

Rationally Engineered Proteins or Antibodies with Absent or Reduced Immunogenicity

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Abstract: One challenge associated with the clinical use of protein therapeutics destined for chronic administration is the potential for the development of unwanted anti-drug immune reactions. The molecular basis for this reactivity is the binding of peptide fragments (epitopes) derived from the breakdown of the protein drug to the HLA receptors expressed by the patient's immune cells. If these epitopes are recognized as "foreign" by the immune system, specific helper T lymphocytes (HTL), are activated, which initiate and direct the formation of antibodies against the protein drug. These antibodies can bind and neutralize the protein drug, resulting in either decreased efficacy or total ineffectiveness of the drug. Moreover, various safety concerns, such as allergic reactions and other adverse events, are also frequently associated with the formation of anti-drug antibodies. Herein, we describe the development of "ImmunoStealth", an integrated bioinformatics, biochemical and cellular immunology approach that specifically addresses the issue of unwanted immune responses against protein therapeutics. Unwanted HTL epitopes are identified using *in silico* sequence analysis methods and high throughput *in vitro* biochemical evaluations and thereafter confirmed using cellular immunogenicity assays. The "offending" epitopes within the drug are then rationally modified to alter their HLA binding capacity, and thus render them non-recognizable by the immune system. This technology will ultimately facilitate the design of safer, more potent and more economical drugs.

Key words: epitope, ImmunoStealth, antibody, HTL

INTRODUCTION

Over the last few years, an ever-increasing number of protein drugs have entered clinical trials or received approval for product registration. Several of these drugs are recombinant hormones, lymphokines and growth factors, such as insulin, factor VIII, interferons (IFN), Interleukin-2 (IL-2) and Granulocyte Macrophage colony stimulating factor (GM-CSF). Antibodies, such as the registered products Remicade (anti-TNF), Rituxan (anti-CD20) and Herceptin (anti-Her2neu) also add to this category. In fact, 30-40% of all drug products currently in development are monoclonal antibodies targeting a wide variety of indications ranging from metabolic disorders to cancer and autoimmune diseases. Finally, as a result of the recent explosion in the volume of sequence and expression data provided by genomic/proteomic projects, new links between protein expression and pathological conditions are being discovered. These advances are predicted to further amplify the number of protein drug candidates developed to treat various pathological conditions.

Protein drugs are accessible to several different therapeutic applications. For example, in cases where pathology is caused by a protein deficiency (e.g factor VIII in hemophiliacs) administration of a recombinant or purified

protein product is often of therapeutic value. By contrast, pathologies associated with over-expression of a given protein may be treated by the administration of monoclonal antibodies directed against the overexpressed protein (e.g. use of Herceptin antibody targeted against Her-2neu protein in breast cancer). In addition, synergistic to the increase in protein drugs and drug candidates is the development of exciting new technologies that allow the engineering of human proteins to achieve novel or dramatically improved pharmacological properties, for example, the development of Lispro, the fast-acting analog of insulin and the development of Proleukin, a single-substitution analog that is a non-aggregated and rapidly acting form of Interleukin-2.

Within this rapidly-growing category of therapeutics, a major and significant complication is frequently encountered, namely the development of immune reactions to the protein drug. The risk is especially high for chronically administered products. The route of delivery also plays an important role in rendering a protein drug immunogenic. Regardless of the cause, following activation of specific immune lymphocytes, a cascade of immune recognition events results in the formation of anti-drug antibodies. These antibodies, in turn, make the drugs less effective or cause other safety issues.

EVIDENCE OF IMMUNE RESPONSES AGAINST PROTEIN DRUGS

What evidence is available regarding the nature and incidence of the development of antibodies against protein

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drugs? A literature survey of the field indicates that immunogenicity issues exist with many of the currently marketed protein therapeutics. Well-documented examples include the development of antibodies against factor VIII in hemophiliacs treated with the drug [1,2], against calcitonin in patients treated for osteoporosis [3-5], against erythropoietin in patients undergoing therapy for chronic renal failure [6,7] and against interferon antibodies in individuals undergoing treatment for multiple sclerosis [8]. In the cases of IFN β and factor VIII, the percentage of individuals developing neutralizing antibodies is as high as 30-40% [1,8]. Furthermore, recent reports regarding the development of antibodies against erythropoietin, which leads to aplasia, provide clear evidence that the development of anti-drug antibodies can cause severe safety problems [6,7].

In case of factor VIII, the predominantly used drug is produced either by recombinant technology though a limited amount is still produced from human plasma. Even though the protein is a self-molecule, antibodies (or so called "inhibitors") develop in a large number of patients. One approach that attempts to circumvent this problem is the development of a factor VIII molecule with a deleted B-domain (Refacto from Pharmacia) with the hope that a smaller molecule may be less immunogenic. However, since it has been reported that the most immunogenic T and B cell epitopes are located within the A3, C1 and C2 domains [9,10], deletion of the B-domain might not lessen immunogenicity. Another approach is the generation of hybrid proteins utilizing both human and porcine sequences [11]. Antibodies from patients treated with the human factor VIII do not recognize this hybrid molecule, however, this molecule might eventually be immunogenic itself due to the non-self nature of the hybrid protein.

Calcitonin is used for treatment of Paget's disease, hypercalcemia and osteoporosis and is a 32- amino acid polypeptide derived from salmon origin. Salmon calcitonin is preferred for therapeutic use over human calcitonin since it is 50-100 times more potent. Salmon calcitonin differs from human calcitonin in 17 out of 32 amino acids, which results in formation of neutralizing antibodies in a large number of patients taking this drug [5]. The antibody response causes some patients to either become resistant to the treatment or results in other side effects [12]. Several chimeric human and salmon analogs have been described with biological activity comparable to salmon calcitonin, however, it is not clear if the use of these chimeric molecules reduces the formation of neutralizing antibodies [5,13].

Human erythropoietin is a heavily glycosylated endogenous protein used for the treatment of anemia in patients with chronic renal failure. Some of the commercially available products include epoetin alpha, epoetin beta and darbepoietin alpha; these products differ from each other in their glycosylation patterns. Recent reports have demonstrated that treatment with recombinant erythropoietin can result in pure-red cell aplasia, resulting in a significant safety risk [6,7,14]. Moreover, it has been established that the development of aplasia was due to the formation of antierythropoietin antibodies. These antibodies cross-reacted with all known brands of erythropoietin and

also recognized deglycosylated epoetin, suggesting that the reactivity was targeted against the protein moiety of the molecule. Since the initial report early this year, several new cases of aplasia have been reported and are being characterized, highlighting the immediacy and importance of addressing this issue in patients undergoing epoetin therapy.

Interferon beta 1b (IFN β) therapy is an effective treatment for patients with relapsing-remitting Multiple Sclerosis. One disadvantage of this treatment is the occurrence of antibodies against IFN β that inhibits its biological activity [8]. These antibodies are neutralizing in nature and patients with these neutralizing antibodies respond to IFN β less well than patients without antibodies [15]. The two commercially available forms are the glycosylated IFN- β_{1a} and the non-glycosylated IFN- β_{1b} . Both forms induce the production of neutralizing antibodies, however, the IFN- β_{1b} molecule is reported to be more immunogenic than the IFN β_{1a} molecule [16]. It has been suggested that this difference may be due to the chemical structure of the former, which can produce aggregates that enhance antibody production, however this explanation has not been demonstrated.

As mentioned above, the field of protein therapeutics is also undergoing a revolutionary change with the advent of protein modification technologies aimed at improving therapeutic proteins. To this effect, several different approaches have been undertaken to analog proteins to improve their pharmacological properties. However, several studies that have demonstrated that even single amino acid residue change can dramatically increase the immunogenicity of protein molecules [17]. Moreover, it has been demonstrated that T lymphocyte reactivity is focused on the region immediately adjacent to the changed amino acid and is not due to any conformational effect [18]. Since these immunogenic regions correspond to short linear stretches of the protein sequence, they can be rationally modified to reduce or eliminate immunogenicity with minimal impact on the structure or function of the molecule. The ImmunoStealth technology is based on this approach.

IMMUNOGENICITY ASSOCIATED WITH MONOCLONAL ANTIBODIES

Monoclonal antibodies can also be powerful immunogens. If antibodies of murine origin are administered to patients, a human anti-mouse response promptly develops leading to inactivation or decreased efficacy of the monoclonal antibody drug [19]. These results provide a clear demonstration that development of anti-drug antibodies can lead to decreased drug efficacy.

Indeed, the immunogenicity of mouse monoclonal antibodies has prompted the development of new technologies aimed at overcoming the problem. Chimeric and humanized antibodies have been developed, in which the immunoglobulin backbone is of human origin, but the variable regions are either of mouse origin, or fully human, respectively. Despite these approaches, neutralizing antibodies (human anti-chimeric antibodies; HACA, or human anti-human antibodies; HAHA) develop in many

instances [20,21]. Routledge *et al.* [22] point out that a common misconception in the field of therapeutic antibodies is the assumption that humanized or chimeric antibodies are less likely to be immunogenic than murine antibodies. It is frequently stated that "chimeric" antibodies are about 25% murine/75% human in sequence, "humanized" about 5% murine/95% human in sequence and "human" are of course 0% murine/100% human in sequence. However, even though a sequence is designated as "murine" there are several segments within the immunoglobulin sequence that have considerable homology to human V-region sequences. Thus a totally murine immunoglobulin sequence might have high homology to human sequences. A careful study of several humanized and chimeric antibodies drug candidates found little overall difference in sequence homology of the humanized and chimeric antibodies versus the original murine antibodies [22-24].

By the same token even minor differences in the CDR region or V region sequences an immune response can develop against the antibody. While the human immune system, is physiologically exposed to large amounts of circulating human Immunoglobulin (Ig), high concentrations of antibody molecules all carrying the same unique CDR regions are detected as a perturbation of immune homeostasis. The fact that the immune system recognizes relatively large amounts of a given antibody as non-self protein was indeed reported early on, and constitutes the foundation of the immune network theory of the immune system [25] developed by N. Jerne in the 1970's, (which earned N. Jerne a Nobel prize for Medicine in 1984). Therefore, the use of chimeric and humanized antibodies is not expected to eliminate unwanted immune responses directed against the antibody therapeutics. Furthermore, even fully human antibodies generated in transgenic mice might be capable of eliciting an immune response as high concentrations of unique CDR regions in such antibodies may not be tolerated by the immune system.

Relative to small molecule therapies, the field of human and humanized antibody therapy is still in its infancy, and thus only limited data regarding the immunogenicity of monoclonal antibodies are available. Moreover, many of the existing antibody therapies are designed to benefit patient populations that may have compromised immune systems (e.g. cancer patients) or in patients undergoing treatment with immunosuppressive drugs (e.g. patients undergoing transplant surgery or suffering from rheumatoid arthritis). Therefore, these patients are less likely to mount anti-antibody responses. We anticipate that, as the number of marketed antibody drugs increases and the indications targeted are expanded to include immunocompetent populations, more information regarding undesirable immune responses to these therapies will become available.

TECHNOLOGIES TO ADDRESS IMMUNOGENICITY

PEGylation of proteins has been developed as a technology to improve pharmacokinetics and potentially reduce immunogenicity [26,27]. This approach has been successful for generation of compounds with reduced

immunogenicity by providing an immunocamouflage [27,28]. However, the challenges to PEGylation technology are increased cost [29] the fact that random attachment of a PEG molecule within a protein can occur around the active site of the molecule and thereby reduce its activity and eventually its efficacy [29]. Moreover, some PEGylated molecules may have side effects that hinder their use as therapeutics [29].

Exon shuffling is another strategy being developed to design proteins with increased activity and low safety risks. This technology alters the position of protein coding regions within a gene to select for improved characteristics. Currently no protein therapeutic modified by this technology has entered the clinic [29].

Antibody therapy with humanized anti-CD4 antibodies can also be used as a strategy to address the issue of immunogenicity. Various CD4 antibodies are being developed currently as therapeutic agents for various disease indications like rheumatoid arthritis and allograft rejection. Such antibodies could be co-administered with the protein therapeutic to decrease the immunogenicity of the protein.

Our group has been interested in developing an alternative based on the molecular mechanisms involved in the development of antibody responses.

FUNDAMENTALS OF THE IMMUNE RESPONSE INVOLVED IN ANTIBODY GENERATION

Except for the rare case of T-cell independent antigens, development of an antibody response to a protein requires activation of a special type of white blood cell, the Helper T lymphocyte (HTL), which directs specific B lymphocytes to produce antibodies [30]. In general, without activation of HTL, little or no antibody is produced [31]. HTL becomes activated when the specific T cell receptor recognizes short (10-15 amino acid residues long) peptide sequences (epitopes) bound to specific cellular receptors called HLA Class II. These HLA molecules are expressed by various cell types including macrophages and B cells. Binding of the epitope to the HLA Class II receptor is necessary (but not sufficient) for HTL activation. Thus, peptide binding capacity is directly and highly correlated to the ability of the peptide to elicit an immune response. If no or low binding capacity exists, no HTL activation can occur, and consequently, no antibody response will develop.

The complex rules for the HLA receptor-peptide interaction have been investigated and deciphered by several groups including our own [32,33]. The peptides binding to a given HLA molecule have distinct sequence characteristics and preferences. For example, amino acids in positions 1 and 6 of the peptide are the primary anchor residues that make direct contact with many HLA molecules Fig. (1). Some of the other amino acids in the peptide also contribute to the overall binding affinity and are known as secondary anchors.

Since 1987, our group has developed strategies to identify and analyze specific epitopes. This technology has

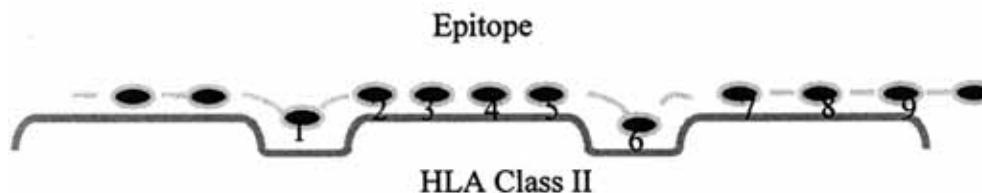


Fig. (1). An epitope bound to its HLA class II molecule.

been applied to the design of specific vaccines and immunotherapies [34-37]. The basic approach that we and others have utilized to identify epitopes [38,39] is based on a combination of *in silico* sequence analysis, high throughput *in vitro* biochemical evaluations and cellular assays to assess immunogenicity. Single or multiple substitution analogs of a given epitope can also be analyzed by the same methods and used to enhance immune recognition (for vaccine purposes) or eliminate immune recognition (in case of unwanted immune responses). The epitope identification process utilized for this purpose is described in detail elsewhere [39].

HLA POLYMORPHISMS AND ITS RELEVANCE TO EPIOTOPE IDENTIFICATION

One element of complexity in the identification of HLA Class II epitopes stems from the fact that HLA molecules are extremely polymorphic. To complicate matters further,

different HLA types are found in varying frequencies amongst different ethnic groups [40] as illustrated in Table I.

To globally address identification and removal of unwanted epitopes, we have standardized a panel of 13 different assays, each specific for a frequent HLA Class II molecule, which effectively covers the worldwide human population.

PREDICTION OF POTENTIAL IMMUNOGENIC EPIOTOPES

As stated above, binding of a peptide to a given HLA molecule is associated with specific sequence characteristics, called motifs or patterns. While a detailed discussion is beyond the scope of this paper, several reviews are available on this topic [32,33]. Motifs can be defined by various methods, such as the sequencing of naturally-occurring ligands of a given HLA molecule [41,42], binding assays

Table I. High Degree of Polymorphism in HLA-DR Molecules

a. DR β 1 gene

Antigen	Phenotypic Frequencies					
	Cauc.	Blk.	Jpn.	Chn.	Hisp.	Avg.
DR1	18.5	8.4	10.7	4.5	10.1	10.4
DR2	22.0	16.5	32.2	23.9	21.3	23.3
DR3	17.7	19.5	0.4	7.3	11.9	1.9
DR4	23.6	6.1	40.4	21.9	24.4	24.4
DR5	19.5	23.0	17.7	35.1	23.7	23.7
DR6	23.8	20.0	24.1	16.1	21.7	21.7
DR7	26.2	11.1	1.0	15.0	14.0	14.0
DR8	5.5	10.9	25.0	10.7	15.1	15.1
DR9	3.6	4.7	14.5	19.9	11.9	11.9
DR10	3.6	1.4	1.2	3.4	2.5	2.5

b. DR β 3/4/5 gene

Antigen.	Phenotypic Frequencies					
	Cauc.	Blk.	Jpn.	Chn.	Hisp.	Avg.
DR1	22.0	16.5	32.2	23.9	21.9	23.3
DR2	54.2	55.2	39.7	53.2	55.4	51.4
DR3	48.9	21.1	59.6	50.9	48.6	45.8

performed with synthetic peptides [43,44], and analysis of phage display libraries[45,46].

DR and DQ motifs are relatively well established [46-48] and these molecules are associated with the majority of epitopes recognized in humans. An example of a motif associated with a particular DR molecule is shown in Fig. (2). This motif consists of a preference for large aromatic or hydrophobic residues (F, M, Y, L, I, V, W) in position 1 and short and/or hydrophobic residues at position 6 of the peptide ligands. Moreover, preferences for certain amino acids in other non-anchor positions are also seen, for example, M is a preferred amino acid at position 2 in the peptide.

To predict *in silico* peptides capable of binding to HLA molecules, various methods have been proposed. These include primary anchor scans [49,50], polynomial functions [51-53], neural nets [54,55] and threading algorithms [55,56]. Experiments comparing the success rate of each suggest that the different methods are roughly equivalent [55]. A good success rate is usually attained with prediction for one particular HLA allele type. However, to accurately assess a peptide's binding capacity across the entire human patient population up to 10 to 15 different predictions may need to be performed. As a result, even a 90% accuracy for a single allele translates to a meager 25% global accuracy for 13 predictions. Even so, for large molecules, *in silico* prediction has proved to be a valid tool to narrow the actual screens with receptor ligand assays. It should also be noted that, in many cases, the use of a set of overlapping peptides spanning an entire region of interest voids the need for *in silico* predictions altogether.

HLA BINDING ASSAYS TO IDENTIFY T CELL EPITOPES

In our experience, the most powerful tool to identify and rationally analog (modify) epitopes is the quantitative

measurement of HLA-peptide binding affinity. T cell activation can occur only if a peptide binds to the HLA allele above a certain affinity threshold [57]. If a peptide does not bind HLA or binds with an affinity below the threshold, no T cell activation can occur [57,58]. The biologically relevant level of binding for HLA molecules has been determined by our group and elsewhere, utilizing a number of different experimental approaches [57,58]. In humans, most epitopes recognized are bound by HLA-DR molecules [57,59,60], even though epitopes bound by HLA DQ and DP molecules have also been described [61,62].

As mentioned above, the issue of HLA polymorphism could potentially complicate the development of an appropriate technology beyond practical feasibility. Indeed, if every different HLA Class II molecule were to recognize a distinct epitope, the number of amino acid changes necessary to introduce in the protein drug to achieve their obliteration might prove impractical. However, it has been observed that a large fraction of immune reactivity is usually directed against one or a few so-called immunodominant epitopes [63-65]. Moreover, our group has demonstrated that immunodominance is usually associated with high affinity binding to multiple HLA Class II molecules [66-68]. Table II shows representative binding data for immunodominant epitopes derived from Tetanus Toxin [66], Influenza Haemagglutinin [67], HCV [68], HBV [69] and Plasmodium Falciparum [70].

For example, the influenza haemagglutinin derived epitope HA 307 binds with high affinity to multiple DR molecules (DR1, DR4, DR5, DR6 and DR7). Thus, obliterating the DR binding capacity of this one epitope would be predicted to reduce immune responses against the Haemagglutinin protein in patients that express these alleles. Thus, our experimental approach entails the rapid identification of "offending" immunogenic and immunodominant epitopes within a protein of interest by performing binding analysis against HLA molecules

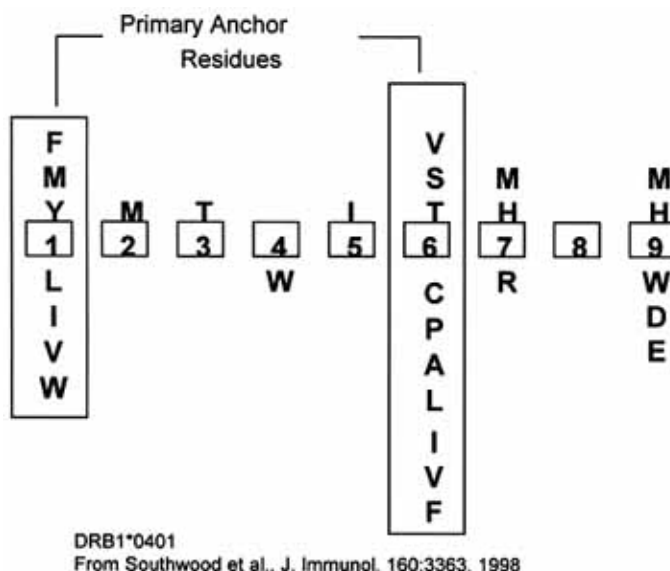


Fig. (2). Example of HLA-DR motif.

Table II. Multiple DR Molecules Bind the Same Immunodominant Epitopes

Epitope	HLA-DR binding capacity (IC50nM)										
	DR1 (*0101)	DR2 (*1501)	DR3 (*0301)	DR4 (*0401)	DR4 (*0405)	DR5 (*1101)	DR5 (*1201)	DR6 (*1302)	DR7 (*0701)	DR8 (*0802)	DR9 (*0901)
Tet Tox 830	23	22750	4891	8036	1462	20	-	4.4	25	49	74
HA 307	5.0	11375	-	45	ND	118	-	8.8	352	1195	ND
HCV NS3 1242	2.9	48	ND	18	1234	103	ND	11	96	60	240
HBV POL 412	2.0	21	-	9.4	47	294	397	135	167	557	682
p. fal. SSP261	26	260	8751	15	317	282	441	3.9	23	41	33

*Shading indicates IC50>100 nM.

representative of the worldwide population. It should be noted that the strategy utilized to identify potential epitopes depends on the specific protein type analyzed. In the case of chimeric or fully human antibodies immune reactivity is expected to be focused on the variable regions of the antibody. In the case of proteins in which changes have been introduced at a few specific positions to generate improved molecules, immune reactivity is typically focused on a 20 to 30 residue region centered around the changes [71,72].

IN DEPTH ANALYSIS OF “OFFENDING” EPITOPES

Once epitope sequences capable of binding to multiple HLA Class II molecules are identified, it may be desirable to verify if these epitopes are indeed the immunodominant, “offending” epitopes responsible for immunogenicity. To accomplish these goals, different strategies can be applied. First, if available, the peripheral blood mononuclear cells (PBMC) of exposed individuals can be assayed for the presence of activated or memory cells recognizing the epitope [34,70]. As an alternative, primary immunogenicity assays utilizing PBMC from unexposed donors can be utilized [73]. Finally, in cases where there is substantial homology between mouse and human molecules, HLA DR transgenic mice [74,75] could be used for immunogenicity testing. Once responses are detected, the next step may involve the determination of the HLA restriction of the immune response. Because of heterozygosity, gene polymorphism and duplication, a given human will usually express 3 to 8 different Class II molecules, in linkage disequilibrium with each other, and potentially also cross-reactive with each other. For this purpose, tools such as blocking reagents, matching cell lines and cell lines transfected with a single HLA Class II are utilized.

ENGINEERING SAFER, MORE POTENT THERAPEUTICS

Following the epitope identification process (through binding analysis and immunogenicity testing), a number of different analog peptides consisting of single amino acid substitutions or changes are produced and assayed in the panel of binding assays specific for the various HLA DR molecules, representative of the worldwide population. This

is an important and unique aspect of our approach because it allows specific testing to determine if the introduced changes do, in fact, lead to reduced or absent binding capacity for multiple molecules. At the same time, the use of binding assays also allows us to guard against the introduction of new HLA reactivities in the analogs generated. For this purpose, systematic testing of the peptides is done against multiple alleles. Finally, loss of immunogenicity can also be verified by use of the primary *in vitro* antigenicity assays or by testing for loss of reactivity against cells from exposed individuals.

The outcome of this series of activities is the definition of a set of analog substitutions recommended for further development in the context of the recombinant protein drug. The bulk of the work to this point is performed in a rapid and inexpensive fashion, taking advantage of assays utilizing short synthetic peptides. It is relevant to emphasize that there is tremendous flexibility in the ImmunoStealth technology in that, not only can it be applied to products that are in clinical development and are experiencing problems with immunogenicity, but can also be applied efficiently during early-stage development to either guide final selection of the lead drug or to custom design the lead drug for low immunogenicity. Following selection of analogs, full-scale versions of the modified protein drug or antibody engineered for reduced or absent immunogenicity are produced to verify that the substitutions introduced did not alter the biological activity of the drug itself, as judged by *in vivo* or *in vitro* potency assays and/or pharmacokinetic and pharmacodynamic studies.

The approach of removal of T-cell epitopes has been applied in animal models to produce an anti-epidermal growth factor receptor antibody with reduced immunogenicity [76]. Potential immunogenic epitopes were identified in the variable region of the parent anti-epidermal growth factor receptor antibody and substituted with amino acids that would disrupt such epitopes, the resulting modified antibody was less immunogenic than the parent antibody in monkeys [76]. The efficacy of this approach has been also demonstrated in a mouse model experiment. In this case, a human antibody was deimmunised with respect to murine MHC II alleles. The resulting antibody which has deimmunised variable regions and murine constant regions did not elicit an immune response when administered into mice [77].

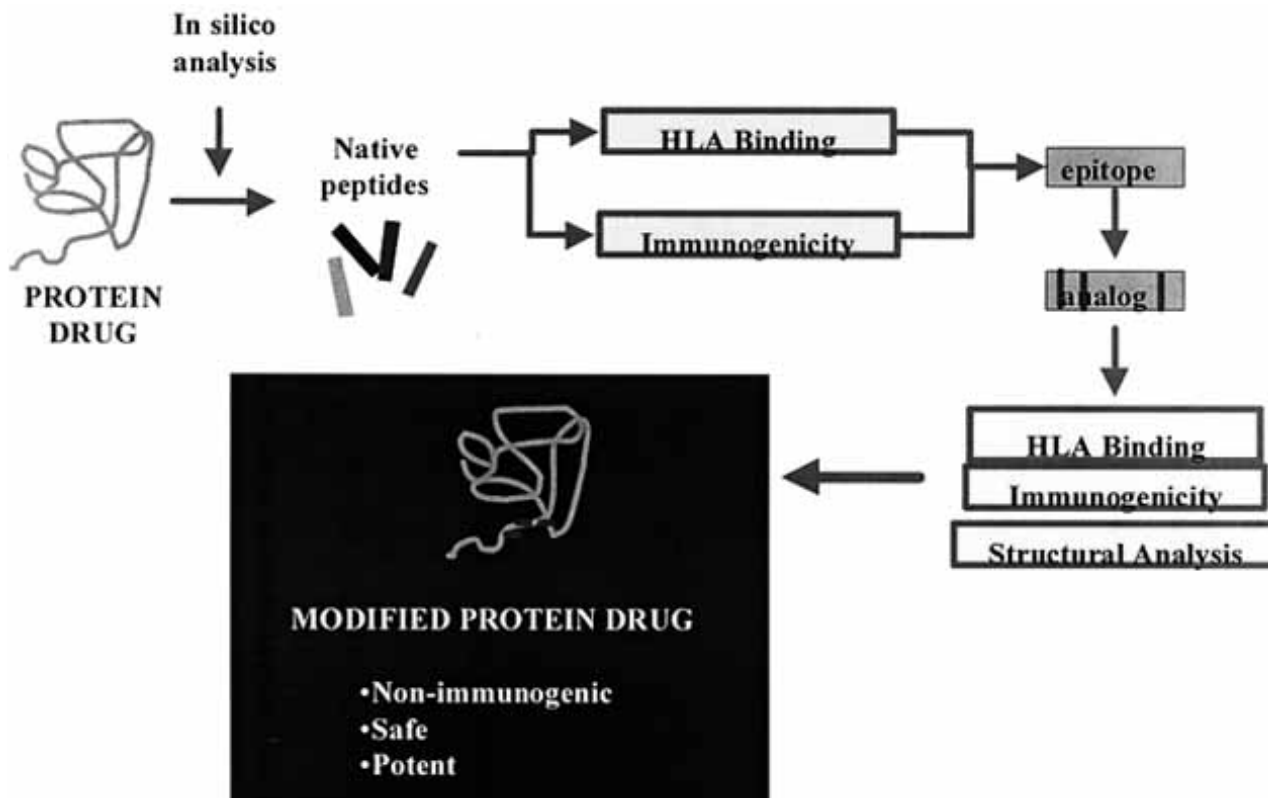


Fig. (3). Description of the ImmunoStealth process.

CONCLUSIONS

In summary, the ImmunoStealth technology addresses a large and growing unmet need. The steps applied in the ImmunoStealth process are outline schematically in Fig. (3).

Our strategy relies on applying our validated Epitope Identification System, to map “offending” epitopes. The use of in silico analysis, a panel of high throughput and quantitative assays coupled with biological and immunological assays and expertise allows the rational selection of epitope analogs. These analogs are then generated and tested for decreased binding and reduced or absent immunogenicity in relevant assay systems. Modified proteins consisting of these analogs and then generated to produce a bioactive and less immunogenic drug. This process results in the selection of protein drugs, including monoclonal antibodies that are clinically safer and more potent therapeutic agents.

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