Polysorbates, Immunogenicity, and the Totality of the Evidence

Edward T. Maggio

Protein aggregation underlies many deleterious effects for biotherapeutics. Principal among those are loss of efficacy, induction of unwanted immunogenicity, altered pharmacokinetics, and reduced shelf life. Consequently, aggregation-preventing surfactants are essential components of many protein formulations. They facilitate the development, manufacture, and stability of dosage forms by helping formulators manage protein aggregation and reduce interactions with container and delivery device surfaces.

Monoclonal antibodies (MAbs) present difficulties with respect to aggregation because they usually require relatively high doses for therapeutic efficacy (1–2 mg/kg). So they need to be concentrated sufficiently to allow for reasonable administration volumes by intravenous infusion or subcutaneous injection. But MAbs are not unique with regard to potential aggregation–related problems. The need for formulation with surfactants transcends many classes and types of biotherapeutics.

Two of the most widely used nonionic surfactants in protein formulations are polysorbate 20 and polysorbate 80 (Tween 20 and Tween 80, respectively). It is estimated that over 70% of the marketed MAb formulations contain one of these two surfactants. Polysorbates are composed of mixtures of structurally related fatty acid esters of polyoxyethylene sorbitan. The principal fatty acids are lauric acid and oleic acid, which make up ~60% of the total fatty acid composition. Esters of fatty acids of different chain lengths make up the remainder of those molecules (**1**). In addition, commercial polysorbate products contain measurable amounts of polyoxyethylene, sorbitan polyoxyethylene, and isosorbide polyoxyethylene fatty acid esters (**2–4**).

Using polysorbates to prevent protein aggregation provides somewhat of a conundrum. Although their usefulness is well accepted, they contain ether linkages (polyoxyethylene moieties) and unsaturated alkyl chains that spontaneously and rapidly auto-oxidize in aqueous solution to protein-damaging peroxides, epoxy acids, and reactive aldehydes, including formaldehyde and acetaldehyde (**5–14**). Those reactive species are detectable in neat polysorbate preparations and — even after purification — they are spontaneously reestablished within a week or two unless oxygen is perfectly excluded (**15, 16**). The auto-oxidation process accelerates once polysorbates are placed in aqueous solution, and reactive species are produced continuously during and after manufacturing and during storage before use.

(WWW.PHOTOS.COM)

Reactive peroxides principally affect methionine, histidine, and tryptophan moieties (**17, 18**). Depending on the local chemical environment, epoxy acids can react with accessible nucleophiles such as those found in lysine, histidine, cysteine, and tyrosine (**19–22**). In turn, aldehydes react with primary amino groups on proteins — a stratagem often used by protein chemists to deliberately induce or increase immunogenicity of proteins (**23–26**).

PRODUCT FOCUS: PROTEINS, ANTIBODIES, PARENTERAL PRODUCTS

PROCESS FOCUS: MANUFACTURING

WHO SHOULD READ: PROCESS DEVELOPMENT, PRODUCT DEVELOPMENT, SUPPLY CHAIN MANAGEMENT, OPERATIONS

KEYWORDS: FORMULATION, EXCIPIENTS, SURFACTANTS, IMMUNOGENICITY, ANALYTICS

LEVEL: INTERMEDIATE

Although aggregation alone can increase immunogenicity, oxidation can exacerbate this problem (**27**).

Oxidation can be a particularly serious problem for biotherapeutics that may be sensitive to oxidative damage because such highly potent proteins are necessarily formulated at relatively low concentrations. For example, Wang et al. describe the dual effects of Tween 80 on the stability of IL-2 (**28**). In that study, the authors demonstrated initial prevention of shaking-induced aggregation by Tween 80, followed by subsequent oxidation and generation of aggregates during storage over two months at 40 °C. Other examples include oxidative degradation of recombinant human ciliary neurotrophic factor (rhCNTF) in solution (**29**) and recombinant human granulocyte colony-stimulating factor (rhG-CSF) in solution during storage by residual peroxides in Tween 80 (**30**).

Because the reactive species are consumed during reaction with protein, they are not easily detected and depending on the relative concentrations of polysorbate and protein may not be present at all as residual free-chemical species. Instead, the chemically modified amino acids must be analyzed to determine the extent of modification. Increases in immunogenicity can be detected only using a suitable in vivo challenge model in a manner similar to how immunogenicity is assessed in patient samples during clinical trials.

Immunogenicity Concerns and FDA Guidance to Industry

Because of the serious nature and unique problems associated with development of unwanted immunogenic responses to protein therapeutics, regulatory interest and concerns by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) (**31**, **32**) have significantly increased. In May 2012, the nature of this problem was distilled succinctly in testimony at the FDA Public Hearing on Biosimilars by Richard Dolinar, head of the Alliance for Safe Biologic Medicines (an organization composed of innovative biotechnology companies, patients, physicians, and others focused on insuring patient safety for biosimilars). During that testimony, he said:

> Unwanted immunogenicity is the preeminent safety challenge associated with all biological therapeutics and can result in unexpected and sometimes severe adverse effects. Complicating matters, side-effects may only appear in patients after higher doses or prolonged duration of treatments and may be attributed to a number of patient, disease, or product related factors.

(The full testimony is available online at www.safebiologics.org/pdf/FDA/ASBM-Testimony.pdf.)

Antidrug antibodies may neutralize the biological effect of protein therapeutics by steric or allosteric interference at a site of action. They also may accelerate drug clearance from systemic circulation (**33**). Of equal or potentially greater concern, is the possibility that such antibodies could act as autoantibodies against corresponding or related naturally occurring proteins (**34**, **35**). Such action compromises the ability of a patient's own residual biological effector proteins to function.

Development of red-cell aplasia resulting from administration of certain formulations of recombinant erythropoietin is perhaps the best known example of potential serious effects of unwanted immunogenicity (**36, 37**). Nonetheless, the increased immunogenicity of beta interferon that results from protein aggregation and the demonstrated reduction in immunogenicity by prevention or reversal of aggregation is highly informative (**38, 39**). MAbs — an important and rapidly growing segment of biotherapeutics — pose particular aggregation and associated immunogenicity difficulties because they typically contain between two and eight aggregation-prone structural motifs (**40**).

Whether immunogenicity is caused by aggregation or chemical modification, nondamaging alternatives to polysorbate 20 and polysorbate 80 are urgently needed. That importance is highlighted in the FDA's Guidance for Industry document titled, *Scientific Considerations in Demonstrating Biosimilarity to a Reference Product* (**31**):

> Immune responses may affect both the safety and effectiveness of the product by, for example, altering PK, inducing anaphylaxis, or promoting development of neutralizing antibodies that neutralize the product as well as its endogenous protein counterpart. Thus, establishing that there are no clinically meaningful differences in immune response between a proposed product and the reference product is a key element in the demonstration of biosimilarity.

In 2009, the EMA's Committee for Medicinal Products for Human Use (CHMP) issued marketing authorization guidelines providing general recommendations on how to assess an unwanted immune response following administration of a biotherapeutic drug (**41**).

Concepts surrounding regulatory treatment of biosimilars are still in the formative stage both at the FDA and at the EMA. Assessing equivalence of different excipients in the stabilization of biotherapeutics has not been well described but will necessarily be an integral part of the regulatory landscape. The FDA has indicated that it expects drug companies to conduct at least one clinical study that includes a comparison of the immunogenicity of a proposed product to that of a reference product. Although there may be flexibility with respect to the extent and timing of such studies (e.g., premarket testing versus pre- and postmarket testing), discovery of nonequivalent immunogenicity in postmarket studies necessitating reformulation and repeat clinical studies would be a major financial setback for a sponsoring company.

Well-documented variability in reactive impurity content of different batches of polysorbates and the spontaneous progressive degradation that occurs during product storage would seem to make it very difficult — if not impractical — to demonstrate equivalent immunogenicity between an innovator biotherapeutic and that of a biosimilar that incorporates a polysorbate or other polyoxyethylene-based surfactant (**42**). Such difficulty arises because the immunogenicity of both comparator and test article would essentially be "moving targets" as spontaneous oxidation proceeds over time.

The FDA has indicated that it shall consider the "totality of the evidence" (**31**) in determining bioequivalence of subsequently developed biosimilars. The agency will certainly evaluate factors such as biological activity and immunogenicity (or lack thereof) observed during the course of preclinical and clinical development.

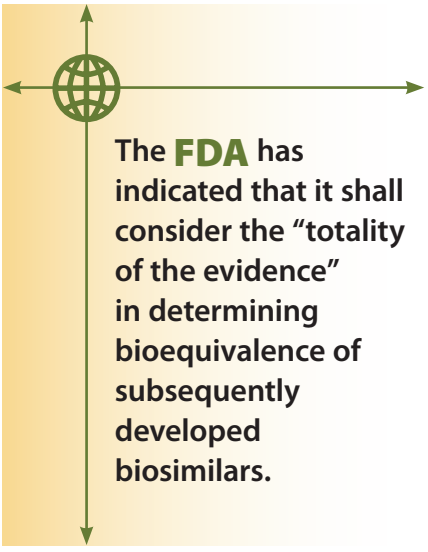
It remains an open question as to how the FDA or the EMA will view the continued use and possible replacement of polysorbates with alternative nonoxidizing surfactants in clinical practice as they become available in the future. Chemical analysis for detection of oxidation products of methionine, tryptophan, or histidine or addition products formed at primary amino groups, thiols, or phenolic hydroxyl groups could provide a definitive and quantitative basis for comparison. They may be included in required testing as long as polysorbates or other polyoxyethylene-based surfactants are used in biotherapeutic formulations. Replacing polysorbates with surfactants that do not lead to progressive protein degradation over time would make demonstration of bioequivalence with respect to immunogenicity more manageable. It could in effect yield biosimilars that are truly superior to innovator products with respect to immunogenicity, stability, and shelf life.

Alternative Excipients

Potential problems associated with using polysorbates and other polyoxyethylene-containing surfactants make clear the need for alternative surfactants that prevent aggregation while not introducing unintended protein damage. A number of established alternatives are useful as aggregation preventers, including sugars and other polyols, polymers, cyclodextrins, amino acids, and of course surfactants. Among various sugars, sucrose and trehalose appear to be the most commonly used stabilizers.

Polyethylene glycols (PEGs) are perhaps the most often used polymers. Available in different molecular-weight ranges, stabilization seems to depend idiosyncratically on the protein and size of PEGs. PEGs are essentially composed of polyoxyethylene moieties and thus are also subject to auto-oxidation.

Among the cyclodextrins, hydroxypropyl-beta-cyclodextrin appears to be a particularly useful stabilizer because it is both a good solubilizing agent and considered safe for parenteral administration (**43, 44**). The amino acids histidine, glycine, lysine, aspartic acid, and glutamic acid are effective alone or in combination with other excipients in certain applications (**45**). The stabilization effects of most excipients can be idiosyncratic in that an excipient that is stabilizing for one protein may be inactive or destabilizing for a different protein. Selection and optimization of the best excipient(s) for each specific protein should be explored in a typical matrix fashion. Nevertheless, as a generalization, among the various



absorption-preventing excipients being used at present, surfactants appear to be the most broadly applicable and predictable in their functional response.

One of the most promising alternatives is a class of non–ether-based alkylsaccharide surfactants that offer significant improvements in stability and reduced immunogenicity while completely circumventing the oxidative damage problems of polysorbates or other polyoxyethylene-based surfactants (**38, 46**). Alkylsaccharides are nonionic surfactants composed of a sugar moiety coupled to an alkyl chain. Unlike polysorbates (which are heterogeneous mixtures of related chemical species with potentially substantial lot-to-lot variability in composition, though presumably within some defined range set by each individual manufacturer), alkylsaccharides can be prepared as single chemical species. As such, their quality control specifications and quality assurance analyses are greatly simplified. A number of different alkylsaccharides are highly effective transmucosal absorption enhancers (**47–49**). Some alkylsaccharides are also highly effective in preventing aggregation for a number of protein molecules (**38, 50**).

Chemists have a variety of options when creating a chemical link between the sugar moiety and the alkyl chain. Glycosides, esters, thioesters, and amides are among the choices that have been examined. Glycosides offer the advantage of excellent stability over a broad pH range. Because they do not contain hetero atoms such as nitrogen or sulfur, they metabolize cleanly to the free sugar and the corresponding long-chain alcohols.

A study using dodecyl maltoside as an aggregation preventer demonstrated that administration of beta interferon does not elicit an immune response in the experimental allergic encephalomyelitis (EAE) animal model of multiple sclerosis (MS) (whether administered by injection or intranasally). By contrast, beta interferon administered without added dodecyl maltoside either by injection or intranasally in the same animal model led to a substantial immune response during the 30-day study (**38**). If a similar result could be demonstrated in patients, the clinically useful lifetime of beta interferon (which is currently limited by development of neutralizing antibodies over the course of therapy) could possibly be extended. Both nasal and injected beta interferons 1a and 1b were effective in reducing neuronal damage in a mouse MS model. Studies have also reported stabilization of other proteins, including MAbs, human growth hormone, recombinant human insulin, and parathyroid hormone (PTH) 1-34 and PTH 1-31(cyclic) under various solution conditions using light scattering to measure aggregation (**11**).

Alkylsaccharides represent just one class of non-ionic non–ether-based surfactants that offer a potential alternative to the use of polyoxyethylene-containing surfactants in biotherapeutic formulations. The chemical diversity of potential alternatives is essentially unlimited, and we can reasonably expect that ongoing and future research will result in additional alternatives to address the need for aggregation prevention without concomitant oxidative damage.

References

1 Kerwin BA. Polysorbates 20 and 80 Used in the Formulation of Protein Biotherapeutics: Structure and Degradation Pathways. *J. Pharm. Sci.* 97(8) 2008: 2924–2935.

2 Ayorinde FO, et al. Analysis of Some Commercial Polysorbate Formulations Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 14(22) 2000: 2116–2124.

3 Brandner JD. The Composition of NF-Defined Emulsifiers: Sorbitan Monolaurate, Monopalmitate, Monostearate, Monooleate, Polysorbate 20, Polysorbate 40, Polysorbate 60, and Polysorbate 80. *Drug. Dev. Ind. Pharm.* 24(11) 1998: 1049–1054.

4 Frison-Norrie S, Sporns P. Investigating the Molecular Heterogeneity of Polysorbate Emulsifiers by MALDI-TOF MS. *J. Agric. Food Chem.* 49(7) 2001: 3335–3340.

5 Donbrow M, Azaz E, Pillersdorf A. Autoxidation of Polysorbates. *J. Pharm. Sci.* 67(12) 1978: 1676–1681.

6 Donbrow M, Hamburger R, Azaz E. Surface Tension and Cloud Point Changes of Polyoxyethylenic Nonionic Surfactants During Autoxidation. *J. Pharm. Pharmacol.* 27(3) 1975: 160–166.

7 Donbrow M, et al. Development of Acidity in Nonionic Surfactants: Formic and Acetic Acid. *Analyst* (Lond.) 103(1225) 1978: 400–402.

8 Ha E, Wang W, Wang YJ. Peroxide Formation in Polysorbate 80 and Protein Stability. *J. Pharm. Sci.* 91(10) 2002: 2252–2264.

9 Wasylaschuk WR, et al. Evaluation of Hydroperoxides in Common Pharmaceutical Excipients. *J. Pharm. Sci.* 96(1) 2007: 106–116.

10 Liu JY, Li YZ, Chang WB. Measurement of the Peroxidation of Brji-35 in Aqueous Solution By Hemin and Horseradish Peroxidase Catalyzed Fluorogenic Reaction. *J. Analytical Chem.* 365(5) 1999: 448–451.

11 Maggio ET. Polysorbates, Peroxides, Protein Aggregation, and Immunogenicity: A Growing Concern. *J. Excip. Food Chem.* 3(2) 2012: 45–53.

12 Ding S. Quantitation of Hydroperoxides in the Aqueous Solutions of Non-Ionic Surfactants Using Polysorbate 80 As the Model Surfactant. *J. Pharm. Biomed. Anal.* 11(2) 1993: 95–101.

13 Nassar MN, et al. Influence of Formaldehyde Impurity in Polysorbate 80 and PEG-300 on the Stability of a Parenteral Formulation of BMS-204352: Identification and Control of the Degradation Product. *Pharm. Dev. Technol.* 9(2) 2004: 189–195.

14 Kishore RS, et al. The Degradation of Polysorbates 20 and 80 and Its Potential Impact on the Stability of Biotherapeutics. *Pharm. Res.* 28(5) 2011: 1194–1210.

15 Ray WJ Jr, Puvathingal JM. A Simple Procedure for Removing Contaminating Aldehydes and Peroxides from Aqueous Solutions of Polyethylene Glycols and of Nonionic Detergents That Are Based On the Polyoxyethylene Linkage. *Anal. Biochem.* 146(2) 1985: 307–312.

16 Kumar V, Kalonia DS. Removal of Peroxides in Polyethylene Glycols By Vacuum Drying: Implications in the Stability of Biotech and Pharmaceutical Formulations. *AAPS PharmTechSci.* 7(3) 2006: E47–E53.

17 Ji JA, et al. Methionine, Tryptophan, and Histidine Oxidation in a Model Protein, PTH: Mechanisms and Stabilization. *J. Pharm. Sci.* 98(12) 2009: 4485–4500.

18 Simat TJ, Steinhart H. Oxidation of Free Tryptophan and Tryptophan Residues in Peptides and Proteins. *J. Agric. Food. Chem.* 46(2) 1998: 490–498.

19 Mateo C, et al. Advances in the Design of New Epoxy Supports for Enzyme Immobilization-Stabilization. *Biochem. Soc. Trans.* 35 2007: 1593–1601.

20 Grazú V, et al. Novel Bifunctional Epoxy/Thiol-Reactive Support to Immobilize Thiol Containing Proteins By the Epoxy Chemistry. *Biomacromolecules* 4(6) 2003: 1495–501.

21 Chu JW, et al. *Understanding Oxidative Instability of Protein Pharmaceuticals;* http://hdl.handle.net/1721.1/3955.

22 Milzani A, et al. The Oxidation Produced by Hydrogen Peroxide on Ca-ATP-G-actin. *Protein Science* 9(9) 2001: 1774–1782.

23 Thiele GM, et al. Soluble Proteins Modified with Acetaldehyde and Malondialdehyde Are Immunogenic in the Absence of Adjuvant. *Alcohol Clin. Exp. Res.* 22(8) 1998: 1731–1739.

24 Moghaddam AE, et al. Reactive Carbonyls Are a Major Th2-Inducing Damage-Associated Molecular Pattern Generated By Oxidative Stress. *J. Immunol.* 187(4) 2011: 1626–1633.

25 Allison ME. Fearon DT. Enhanced Immunogenicity of Aldehyde-Bearing Antigens: A Possible Link between Innate and Adaptive Immunity. *Eur. J. Immunol.* 30(10) 2000: 2881–2887.

26 Lagergård T, et al. Formaldehyde Treatment Increases the Immunogenicity and Decreases the Toxicity of *Haemophilus ducreyi* Cytolethal Distending Toxin. *Vaccine* 25(18) 2007: 3606–3614.

27 van Beers MMC, et al. Oxidized and Aggregated Recombinant Human Interferon Beta Is Immunogenic in Human Interferon Beta Transgenic Mice. *Pharm. Res.* 28(10) 2011: 2393–2402.

28 Wang W, Wang YJ, Wang DQ. Duel Effects of Tween 80 on Protein Stability. *Intl. J. Pharmaceut.* 347(1–2) 2008: 31–38.

29 Knepp VM, et al. Identification of Antioxidants for Prevention of Peroxide-Mediated Oxidation of Recombinant Human Ciliary Neurotrophic Factor and Recombinant Human Nerve Growth Factor. *J. Pharm. Sci. Technol.* 50(3) 1996: 163–171.

30 Herman AC, Boone TC, Lu HS. *Characterization, Formulation, and Stability of Neupogen (Filgrastim): A Recombinant Human Granulocyte-Colony Stimulating Factor.* Plenum Press: New York, NY, 1996.

31 Guidance for Industry: Scientific Considerations in Demonstrating Biosimilarity to a Reference Product. Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research. February 2012; www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm.

32 Sharma B. Immunogenicity of Therapeutic Proteins. Part 1: Impact of Product Handling. *Biotechnol. Adv.* 25(3), 2007: 310–317.

33 Baker MP, Jones TD. Identification and Removal of Immunogenicity in Therapeutic Proteins. *Curr. Opin. Drug Discov. Devel.* 10(2) 2007: 219–227.

34 Sauerborn M, Schellekens H. B-1 Cells and Naturally Occurring Antibodies: Influencing the Immunogenicity of Recombinant Human Therapeutic Proteins? *Curr. Opin. Biotechnol.* 20(6) 2009: 715–721.

35 Wang X, Sing SK, Kumar S. Potential Aggregation-Prone Regions in Complementarity-Determining Regions of Antibodies and Their Contribution Towards Antigen Recognition: A Computational Analysis. *Pharm.Res.* 27(8) 2010: 1512–1529.

36 Casadevall N, et al. Pure Red-Cell Aplasia and Antierythropoietin Antibodies in Patients Treated with Recombinant Erythropoietin. *N. Engl. J. Med.* 346(7) 2002: 469–475.

37 Rossert J, Casadevall N, Eckardt K. Anti-Erythropoietin Antibodies and Pure Red Cell Aplasia, *J. Am. Soc. Nephrol.* 15(2) 2004: 398–406.

38 Rifkin RA, et al. *n*-Dodecyl-β-D-Maltoside Inhibits Aggregation of Human Interferon-β-1b and Reduces Its Immunogenicity, *J. Neuroim. Pharmacol.* 6(1) 2011: 158–156.

39 van Beers MMC, et al. Aggregated Recombinant Human Interferon Beta Induces Antibodies but No Memory in Immune-Tolerant Transgenic Mice. *Pharm. Res.* 27(9) 2010: 1812–1824.

40 Wang X, Singh SK, Kumar S. Potential Aggregation-Prone Regions in Biotherapeutics: A Survey of Commercial Monoclonal Antibodies. *mAbs* 1(3) 2009: 254–267.

41 Jahn EM, Schneider CK. How to Systematically Evaluate Immunogenicity of Therapeutic Proteins: Regulatory Considerations. *N. Biotechnol.* 25(5) 2009: 280–286.

42 Erlandsson B. Stability-Indicating Changes in Poloxamers: The Degradation of Ethylene Oxide-Propylene Oxide Block Copolymers at 25 and 40 °C. *Polymer Degrad. Stability* 78(3) 2002: 571–575.

43 Serno T, et al. Inhibition of Agitation-Induced Aggregation of an IgG-Antibody By Hydroxypropyl-Beta-Cyclodextrin. *J. Pharm. Sci.* 99(3) 2010: 1193–1206.

44 Brewster ME, et al. Use of 2–Hydroxypropyl-Beta-Cyclodextrin As a Solubilizing and Stabilizing Excipient for Protein Drugs. *Pharm. Res.* 8(6) 1991: 792–795.

45 Jensen WA, et al. Stability Studies on Pig Heart Mitochondrial Malate Dehydrogenase: The Effect of Salts and Amino Acids. *Biochim. Biophys. Acta.* 1296, 1996: 23–34.

46 Maggio ET. Use of Excipients to Control Aggregation in Peptide and Protein Formulations. *J. Excip. Food. Chem.* 1(2) 2010: 40–49.

47 Pillion DJ, et al. Insulin Delivery in Nosedrops: New Formulations Containing Alkylglycosides. *Endocrinology* 135(6) 1994: 2386–2391.

48 Ahsan F, et al. Enhanced Bioavailability of Calcitonin Formulated with Alkylglycosides Following Nasal and Ocular Administration in Rats. *Pharm. Res.* 18(12) 2001: 1742–1746.

49 Pillion DJ, et al. Synthetic Long-Chain Alkyl Maltosides and Alkyl Sucrose Esters as Enhancers of Nasal Insulin Absorption. *J. Pharm. Sci.* 91(6) 2002: 1456–1462.

50 Maggio ET. Novel Excipients Prevent Aggregation in Manufacturing and Formulation of Protein and Peptide Therapeutics. *BioProcess Int.* 6(10) 2008: 58–65.

For Further Reading

Sauerborn M, et al. Immunological Mechanism Underlying the Immune Response to Recombinant Human Protein Therapeutics. *Pharmacol. Sci.* 31(2) 2010: 53–59.

Edward T. Maggio, *PhD, is president and chief executive officer at Aegis Therapeutics, LLC16870 W. Bernardo Drive, Suite 390, San Diego, CA 92127; 1-858-618-1400, ext 101; fax: 1-858-618-1441; emaggio@aegisthera.com.*