21, we estimate that the current global supply of natural QS-21 is approximately 6 million individual 100 µg doses. This extremely optimistic estimate does not approach qualification as an adequate supply given the many potential indications to which QS-21-adjuvanted vaccines would be applied. If a QS-21-containing vaccine was US FDA approved for widespread clinical use, this demand would greatly increase and supply from natural sources might no longer suffice.

Enhancing the potential of QS-21 as a vaccine adjuvant through chemical synthesis

The challenge of isolating highly purified QS-21 directly from the natural source with batch-to-batch consistency in composition has led to alternate methods of procurement of this saponin adjuvant. One attractive means to address this crippling impasse is through the chemical synthesis of the fraction's active molecular constituents. However, despite the clinical potential of QS-21, a surprising dearth of chemical synthesis efforts have been

reported toward these saponins [43–45]. Over a decade-long effort, we have accomplished the first and only chemical synthesis of the principal isomeric constituents of QS-21, namely QS-21-Api [46,47] and QS-21-Xyl [48]. In addition, each of these synthetic saponins was verified to exhibit comparable adjuvant activity *in vivo* in preclinical evaluations employing the GD3–KLH melanoma conjugate antigen [49]. This validation of both structural and biological equivalence of our synthetic versus our naturally-derived QS-21 fraction now overcomes the procurement challenges with natural QS-21. Moreover, a second generation synthesis for the saponin adjuvant has recently been developed in which the entire prosapogenin active portion of the QS-21 constituents can be isolated in pure form and incorporated into a robust semi-synthesis strategy [50]. This more advanced approach allows for isolation of half of the molecule in yields that exceed that of QS-21 isolation by at least two orders of magnitude, and enables increased efficiency of the synthesis by more than 25% compared with the original synthetic protocol. Most importantly, our synthetic QS-21 (SQS-21) offers consistency in batch-to-batch composition and is currently the only means by which to acquire the saponin adjuvant in molecular homogeneous form, devoid of naturally derived trace impurities. A pilot clinical trial has recently been completed employing SQS-21 as the adjuvant for a bivalent melanoma conjugate vaccine (GD3–KLH plus GD2–KLH). Mild toxicity and potent immune responsiveness comparable to the same vaccine with natural QS-21 have been demonstrated.

The chemical insights gained from our synthetic efforts toward SQS-21 have led to the emergence of added benefits, namely the application of our saponin chemistry to design and construct novel adjuvants that may overcome the hydrolytic and toxic liabilities associated with QS-21. For example, the acyl-fucose ester linkage within the QS-21 saponins is known to be susceptible to spontaneous hydrolytic scission, contributing to *in vitro* (and likely *in vivo*) decomposition with potentially negative effects on dose management and tolerability. To circumvent these liabilities, a series of non-natural SQS saponin adjuvants was designed and synthesized (Figure 2). The first, SQS-0101, incorporated rather conservative structural modifications in which the labile ester groups within the acyl chain are replaced by more hydrolytically robust amide linkages. More profound structural changes took the form of SQS-0102 and SQS-0103, which incorporate highly simplified lipophilic acyl chains, either glycosylated or nonglycosylated, respectively. Importantly, when the immunopotentiating ability of each of these novel adjuvants was evaluated in mice with the GD3–KLH melanoma conjugate antigen, all SQS saponins exhibited adjuvant activities rivaling that of our naturally derived QS-21 [50]. In addition, differential toxicity profiles were observed, whereby SQS-0102 exhibited markedly enhanced toxicity, yet SQS-0101 and SQS-0103 elicited significantly reduced toxicity. These promising results highlight the exciting prospect of designing improved saponin adjuvants based on the Quillaja molecular skeleton (i.e., enhancing potency with attenuation of toxicity).

Expert commentary

Vaccines will continue to grow into an ever wider landscape of prophylactic and therapeutic applications for the prevention of acute infectious diseases as well as the treatment of chronic infections and other diseases such as Alzheimer's disease and cancer. In both the infectious disease and the cancer settings, the immunological adjuvant selected has been demonstrated to be critically important. Saponins such as QS-21 have proven to be among the most potent immunological adjuvants. QS-21 has been used in over 80 clinical studies involving approximately 15,000 patients across over 20 indications, including malaria, hepatitis, Alzheimer's disease and especially cancer. Although QS-21 has generally outperformed the other classes of adjuvants, there are problems with the continued focus on natural QS-21 as an adjuvant in vaccines against cancer and other diseases. These include local and systemic toxicity that limits dose, chemical instability (especially when not

refrigerated) and its increasingly limited availability from natural sources. The recent validation of both structural and biological equivalence of synthetic QS-21 saponin molecules now overcomes the procurement challenges with natural QS-21 and has made it possible to address some of the problems associated with its use. Synthetic QS-21 congeners have been recently developed that overcome stability concerns and have improved potency/toxicity ratios in preclinical settings while offering unparalleled purity. These promising results allude to the exciting prospect of improved saponin adjuvants capable of further augmenting the potency of vaccines against cancer and a variety of other diseases.

Five-year view

Following sipuleucel-T's approval in April 2010, it is likely that within the next 5 years, one or more additional therapeutic cancer vaccines will be approved for use by the US FDA. These vaccines may utilize direct immunization or adoptive cytotoxic lymphocyte transfer. Based upon current clinical trial progressions the most likely indications to get approval in this time frame will be for melanoma, breast cancer or lung cancer. These vaccines will undoubtedly utilize recombinant and synthetic antigens. They will also require immunogenic carrier proteins as well as a potent adjuvant to elicit the most robust immune response.

It is also likely within the next 5 years that additional adjuvants will be approved for use by the FDA. Based upon current clinical trial progressions, the most likely novel adjuvant-containing vaccine to achieve approval will contain a saponin. Over the past two decades, saponin adjuvants have emerged as leading adjuvant candidates. QS-21 in particular has been widely used in developing vaccine platforms, particularly for malaria, hepatitis and cancer. Due to the late stage of development, we believe the vaccine most likely to fulfill this criteria is GlaxoSmithKline's RTS,S malarial vaccine, which is currently in Phase III studies in children and contains QS-21 as part of its adjuvant formulation. Due to the robust development of cancer vaccines containing saponins, such as the Gynecologic Oncology Group's Phase III ovarian/fallopian/peritoneal cancer vaccine, saponins will no doubt be one of the first alternative adjuvants approved for use in the USA.

Synthetic methods will greatly ease procurement issues associated with QS-21. Although replacing natural QS-21 with synthetic QS-21 in ongoing trials may be more difficult for vaccine manufactures than initiating new trials with synthetic QS-21, owing to the limitations of natural saponins and purification, it is likely that vaccine manufacturers will rely more and more upon a synthetic QS-21 replacement in the near future. Furthermore, the chemical insights gained from our efforts have led us to develop novel adjuvant compounds that do not exist in nature. Synthetic saponin adjuvants will provide the vaccine industry with the capability of having extremely safe and highly efficacious adjuvants that are shelf stable and easy to deploy throughout the world. These adjuvants will be able to deliver multifold improvements in efficacy, while simultaneously improving safety and reducing local reactogenicity. Although 5 years is an optimistic timeline for the approval of a non-natural saponin adjuvant, we believe that multiple clinical trials will open within this time that utilize a synthetic saponin analog, in both therapeutic and prophylactic settings. We also believe that one of these novel compositions will gain approval for use within the next 10 years. We also hold the optimistic belief that synthetic saponin adjuvants will gain approval for routine pediatric use in prophylactic indications as well.

Perhaps most importantly, the observable structural activity relationships made possible by synthetic saponin compounds will help the research community to better understand the mechanisms driving adjuvant activity, and allow for the design of a completely new generation of small molecule adjuvants.

Dedication

This work is dedicated to the memory of our deceased friend and colleague, David Y Gin.

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Key issues

- Adjuvants are critical components in the vaccine industry.
- Vaccines against cancer using both cancer antigen–carrier protein conjugates and a potent adjuvant have resulted in the highest immune responses.
- The natural product known as QS-21 is an important lead investigational adjuvant exhibiting exceptional adjuvant properties for a range of antigens.
- In both the infectious disease and cancer settings, the adjuvant is critically important and QS-21 has proven to be among the most potent.
- There are significant challenges in obtaining natural QS-21.
- The recent synthesis of QS-21's active molecules has provided a robust method to produce this leading vaccine adjuvant at high purity as well as to produce novel synthetic saponin adjuvants for use in a variety of formulations.

Figure 1.
The principal molecular constituents of QS-21.

Figure 2.
Novel, stable, non-natural, potent synthetic saponin adjuvants.