(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 September 2004 (30.09.2004)

PCT

(10) International Publication Number
WO 2004/082627 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number:
PCT/US2004/008083

(22) International Filing Date: 17 March 2004 (17.03.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/455,457 18 March 2003 (18.03.2003) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

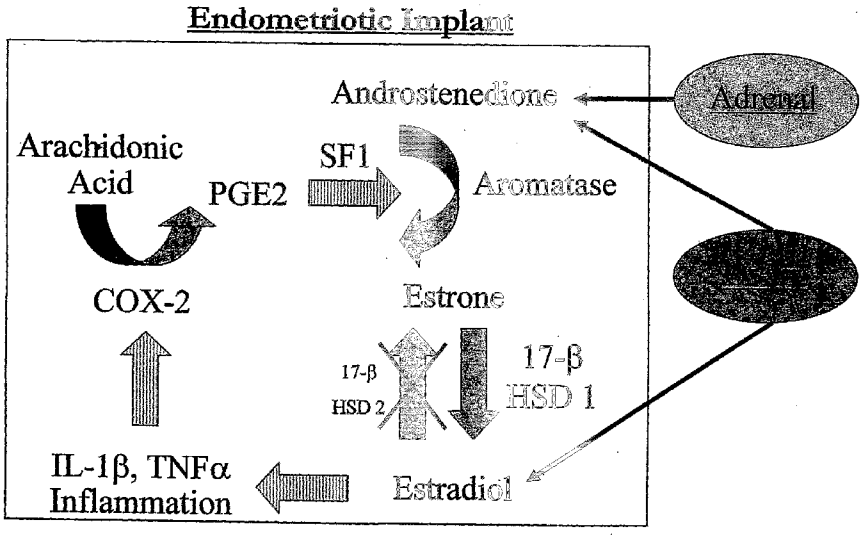
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *without international search report and to be republished upon receipt of that report*

[Continued on next page]

(54) Title: ENZYME REPLACEMENT THERAPY WITH 17-β-HYDROXYSTEROID DEHYDROGENASE-TYPE 2



(57) Abstract: Compositions and methods for regulating the intra-conversion of estradiol/estrone or testosterone/androstenedione are provided herein. The compositions include a liposome carrier component; and a moiety externally attached thereto, which is targeted for a site in the body associated with aberrant production of estradiol or testosterone, or leaky blood vessels thereat. The inventive compositions also include an isolated or recombinant HSD-Type 2 polypeptide or nucleic acid form that is encapsulated by the liposome carrier component. The compositions are of use in overcoming the deficient expression of 17-β-hydroxysteroid dehydrogenase-Type 2 in endometriotic tissues. An HSD-Type 2 polypeptide or nucleic acid form can be delivered to a target cell, enzyme, or cytokine at the endometriotic implant site, or to leaky blood vessels within the implant. A label capable of external detection can be further included in the composition.

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ENZYME REPLACEMENT THERAPY WITH 17-β-HYDROXYSTEROID DEHYDROGENASE-TYPE 2

FIELD OF THE INVENTION

The present invention is directed to pharmaceutical compositions useful for treating a disorder associated with aberrant production of estradiol or testosterone. The present invention provides endometriotic implant-targeted delivery systems useful in the treatment and detection of endometriosis.

BACKGROUND OF RELATED TECHNOLOGY

Endometriosis is a disorder which is characterized by the presence, growth and progression of endometrial glands and stroma outside the uterine cavity. This disorder is linked to both pelvic pain and infertility. For example, it is estimated that 25-50% of infertile women have endometriosis. It is further estimated that 70% of women with endometriosis are infertile. Moreover, the prevalence of endometriosis of reproductive age women is believed to be as high as 10% as described by Aral and Cates, Journal of the American Medical Association 250: 2327-2331 (1983).

Estrogen in endometriotic lesions comprises both estrone (a weak estrogen) and estradiol (a potent estrogen). Figure 1 schematically depicts a biosynthetic pathway for estrogen formation in an endometriotic implant. Estrogen is an important factor known to stimulate the growth of endometriosis. It is the estradiol that primarily affects an endometriotic lesion. In a reproductive age woman, circulating estradiol is secreted directly from the ovary in a cyclic fashion. In the early follicular phase and post-menopause, extra-ovarian tissues, such as adipose and skin tissue (for e.g., skin fibroblasts), are the most important sources for circulating estradiol. Estradiol is also produced locally in the endometriotic implant in both reproductive age women, as well as post-menopausal women.

Referring to Figures 1 and 2, the most important precursor of estrogen is androstenedione, which mainly originates from the adrenal gland and ovaries and is converted to estrone through the action of the enzyme aromatase, also referred to as CYP 450-19. Notably, the estrone produced is only very weakly estrogenic and can be

subsequently reduced to estradiol in peripheral tissues and the endometriotic implant (Figure 1), through the actions of 17-β-hydroxysteroid dehydrogenase-type 1 (HSD-Type 1). See, for example, Zeitoun et al., Molecular Endocrinology 13: 239-253 (1999). Estradiol can be inactivated by conversion to estrone in epithelial cells of the eutopic endometrium. This inactivation is catalyzed by another 17-β-HSD isozyme, 17-β-hydroxysteroid dehydrogenase-type 2 (HSD-Type 2). However, as depicted in Figure 1, in endometriotic tissues, the potent estrogen estradiol is minimally metabolized because of a lack of HSD-Type 2, leading to increased levels of estradiol. Moreover, estradiol and cytokines, such as IL-1β and TNF-α, which are increased in endometriosis due to inflammation, induce cylco-oxygenase-2 (COX-2), leading to elevated levels of PGE_2 in the endometriotic tissue as described by Huang, et al., American Society for Reproductive Medicine 5: (Abstract) (1996). Over-expression of aromatase is stimulated by PGE_2, CREB and transcription factor SF_1 in endometriotic stromal cells. See Noble et al., Endocrinology and Metabolism 82: 600-606 (1997).

It has recently been demonstrated that significant levels of aromatase activity and mRNA are present in the stromal cells of pelvic endometrial implants, whereas aromatase expression was minimal in the eutopic endometrium of women with endometriosis as described by Noble et al. in Endocrinology and Metabolism 81: 174-179 (1996) and in Endocrinology and Metabolism 82: 600-606 (1997).

The aberrant expression of aromatase and its stimulation by PGE_2 in endometriotic tissues results in local production of estrogen, which induces PGE_2 synthesis and establishes a positive feedback loop for continuous estrogen formation in endometriosis. Moreover, the lack of HSD-Type 2 in endometriotic tissues gives rise to increased local concentrations of estradiol, which cannot be reoxidized into estrone anymore. Elevated estradiol, in turn, promotes the growth of endometriotic tissue and, in addition, promotes local PGE_2 synthesis in stromal cells. Since PGE_2 is the most potent inducer of aromatase in endometriosis, this completes the positive feedback cycle that favors increased levels of estradiol in endometriosis.

Approaches currently in use to treat endometriosis are not optimal. These approaches include a laparoscopic approach, stand-alone aromatase inhibitors, oral contraceptives, tamoxifene, steroid hormones, proteins and antibodies, cytokines, IL-6 like polynucleotides and polypeptides and COX-2 inhibitors. A disadvantage of the laparoscopic approach is that

it fails to detect endometriotic implants which are hidden behind organs or transparent, and/or located outside the abdominal region. A disadvantage of stand-alone aromatase inhibitors is that they lack specificity for endometriotic tissues and thus, in addition to affecting the level of estradiol produced locally in the endometriotic implant, they also affect circulating estradiol production. Therefore, the stand-alone aromatase inhibitors also block the ovarian functions, thereby creating an artificial menopause and contributing to significant bone loss. Moreover, tamoxifene, steroid hormones, and cytokines such as IL-6, TNF-α and enzymes like COX-2, are also not specific to endometriosis, and some steroid hormones are metabolized before reaching their target. Furthermore, endometriosis treatment with oral contraceptives is only successful in a limited percentage of women.

Referring to Figure 2, androstenedione is also an important precursor of estradiol *via* the production of testosterone. The male sexual hormone testosterone is the intermediate molecule obtained by the reduction of androstenedione through the action of the 17β-HSD Type 1 enzyme, and also Type 3 and Type 5 (Labrie, Steroids 62: 148-158 (1997) and Figure 2). As we've already seen in the metabolism of estrone to estradiol (Figure 1), these reactions are limited to an equilibrium. Testosterone can further be reduced to dihydrotestosterone (DHT) through the action of 5α-reductase or to estradiol by aromatase (CYP 450-19). It is believed that the lack of 17β-HSD Type 2 enzyme re-oxidizing testosterone into androstenedione could lead to a production of an excess of testosterone and eventually of DHT if 5α-reductase is present in sufficient amount or in excess. Moreover, if aromatase is over-expressed, this would also lead to the production of an excess of estradiol, a likely prostate carcinogen in aging males. It is known that many organs in the body are composed of cells that respond to, or are regulated by, exposure to testosterone. Cells in the prostate have testosterone receptors that, when exposed to testosterone, stimulate the cells to grow. When cells that have testosterone receptors become cancerous, the growth of these cancer cells can be increased by exposure to testosterone. Therefore, an approach consisting of *in situ* regulation of the metabolism of testosterone may be beneficial in the treatment and/or prevention of diseases (prostate cancer, benign prostatic hyperplasia, etc.) or disorders (hair loss, etc.) where amounts of testosterone and/or DHT have to be controlled or reduced.

Gene therapy refers to therapy performed by administering to a patient an expressed or expressible nucleic acid. For example, either an isolated or recombinant protein that mediates a therapeutic effect is administered or the nucleic acid producing the encoded

therapeutic protein is administered. One problem associated with the use of nucleic acids and proteins in gene therapy approaches has been the relatively poor ability of these agents to cross the cell membrane. Another problem has been that proteins and nucleic acids can interact with a variety of extracellular molecules which can alter their bioavailability. Furthermore, proteins and nucleic acids are susceptible to degradation in biological fluids and they display pharmokinetics which may not be optimal for some therapeutic applications. One approach to overcome these problems has been to administer these molecules in vivo in the presence of a lipid vesicle, such as a liposome.

Liposomes are spherical vesicles prepared from either natural or synthetic phospholipids or cholesterol. These vesicles can be composed of either one (unilamellar liposomes) or several (oligo-or multilamellar liposomes) lipid bilayers surrounding internal aqueous volumes. It is known to entrap drugs, proteins and nucleic acids within the internal aqueous space of a liposome. For example, polynucleotide-liposome compositions are known which include a polynucleotide entrapped in the aqueous interior of neutral liposomes formed from neutral vesicle-forming lipids. See Juliano and Akhtar, Antisense Res. Dev. 2: 165-176 (1992); Ropert, et al., Pharm. Res. 10(10): 1427-1433 (1993) and Szoka and Papahadjopoulos, Proc. Natl. Acad. Sci. USA 75(9): 4194-4198 (1978). Moreover, U.S. Patent No. 5,567,433 discloses a liposome preparation including encapsulated granulocyte-colony stimulating factor (G-CSF), a relatively unstable protein. In addition, U.S. Patent No. 4,241,046 describes a method for encapsulating an enzyme within a synthetic liposome, the product liposomes being useful for enzyme replacement therapy. Liposomes allow the parenteral administration of the therapeutic agent. On the cellular level, liposomes interact with cell membranes by adsorption, endocytose, membrane fusion, and lipid exchange, or by a combination of these mechanisms as described by Pagano and Weinstein in Ann. Rev. Biophys. Bioeng. 7: 435 (1978). Fast elimination of the therapeutic agent and its metabolism can be impeded by shielding the therapeutic agent in a liposome. See, for example, Schwendener, et al., Biochim. Biophys. Acta, 1026: 69-79 (1990) and Schwendener, Chimia 46: 69-77 (1992). Moreover, antibodies can be covalently attached to the outer surface of the liposomes to enable the specific binding to defined targets.

Research is currently being carried out to develop radiolabeled antibodies or fragments thereof which are specific to endometriosis. For example, U.S. Patent Nos. 5,776,095 and 5,776,093 each disclose the use of radiolabeled antibody or antibody

fragments specific for endometriotic tissues for endometriosis detection and/or therapy. They further disclose that labeled antibodies and antibody fragments specific for a targeted tissue or organ may be conjugated to a drug. From a diagnostic standpoint, a disadvantage of this approach is that it is extremely difficult to bind only one radioactive atom to proteins, antibodies or polypeptides in a time compatible with its half-life, which is one of the necessary conditions for performing a meaningful quantitative evaluation of endometriosis with a radiolabeled compound. Moreover, from a therapy standpoint, this approach would let the organs surrounding the endometriotic implants to be irradiated, as well.

There is a need in the art for therapeutic agents that are targeted for sites in the body associated with the aberrant production of estradiol. For example, it would be desirable to target endometriotic tissues where the oxidation of estradiol to estrone *via* 17β-HSD type 2 is impaired. In particular, there is a need for therapeutic agents which are specific to endometriotic tissue, which would be useful for the treatment of reproductive age women, as well as post-menopausal women suffering from this disorder, and which would not suffer from the disadvantages described above. The advantage of specifically targeting endometriotic tissue is that the agent would not alter the function of other estrogen-responsive tissues. With the high specificity of a new compound for endometriotic tissues, the long term and/or repeated therapy programs would be possible, when necessary. In particular, there is a need for a therapeutic agent useful for overcoming the abnormality in endometriosis which impairs the oxidation of estradiol to estrone, i.e. deficient HSD-Type 2 expression.

There is a further need in the art for therapeutic agents that are targeted for sites in the body associated with the aberrant production of testosterone. For example, there is a need for therapeutic agents that target sites where oxidation of testosterone into androstenedione *via* 17β-HSD Type 2 occurs or is impaired, so as to control or reduce amounts of testosterone and/or DHT.

SUMMARY OF THE INVENTION

The present invention provides a composition useful in the treatment of disorders associated with aberrant production of estradiol or testosterone. The inventive compositions can be used to reduce levels of estradiol in endometriotic tissues, which are deficient in HSD-type 2 expression. In particular, by delivering HSD-type 2 in a targeted fashion to the

endometriotic implants, estradiol can be re-oxidized into estrone. Alternatively, the compositions can be used to reduce or control levels of testosterone by re-oxidizing testosterone into androstenedione. The compositions can also be used to reduce or control levels of estradiol in the ovaries of women by re-oxidizing estradiol into estrone.

The inventive composition includes a liposome carrier component and an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, which is encapsulated by the liposome carrier component. The inventive composition further includes a moiety, which is attached to the liposome component. The moiety can be targeted for a site in the body associated with aberrant production of estradiol or testosterone and/or for leaky blood vessels at that site.

For example, provided is a pharmaceutical composition targeted for endometriotic implants that can overcome the abnormality in endometriosis which impairs the oxidation of estradiol to estrone, i.e., deficient HSD-type 2 expression. The composition includes a liposome carrier component having an external phospholipid layer and an internal phospholipid layer; a moiety targeted for an endometriotic implant or leaky blood vessels within the implant; and an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form that is encapsulated by the liposome carrier component. The moiety is chemically bound to the external phospholipid layer in this embodiment.

The invention further provides methods for treating a disorder associated with aberrant production of estradiol or testosterone, such as, but not limited to endometriosis. The method includes administering to a patient a therapeutically effective amount of a composition including a liposome carrier component; a moiety, which is attached to the liposome component; and an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, which is encapsulated by the liposome carrier component. The moiety is targeted for a site in the body associated with aberrant production of estradiol or testosterone, or for leaky blood vessels at that site. For example, the composition administered can be used to treat endometriosis, where the moiety attached to the surface f the liposome is targeted for an endometriotic implant or for leaky blood vessels within the implant.

Also provided by the present invention is a method for detecting an endometrial implant and/or diagnosing the presence of endometriosis in a patient. The method includes administering to a patient a composition including a liposome carrier component; a moiety targeted for an endometriotic implant or leaky blood vessels within the implant, which is attached to the liposome carrier component; and an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form that is encapsulated by the liposome carrier component; and a label capable of external detection. The label is proximate to or covalently bound to the HSD-Type 2 polypeptide or nucleic acid form. The method further includes monitoring the presence of the composition in the endometriotic implant by detecting the label.

Another aspect of the present invention relates to a method of preparing a composition useful in the treatment of a disorder associated with aberrant production of estradiol or testosterone, such as, but not limited to, endometriosis. The method includes the steps of providing a liposome component having an external phospholipid layer and an internal phospholipid layer; and attaching a moiety to the external layer to form a targeted liposome component. The moiety is targeted for a site in the body associated with the aberrant production of estradiol or testosterone, or for leaky blood vessels at that site. The moiety can be targeted for an endometriotic implant or leaky blood vessels within the implant, for example. The method further includes combining the liposome component with an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form under suitable conditions for the polypeptide or nucleic acid to form become encapsulated by the liposome component.

The present invention further provides a method of regulating the intra-conversion of estradiol/estrone or testosterone/adrostenedione in a patient. This method includes administering an effective amount of a composition including a liposome carrier component; and an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, which is encapsulated by the liposome carrier component.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 schematically depicts a biosynthetic pathway for estrogen formation in an endometriotic implant.

Figure 2 schematically depicts the metabolism of androstenedione to estradiol.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the terms "endometriotic tissue", "endometriotic implant", "endometriosis", and the like refer to ectopic endometrium-like tissues outside the uterine cavity.

The term "endometrium" refers to the eutopic or intra-uterine endometrial tissue in its normal location.

"Gene therapy" as used herein refers to therapy performed by administering to a patient an expressed or expressable nucleic acid. For example, either an isolated or recombinant protein that mediates a therapeutic effect is administered or the nucleic acid producing the encoded therapeutic protein is administered.

As used herein, a "17-β-hydroxysteroid dehydrogenase-type 2 nucleic acid form" can include any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA, or modified RNA or DNA. For example, a polynucleotide can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions and RNA that is a mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. A polynucleotide may also contain one or more modified bases, or DNA or RNA backbones modified so as to improve stability or for other reasons. "Modified" bases may include, for example, tritylated bases and unusual bases such as inosine. Since a variety of modifications can be made to DNA and RNA, the term nucleic acid form embraces all polynucleotides that are chemically, enzymatically, or metabolically modified forms.

As used herein, a "17-β-hydroxysteroid dehydrogenase-type 2 polypeptide" embraces the 17-β-hydroxysteroid dehydrogenase-type 2 enzyme represented by SEQ ID NO: 1, or a functional fragment or variant thereof. For example, a functional HSD-Type 2 polypeptide would be functional if it exhibited enzymatic activity similar to, but not necessarily identical to, the activity of the HSD-Type 2 enzyme having the Enzyme Classification Number EC No. 1.1.1.62, which is represented by SEQ ID NO: 1. Such activity can be measured in a

particular biological assay, with or without dose dependency. In the case where dose dependency does exist, it need not be identical to that of the wild-type enzyme, but rather substantially similar to the dose-dependence in a given activity as compared to the wild-type enzyme, i.e., the candidate polypeptide will exhibit greater activity or not more than about 25-fold less and, preferably, not more than about 10-fold less activity, and most preferably, not more than about 3-fold less activity relative to the wild-type enzyme having EC No. 1.1.1.62. As described in further detail below, the HSD-Type 2 polypeptide may be part of a larger protein, such as a fusion protein.

"Variant" refers to a nucleic acid or polypeptide form which differs from the wild-type HSD-Type 2 enzyme or the nucleic acid encoding the enzyme, but retains essential properties thereof. Generally, variants are overall closely similar, and, in many regions, identical to the HSD-Type 2 wild-type nucleic acid or polypeptide form.

The present invention encompasses compositions and methods useful for regulating the intra-conversion of estradiol/estrone or testosterone/adrostenedione. For example, the invention relates to liposome compositions for use in overcoming the deficient expression of 17-β-hydroxysteroid dehydrogenase-type 2 in endometriotic tissues. This enzyme is referred to herein as HSD-Type 2 or 17-β-HSD-Type 2.

The present invention is based on the ability of HSD-Type 2, generally represented by Gen Bank Accession Number NP 002144 (homo sapiens), and having the enzyme classification number EC 1.1.1.62 (SEQ ID NO: 1), to mediate the oxidation of estradiol to estrone or to mediate the oxidation of testosterone to adrostenedione. The Gen Bank Accession Number of the homo sapiens gene encoding this enzyme corresponds to NM 002153 (SEQ ID NO: 2).

As discussed above, one problem associated with the use of nucleic acids and proteins in gene therapy has been the poor ability of these agents to cross the cell membrane. Moreover, both proteins and nucleic acids can interact with extracellular molecules, thus altering their bioavailability. In addition, proteins and nucleic acids can be degraded in biological fluids. The present invention overcomes these difficulties by administering the protein or nucleic form of HSD-Type 2 in the presence of a targeted or stealth liposome or combination thereof, the product liposomes being useful for enzyme replacement therapy.

The present invention solves a need in the art by providing a therapeutic liposome composition which is specifically targeted to endometriotic implants. However, it is also well within the scope of the present invention that these liposome compositions may be designed to target any tissue including ovarian tissue, where regulation of the intra-conversion of estradiol/estrone may be needed and where administering either the protein or nucleic acid form of HSD-Type 2 would be of therapeutic benefit. It is further well within the scope of the present invention that the inventive liposome compositions may be designed to target any tissue, including prostatic tissue, where regulation of the intraconversion of testosterone/androstenedione may be needed and where administering the protein or nucleic acid form of HSD-type 2 would be of therapeutic benefit.

In the present invention, liposomes can be loaded with protein, polypeptides, or nucleic acids, such as expression vectors. In general, these agents potentiate or mimic the ability of HSD-Type 2 to modulate the oxidation of estradiol to estrone or to modulate the oxidation of testosterone to androstenedione. It is noted that it is well within the contemplation of the present invention that a functional variant of either HSD-Type 2 or a nucleic acid encoding this variant can be administered.

The liposome compositions of the present invention satisfy a need in the art by providing new compositions which are useful in the treatment and diagnosis of disorders associated with altered expression or activity of HSD-Type 2. Any means of altering expression or activity of HSD-Type 2 will have important consequences for the treatment of endometriosis, as well as other disorders.

It is noted that, unless indicated otherwise, the HSD-Type 2 forms include HSD-Type 2 polypeptides, fragments or mutants thereof, polypeptides and fragments which are of the same superfamily as the HSD-Type 2 protein, as well as all relevant nucleic acid sequences.

A clear role of HSD-Type 2 in regulating the balance of estrogen levels is known. For example, it is known that estradiol can be inactivated by conversion to estrone in epithelial cells of the eutopic endometrium, this inactivation being catalyzed by HSD-Type 2. However, in endometriotic tissues, the potent estrogen estradiol is minimally oxidized because of a lack of HSD-Type 2. This leads to increased local levels of estradiol. Elevated estradiol promotes the growth of endometriotic tissue, and, in addition, promotes local PGE_2

synthesis in stromal cells. Since PGE_2 is an important inducer of aromatase in endometriosis, this further favors increased levels of estradiol in endometriosis. Therefore, a therapeutic approach which seeks to replace deficient expression and/or activity of HSD-Type 2 is likely to be of benefit to women suffering from endometriosis or other disorders where the ability to oxidize estradiol to estrone is deficient.

In one embodiment of the present invention, the liposome carrier component comprises an external phospholipid layer and an internal phospholipid layer. The HSD-Type 2 polypeptide or nucleic acid form can likely be encapsulated regardless of the degree of ionicity of the liposome.

The liposome compositions of the present invention include a moiety targeted for a site in the body associated with aberrant production of estradiol or testosterone. In one embodiment, the moiety is chemically bound to the external phospholipid layer. The moiety may be a polymer, peptide, polypeptide, protein, or glycoprotein. In one desired embodiment, the attached moiety is an attached protein or protein fragment. Desirably, the moiety is an antibody Fab fragment. In one preferred embodiment, the moiety is targeted for an endometriotic implant or leaky blood vessels within the implant and is used to treat endometriosis. The moiety may be specific for endometriotic cells in the implant. For example, the moiety may bind to a marker produced by or associated with an endometriotic cell. The attached moiety can be specific for a cytokine, cell, or enzyme which is present in increased amounts in the implant as compared to normal tissues. Moreover, the attached moiety can be specific for a cytokine, cell, or enzyme present in increased amounts in response to inflammation in the endometriotic implant.

Similarly, for the treatment of disorders associated with aberrant production of testosterone, such as prostate cancer or benign prostate hyperplasia, the attached moiety can be specific for a cytokine, cell, or enzyme present in increased amounts in response to inflammation at the affected site. For example, the elaboration of inflammatory cytokines, such as IL-1 and TNF-α in the prostate cancer tumor microenvironment is common. Therefore, an aromatase-inhibiting liposome composition according to the present invention including a moiety specific for one of these inflammatory cytokines is likely to be useful for treatment.

Desirably, a liposome carrier component including an attached protein or protein fragment (e.g. an antibody fragment) would be present in suitable amounts to obtain specific binding to target cells. Targeting to body sites associated with aberrant production of estradiol or testosterone may also occur through the normal bio-distribution of liposomes after *in vivo* administration. This can be accomplished by providing a hydrophilic polymer chain as the moiety attached to the liposome carrier component. For example, in one embodiment, the hydrophilic polymer chain is a polyethylene glycol (PEG) chain which has a prolonged circulation time within the blood stream. Liposomes comprising PEG chains on their surface are capable of exiting through leaky or open-ended blood vessels located in the endometriotic tissue site as determined by the present inventor.

Furthermore, it has been shown previously that therapeutic agents delivered *via* liposomes containing a hydrophilic polymer coating concentrate in tumor tissues. For example, high drug accumulation has been shown to occur in human prostate carcinoma xenograft after administration of liposome-encapsulated doxorubicin. Therefore, the present inventor believes that liposomes provided with attached hydrophilic polymer chains, such as PEG, are likely to be useful for delivering an encapsulated HSD-type 2 form to prostatic tumor tissue, for example.

In one embodiment, the liposome carrier component including an attached moiety is present in amounts about 1 to about 1,500 nM. In another embodiment, the liposome carrier component including an attached moiety is present in amounts of about 10 to about 150 nM.

Liposome Components and Their Preparation

The liposome components may be prepared by a variety of techniques, such as those detailed in Szoka, et al., Biochem. Biophys. Acta 601: 559-571 (1980). Multilamellar vesicles (MLV's) can be formed by simple liquid-film hydration techniques. Briefly, a mixture of liposome-forming lipids of the type listed below are dissolved in a suitable organic solvent and subjected to evaporation in a vessel to form a thin film, which is then covered by an aqueous medium. The liquid film hydrates to form MLVs, typically with sizes between about 0.1 to 10 microns.

Suitable liposome components of the present invention are composed primarily of vesicle-forming lipids. Such a vesicle-forming lipid is one which (a) can form spontaneously

in bilayer vesicles in water, as exemplified by the phospholipids, or (b) can be stably incorporated into the lipid bilayer. The vesicle-forming lipids of this type are preferably ones which have two hydrocarbon chains, typically acyl chains, and a head group, either polar or non-polar. Some preferred diacyl-chain lipids for use in the present invention include diacyl glycerol, phosphatidylethanolamine (PE), diacyl aminopropane diols, such as distearyl aminopropanediol (DS) and phosphatidylglycerol (PG).

The vesicle-forming lipid is selected to achieve a specified degree of fluidity or rigidity, to control the stability of the liposome in the serum and to control the rate of the release of the entrapped agent in the liposome. The rigidity of the liposome, as determined by the vesicle-forming lipid, may also play a role in the fusion of the liposome to a targeted cell.

In one embodiment of the invention, the liposomes are prepared with a relatively rigid lipid to impart rigidity to the lipid bilayer. In a preferred embodiment, the vesicle-forming lipid is distearyl phospatidyl choline (DSPC).

In another embodiment of the invention, the lipids forming the bilayer vesicle, i.e., liposome, are effective to impart a positive liposome-surface charge. Such lipids include those typically referred to as cationic lipids, which have a lipophilic moiety such as sterol, an acyl or diacyl chain, and where the lipid has an overall net positive charge. Exemplary cationic lipids include 1,2-dioleyloxy-3-(trimethylamino) propane (DOTAP); N-[1-(2,3,-ditetradecyloxy) propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide (DMRIE); N-[1-(2,3,-dioleyloxy) propyl]-N,N-dimethyl-N-hydroxyethylammonium bromide (DORIE); N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA); 3β[N-(N',N'-dimethylaminoethane) carbamoly] cholesterol (DC-Chol); and dimethyldioctadecylammonium (DDAB).

The cationic vesicle-forming lipid may also be a neutral lipid, such as dioleoylphosphatidyl ethanolamine (DOPE) or an amphipathic lipid, such as a phospholipid, derivatized with a cationic lipid, such as polylysine or other polyamine lipids. For example, the neutral lipid (DOPE) can be derivatized with polylysine to form a cationic lipid.

Suitable methods for preparing liposome components suitable for the present invention are provided in U.S. Patent Publication No. 2002/0172711 A1, the entire contents of which are herein incorporated by reference.

Liposome-Encapsulated Polypeptides

According to the present invention, there is provided a targeted liposome composition that includes as one of its components an isolated or recombinant HSD-Type 2 polypeptide or a functional or mutant analog thereof. In one embodiment, the polypeptide includes an amino acid sequence classified under EC 1.1.1.62 (SEQ ID NO: 1), or fragments thereof. The liposome composition is a pharmaceutical composition useful for parenteral administration of the therapeutic polypeptide. The pharmaceutical composition may further comprise a biocompatible pharmaceutical carrier. This may, in one embodiment, be clinical grade sterile water. However, any acceptable biocompatible pharmaceutical carrier may be useful such as, but not limited to, saline, buffered saline and dextrose.

As described above, useful HSD-Type 2 polypeptides are those capable of mediating or modulating the oxidation of estradiol to estrone. Other useful HSD-type 2 polypeptides are those capable of mediating or modulating the oxidation of testosterone to androstenedione. It is well within the contemplation of the present invention that a derivative or only a fragment of the HSD-Type 2 protein may be necessary for functional activity. Derivatives can include naturally occurring or synthetic modifications, such as, but not limited to, methylation or phosphorylation. It is further contemplated that a variant form of HSD-Type 2 may be a useful component of the inventive liposome compositions. For example, a variant form of HSD-Type 2 can be one which maintains wild-type enzymatic activity. Alternatively, the variant form may be useful for potentiating or inhibiting the conversion of estradiol to estrone.

The invention further provides for target liposome compositions including encapsulated fusion proteins. For example, a suitable fusion protein may include an HSD-Type 2 polypeptide, or variant thereof, and a second polypeptide having an amino acid sequence unrelated to the amino acid sequence of the HSD-Type 2 polypeptide. For example, in one desired embodiment, the second polypeptide functions as a detectable label for detecting the presence of the fusion protein, or as a matrix-binding domain for immobilizing the fusion protein to facilitate protein purification prior to its encapsulation by

the liposome.

The invention includes functional equivalents of the HSD-Type 2 enzyme. Desirably, a protein or polypeptide is a functional equivalent if its amino acid sequence is at least approximately 60% identical, preferably at least approximately 70% identical.

It is preferred that the HSD-Type 2 polypeptide or variant thereof is derived from a mammalian species. Mammals include laboratory animals, such as rats, mice, and rabbits; farm animals, such as cows, pigs, horses and sheep; pet animals, such as dogs and cats; and primates, such as monkeys, orangutans, apes and humans. The preferred mammals include mice or humans.

The HSD-Type 2 polypeptide and variants thereof are preferably isolated. By the term "isolated," it is meant that the HSD-Type 2 polypeptide or variants thereof are partially purified or purified to homogeneity. The polypeptide is considered partially purified if it is at least 25%, preferably at least approximately 50%, more preferably at least approximately 75%, most preferably at least approximately 90% and optimally at least approximately 90% free of other proteins. The polypeptide is considered to be purified to homogeneity if it exhibits a single band by SDS page. As described above, purification may be achieved, at least in part, by immobilizing a fusion protein encoding the HSD-Type 2 polypeptide onto a particular matrix. For example, the fusion protein may encode a matrix-binding domain for immobilizing the fusion protein onto the matrix. Such methods of purification are well known in the art.

Liposome-Encapsulated Nucleic Acid Molecules

The present invention further provides a targeted liposome composition that includes as one of its components an HSD-Type 2 nucleic acid form which encodes the HSD-Type 2 polypeptide, or variants thereof. The invention further encompasses nucleic acid forms which encode only a portion of the HSD-Type 2 polypeptide, as well as any derivatives thereof. For example, a useful cDNA corresponds to bases 168-1331 from the homo sapiens gene, generally represented by Gen Bank Accession No. NM 002153 and SEQ ID NO: 2. Moreover, any portion of the gene sequence represented by Gen Bank Accession No. NM 002153 (SEQ ID NO: 2) may be useful for production of the polypeptide fragments described herein.

In one preferred embodiment, the nucleic acid encoding the HSD-Type 2 polypeptide, variant versions, or fragments thereof, includes a 5' or 3' regulatory sequence. Desirably, the regulatory sequence is operably linked to nucleic acid sequence encoding an HSD-Type 2 polypeptide or fragment thereof. As described above, the nucleic acid form would desirably encode an HSD-Type 2 polypeptide or fragment which can successfully be used to overcome the deficient expression of HSD-Type 2 in endometriotic tissues. Alternatively, the expressed polypeptide or fragment can be successfully used to control or reduce testosterone levels in tissues where the oxidation of testosterone to androstenediol is impaired. It is desired that the nucleic acid form is derived from a mammal, preferably a human.

Also included within the scope of the present invention are the use of liposome-encapsulated, altered nucleic acid sequences encoding HSD-Type 2 polypeptide or a fragment thereof. Such alterations may include, but are limited to, deletions, insertions, or substitutions of different nucleotides, promoters and/or transcription factors resulting in a nucleic acid form that encodes the same or a functionally equivalent HSD-Type 2 polypeptide. To this end, the encoded protein may also contain deletions, insertions, or substitutions of amino acid residues which produce a silent change and results in a functionally equivalent HSD-Type 2 protein. Such altered nucleic acid sequences and their encoded proteins may be useful for therapeutic purposes.

Also included within the scope of the invention are altered nucleic acid sequences resulting from at least one mutation in a nucleic acid sequence, which altered nucleic acid sequences may result in an HSD-Type 2 polypeptide whose structure or function is altered. Such altered nucleic acid forms and the polypeptides resulting from them may be useful in the area of diagnosis of a particular disorder, such as endometriosis, or design of therapeutic agents against such a disorder.

It is noted that nucleic acid forms encapsulated by the liposomes encompassed by this invention include, but are not limited to, all functional equivalents of any of the sequences described above. For example, a nucleic acid form would be a functional equivalent if its nucleic acid sequence is at least approximately 60% identical, preferably at least approximately 70% identical, most preferably at least approximately 80% identical, especially preferably at least approximately 90% identical, optimally at least approximately 95% identical, and especially optimally at least approximately 98% identical. Percent

identity between the two strands of sequences is calculated by juxtaposing the two strands so as to achieve the highest possible identify of residues. Software is available to aid in the alignment.

Preparing HSD-Type 2 Polypeptide and Nucleic Acid Forms

The HSD-Type 2 polypeptides included in the liposome compositions of the present invention, variant versions, or fragments thereof, and DNA encoding the same may chemically synthesized by methods known in the art. Suitable methods for synthesizing polypeptides are described by Stewart and Young in "Solid Phase Peptide Synthesis," Second Edition, Pierce Chemical Co. (1984) and in Solid Phase Peptide Synthesis, Methods Enzymol., 289: Academic Press, Inc., NY (1997). Suitable methods for synthesizing DNA are described by Caruthers in Science 230: 281-285 (1985) and DNA Structure, Part A: Synthesis and Physical Analysis of DNA, Lilley, D.M.J. and Dahlberg, J.E. (Eds.), Methods Enzymol. 211: Academic Press, Inc., NY (1992). The subject matter of all of the aforementioned citations are incorporated herein by reference. HSD-Type 2 polypeptides may also be prepared by providing DNA that encodes the polypeptides, mRNA and/or promoter(s); amplifying or cloning the DNA in a suitable host; expressing the DNA in a suitable host; and harvesting the polypeptide. For example, the HSD-Type 2 enzyme or a fragment thereof may be translated either directly or indirectly from a cDNA encoding the HSD-Type 2 amino acid sequence.

The DNA encoding HSD-Type 2 polypeptides for use in liposome compositions of the present invention may be replicated and used to express recombinant protein following insertion into a wide variety of host cells in a wide variety of cloning vectors.

Cloning vectors may comprise segments of chromosomal, non-chromosomal, and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from *E. coli*, such as col E1, pCR 1, pBR 322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivates of phage DNA, such as M13 fd and other filamentous single-stranded DNA phages.

The host may be prokaryotic or eukaryotic. The DNA may be obtained from natural sources and, optionally, modified by, for example, site-specific mutagenesis. The genes may also be synthesized from the individual nucleotides in whole or in part. Synthetic methods,

such as solid phase methods, are known in the art, such as those described by Caruthers in Science 230: 281-285 (1985) and DNA Structure Part A: Synthesis and Physical Analysis of DNA, Lilley, D.M.J. and Dahlberg, J.E. (Eds.), Methods Enzymol. 211: Academic Press, NY (1992).

In the present invention, an expression vector may be used to express in a host cell an HSD-Type 2 polypeptide, variant thereof, or a fragment thereof such as described above. In one embodiment, an expression vector capable of producing the HSD-Type 2 gene product may be encapsulated in the liposome. After administering this liposome composition *in vivo*, the HSD-Type 2 gene is expressed in the endometriotic host cells, for example. This technique should provide for the stable transfer of the nucleic acid to the targeted cell in the endometriotic implant, so that the nucleic acid is expressible by the cell and, preferably, heritable and expressible by its cell progeny. In an alternative embodiment, the HSD-Type 2 polypeptide is first produced *ex vivo* in a host cell, such as *E. coli*, after which time it is recovered from the host cell culture and purified prior to liposome encapsulation. In this instance, it is the HSD-Type 2 polypeptide which is administered *in vivo*.

Vectors for expressing proteins in bacteria, especially *E. coli*, are also known. Such vectors include the pK 233 (or any of the *tac* family of plasmids), T7, pBluescript II, bacteriophage lambda, *ZAP*, and lambda P_L (See Wu, R. (Ed.), Recombinant DNA Methodology II, Methods in Enzymol., Academic Press, Inc., NY, 1995). Examples of vectors with expressed fusion proteins are PATH vectors described by Dieckmann and Tzagoloff in J. Biol. Chem. 260: 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a poly-linker at the carboxy terminus. Other expression vector systems are based on β-galactosidase (pEX); maltose binding protein (pMAL); glutathione-S-tranferase (pGST or pGEX) – see Smith, D.B., Methods Mol. Cell Biol. 4: 220-229 (1993); Smith, D.B. and Johnson, K.S., Gene 67: 31-40 (1988); and Peptide Res. 3: 167 (1990), and TRX (thioredoxin) Fusion Protein (TRX FUS) – see La Vallie, R. et al., Bio/Technology 11: 187-193 (1993).

Vectors useful for cloning and expression in yeast are available. Suitable examples are 2μm circle plasmid, Ycp50, Yep24, Yrp7, Yip5, and pYAC3.

Suitable cloning/expression vectors for use in mammalian cells are also known. Such vectors include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV) retrovirus-derived DNA sequences. Any such vectors, when coupled with vectors derived from a combination of plasmids and phage DNA, i.e. shuttle vectors, allow for the isolation and identification of protein coding sequences in prokaryotes.

Further eukaryotic expression vectors are known in the art (e.g., P.J. Southern and P. Berg, J. Mol. Appl. Genet. 1: 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1: 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159: 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159: 601-664 (1982); S.I. Scahill et al, "Expression And Characterization Of The Product Of A Human Immune Interferon DNA Gene In Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80: 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77: 4216-4220 (1980).

It is noted that expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene insures growth of only those host cells which express the insert. Typical selection genes encode proteins that: (a) confer resistance to antibiotics or other toxic substances (e.g. ampicillan, neomyicin, methotrexate, etc.); (b) compliment auxotrophec deficiencies or (c) supply critical nutrients not available from complex media, e.g. gene encoding d-alanine, racenase for Basillia. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art.

The expression vectors useful in preparing the liposome compositions of the present invention preferably contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the *lac* system, the *trp* system, the *tac* system, the *trc* system, the *tet* system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for

3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters or SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Once the HSD-Type 2 gene is cloned into such an expression vector, the gene product may be produced in a suitable expression host in either a constitutive or inducible manner. Useful expression hosts include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, *E. coli*, such as *E. coli* SG-936, *E. coli* HB 101, *E. coli* W3110, *E. coli* X1776, *E. coli* X2282, *E. coli* DH1, *E. coli* DH5αF', and *E. coli* MRCl, *Pseudomonas*, *Bacillus*, such as *Bacillus subtilis*, and *Streptomyces*. Suitable eukaryotic cells include yeasts and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells.

Host cells which contain the nucleic acid sequence encoding HSD-Type 2 and express HSD-Type 2 may be identified by a variety of procedures known to those skilled in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane and solution based technologies for the detection and/or quantification of nucleic acid or proteins.

A suitable method for producing an HSD-Type 2 polypeptide or fragment or derivative thereof for liposome encapsulation includes: (a) culturing the host cell described above under conditions suitable for the expression of the polypeptide or fragment thereof; and (b) recovering the polypeptide or fragment thereof from the host cell culture. It is noted that expression vectors may include secretion signals where appropriate which allow the protein to cross and/or lodge in cell membrane or to be secreted from the cell. Such secretion would facilitate recovery of the polypeptide or fragment thereof from the host cell culture. Such vectors containing secretion signals may be prepared by means as standard recombinant techniques well known in the art and discussed, for example, in Ausubel, F.M. et al. (Eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York, (1999).

The HSD-Type 2 polypeptides for liposome encapsulation can be recovered from: products purified from natural sources, including bodily fluids, tissues and cells, whether

directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, as described above. The HSD-Type 2 polypeptide may be purified using standard known techniques. Some examples of suitable techniques include, for example, gel purification, column chromatography, or electrophoretic methods. Recombinant constructions may be used to join sequences encoding HSD-Type 2 to nucleic acid sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification or immobilized immunoglobulin, and the domain utilized in the flags-extension/affinity purification system (Immunex Corp., Seattle, WA). Moreover, signal sequences may be used to facilitate the export of HSD-Type 2 into a cell culture supernatant to facilitate purification of the protein.

As described above, the expression vector allows for the translation of HSD-Type 2 protein domains which are capable of overcoming the deficient expression of HSD-Type 2 in endometriotic tissues.

Ligand Moieties for Attachment to the Liposome Carrier Component

The present invention provides liposome compositions which can be targeted for an endometriotic implant or other tissues where it would be desired to regulate the intraconversion of estradiol/estrone. Alternatively, the liposome compositions can be targeted for tissue were it is desired to regulate the intraconversion of testosterone/androstenedione. The liposome carrier component includes an external phospholipid layer and an internal phospholipid layer. The targeted moiety is preferably bound to the external phospholipid layer. In one embodiment, the moiety is a peptide, polypeptide, protein or glycoprotein. For example, the moiety may be an antibody Fab fragment specific for an endometriotic implant. The moiety may be specific for endometriotic cells within the implant. For example, the moiety may bind a marker produced by or associated with an endometriotic cell. In another embodiment, the moiety is specific for a cytokine, cell, or enzyme which is present in increased amounts in the endometriotic implant as compared to normal tissue. In another embodiment, the moiety is specific for a cytokine, cell or enzyme present in increased amounts in response to inflammation in the implant.

It is further noted that a moiety bound to the external phospholipid layer of the liposome carrier component may be targeted for leaky blood vessels within the endometriotic implant. For example, as will be described in further detail below, the moiety may be a hydrophilic polymer chain. Desirably, the hydrophilic polymer chain is a polyethylene glycol chain. In one preferred embodiment, a protein or protein fragment, such as an antibody Fab fragment specific for the endometriotic implant, is attached to the distal ends of these polyethylene glycol chains.

As described above, a moiety which specifically targets a cytokine, cell or enzyme in the endometriotic implant would be useful in the present invention. For example, it is known that TNF-α is produced following the initial immunologic response subsequent to the inflammation process characteristic of endometriosis lesions. Therefore, an antibody fragment specific for TNF-α (such as Centocor's Remicade) would be useful.

Regarding the targeting of enzymes in the implant, it is known that eosinophil peroxidase (EPO) (an intracellular enzyme that is released from eosinophils as they degranulate) is expressed in human endometriosis specimens. EPO is also present in normal endometrium where degranulation occurs just prior to and during menstruation. Therefore, liposomes which include a moiety capable of specifically binding to EPO would be useful in targeting endometriosis tissue, provided they are not used just prior to and during menstruation. A useful moiety in this respect is described in International Publication No. WO 00/59547.

Moreover, U.S. Patent No. 5,618,680 describes the use of a monoclonal anti-HLA-A, B and C antibody as being useful in the detection and diagnosis of endometriosis. This ligand is specific to MHC-Class I antigens. This patent discloses that a woman with endometriosis has a different expression of the major histo-compatibility complex (MHC) Class I antigens (especially HLA-A, B and C surface antigens) in or on their endometrial cells when compared with the expression of the same antigens of endometrial cells of a healthy woman. The presence of a different expression of these antigens on endometrial cells is therefore predictive of endometriosis. Therefore, it is well within the contemplation of the present invention that ligands specific to MHC-Class I antigens, especially HLA-A, B and C antigens, would be useful as moieties attached to the external phospholipid layer of the liposome components present in the inventive compositions. The entire contents of U.S.

Patent No. 5,618,680 are herein incorporated by reference.

Furthermore, U.S. Patent No. 5,891,644 describes an antibody specific for an isolated chemotactic factor from patients with endometriosis. In particular, the chemotactic factor is a soluble peptide having a molecular weight of about 27 kD, chemotactic to neutrophils and macrophages, and is naturally occurring in the peritoneal fluid of mammals with minimal to moderate endometriosis. Suitable methods for isolating and purifying this chemotactic factor for use as an antigen in generating a suitable antibody for the present invention are described in U.S. Patent No. 5,891,644, the entire contents of which are herein incorporated by reference.

Moreover, it is known that certain matrix metalloproteinases are present in increased amounts in the endometriotic implant in response to inflammation. See, for example, Gynecol. Obstet. Inves. 48, Suppl. 1: 2-13 (1999). In one embodiment, the metalloproteinase which is present in the endometriotic implant in increased amounts, as compared to normal endometrium, is matrix metalloproteinase-7 or matrix metalloproteinase-11. Therefore, a liposome carrier component including an attached moiety targeting a metalloproteinase present in increased amounts in the endometriotic implant in response to inflammation would be useful in the present invention.

Most preferably, suitable antibodies for the liposome compositions of the present invention are human antigen-binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (scFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable regions alone, or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments which further include any combination of variable regions with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, chimeric, murine (e.g., mouse and rat), donkey, rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies that have the amino acid sequence of a human immunoglobulin and include antibodies that are isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express

endogenous immunoglobulins, as described in U.S. Patent No. 5,939,598 to Kucherlapati, et al.

The antibodies useful for the present invention may be monospecific, bispecific, trispecific, or of greater multi-specificity. For example, multi-specific antibodies may be specific for different epitopes of a cytokine, cell, or enzyme which may be present in increased amounts in the implant as compared to normal tissues. Alternatively, an antibody may be specific for both an epitope of a cell in the endometriotic implant, as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material.

Bispecific antibodies, designed with dual antigenic specificities and prepared by chemically linking two different monoclonal antibodies or by fusing two hybridoma cell lines to produce a hybrid-hybridoma, are known. These are described by Brennan, M. et al. in Science 229: 81-83 (1985); by Paulus, H. in Behring Inst. Mitt. 78: 118-132 (1985); by Rammensee, H.G. et al., Eur. in J. Immunol. 17: 433-436 (1987); by Segal, D. et al. in Princess Takamatsu Symp. 19: 323-331 (1988); by Kranz, D. et al. in J. Hematother, 4: 403-408 (1995); and by Morimoto, K. and Inouve, K., J. Immunol. Methods, 224: 43-50 (1999).

An "antibody" in accordance with the present specification is defined broadly as a protein that binds specifically to an epitope. The antibody may be polyclonal or monoclonal. Antibodies further include recombinant polyclonal or monoclonal Fab fragments prepared in accordance with the method of Huse, et al., Science 246: 1275-1281 (1989) and Coligan, J.E. et al. (Eds.) Current Protocols in Immunology, Wiley Intersciences, NY, (1999).

The antibodies for use with the present invention may include derivatives that are modified, i.e. by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotipic response. For example, the antibody derivatives may include antibodies that have been modified by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by techniques which are known such as, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. In addition, the antibody derivative may contain one or more non-classical amino acids.

The targeted antibodies for use in the liposome compositions of the present invention may be generated by any suitable method known in the art. For example, polyclonal antibodies may be isolated from mammals that have been inoculated with the targeted cell marker, cytokine, or enzyme or a functional analog of any of these in accordance with known methods such as those described in Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, NY, (1999). For example, a cytokine, cell, or enzyme can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce production of sera containing polyclonal antibodies specific for the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species, and include, but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, key hole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as bacille Calmette-Guerin (BCG) and corynebacteriumparvum. Such adjuvants are well known in the art.

Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in Nature 256: 495-497 (1975) and by Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); and Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999); as well as the recombinant DNA method described by Huse et al., Science 246: 1275-1281 (1989).

In order to produce monoclonal antibodies, a host mammal is inoculated with an antigen, such as a cytokine, cell marker or enzyme known to be present in the endometriotic implant as described above, and then boosted. Spleens are collected from inoculated mammals a few days after the final boost. Cell suspensions from the spleens are fused with a tumor cell in accordance with the general method described by Kohler and Milstein in Nature 256: 495-497 (1975). See also Campbell, "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al. (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985) and Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Wiley Intersciences, New York, (1999). In order to be useful, an antigen must

contain sufficient amino acid residues to define the epitope of the molecule being detected. If the antigen is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhold limpet hemocyanin and bovine serum albumen. Conjugation may be carried out by methods known in the art. See, for example, Coligan, J.E., et al. (Eds.), Current Protocols in Immunology, Chapter 9, Wiley Intersciences, New York, (1999). One such method is to combine a cysteine residue of the antigen with a cysteine residue on the carrier molecule.

Hydrophilic Polymer Chain Moieties For Attachment to the Liposome Component

In one embodiment of the present invention, the liposome composition has an outer surface coating of hydrophilic polymer chains. For example, suitable liposome compositions comprising an outer surface coating of hydrophilic polymer chains are described in U.S. Patent Publication No. US-2002/0172711 A1. These hydrophilic polymer chains may be releasable. Preferably, the liposomes are designed to have an extended blood circulation time.

It is noted that the hydrophilic polymer chains may be either directly or indirectly linked to the polar head group of a vesicle-forming lipid. For example, the hydrophilic polymer chains may be connected to the liposome lipids, or to hydrophobic chains connected to liposome lipids, desirably by chemically releasable bonds - that is, covalent chemical bonds that can be released by a suitable cleaving agent, such as a reducing agent, a reduced or elevated pH, a hydrolytic enzyme, or a photolytic stimulus. The hydrophilic chains preferably have a surface density sufficient to create a molecular barrier which is effective to substantially prevent the interaction of a serum proteins with the liposome surface. As such, the hydrophilic chain coating is effective to extend the circulation time of the liposomes in the blood-stream for periods of up to several hours to several days. Such an extended circulation time allows the inventive liposome compositions to exit the blood stream to the endometriotic implant from leaky and open-ended blood vessels within the implant.

The hydrophilic polymer chains are preferably composed of polymer chains of polyethylene glycol, polyvinyl pyrrolidone, polyvinyl methylether, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyloxazoline, polyhydroxypropylmethacralimide, polymethacralimide, polydimethylacrylamide, polyhydroxypropyl metharacalate, polyhydroxyethylacrylate, hydroxymethylcellulose, hydroxyethylcellulose, or

polyaspartamide. These polymer chains have a preferred molecular weight of between about 500-10,000 daltons. In preferred embodiments, the hydrophilic polymer chains are polyethylene glycol (PEG) chains. In one embodiment, these hydrophilic polymer chains may be releasably attached to the liposome *via* a reducible chemical linkage. After administering the liposome composition to the subject, a reducing agent, such as cysteine, glutathione or ascorbate, may subsequently be administered to the subject to release the hydrophilic polymer chains. Although not wishing to be bound by any one theory, liposomes which have releasable PEG chains appear to be retained to a greater degree in the target tissue as compared to liposomes which do not have releasable PEG chains. Release of the PEG chain exposes the positive liposome surface charges of cationic liposomes, enhancing binding to the negative cell membranes and improving retention of the liposomes in the tissues.

Preferably, the hydrophilic chains are present in the outer lipid layer of the liposomes in an amount corresponding to about 1-20 mole percent of the liposome surface lipids, with lower molecular weight polymers, e.g., 500 daltons, being present at a higher density, e.g., 20 mole percent, and higher molecular weight polymers, e.g., 10,000 dalton chains, being present at a lower density, e.g., 1-5 mole percent.

In one embodiment of the invention, the hydrophilic polymer chains may include a ligand which can specifically bind to the target endometriotic tissue. For example, an antibody Fab fragment may be attached to the distal ends of PEG chains on the surface of the liposomes. As disclosed in U.S. Publication No. US 2002/0172611 A1, an unshielded ligand may be attached to the hydrophilic polymer coating so as to effect ligand-specific binding to a molecule on the target cell surface prior to chemical release of the hydrophilic polymer coating.

It is known that liposomes having PEG chains attached to the outer surface thereof have prolonged circulation time in the blood stream. They can effectively evade the immune system, which would otherwise attack the liposome soon after injection causing rupture of the liposome and premature release of the therapeutic agent entrapped inside. By increasing the blood circulation time, the therapeutic agent entrapped in the liposome stays within the liposome until it reaches the target endometriotic tissue. It has been shown previously that therapeutic agents delivered *via* liposomes concentrate in tissues with leaky vasculatures. For example, high drug accumulation has been shown to occur in human prostate carcinoma

Xenograft after administration of liposome- encapsulated doxorubicin. Such tumor tissues are characterized by "leaky" or open-ended blood vessels as described by Vaage, J., et al. Cancer 73: 5 (1994). By virtue of their size, liposomes containing a hydrophilic polymer coating on their outer surface do not escape from normal blood vessels. However, many diseases, including endometriosis, are characterized by "leaky" or open-ended blood vessels. Therefore, the present inventor contemplates that liposomes would exit from the blood stream and build up in these leaky or open-ended blood vessel area within the endometriotic implant.

Attachment of a Moiety to the Liposome Component

Suitable means for preparing a coating of hydrophilic polymer chains on the liposomes are provided in U.S. Patent Publication No. 2002/0172711 A1 and in the Examples below.

As described above, in one embodiment of the liposome compositions of the present invention, a liposome that includes a hydrophilic polymer coating, such as PEG, may further include a ligand for targeting the liposomes to a selected cell type, enzyme, or cytokine within a tissue in which metabolism *via* HSD-type 2 occurs or is impaired, such as the endometriotic implant. Preferably, the ligand is an antibody Fab fragment that is bound to the liposome by covalent attachment to the free distal end of a lipid-anchored hydrophilic polymer chain, such as PEG.

In one embodiment of the invention, the hydrophilic polymer chain is PEG, and several methods for attachment of the ligands to the distal end of PEG chains have been described. See, for example, Allen, et al., Biochim. Biophys. Acta. 1237:99-108 (1995); Zalipsky, Bioconj. Chem. 4: 296-299 (1993); Zalipsky, et al., React. Polym. 22: 243-258 (1994); Zalipsky, Bioconj. Chem. 6:150-165 (1995 A); and Zalipsky, Adv. Drug Delivery Rev. 16: 157-182 (1995 B). In these methods, the inert terminal methoxy group of methoxy PEG (mPEG) is replaced with a reactive functionality suitable for conjugation reactions, such as an amino or hydrazide group. The end functionalized PEG is attached to a lipid, typically distearyl phosphatidylethanolamine (DSPE). The functionalized PEG-DSPE derivatives are employed in liposome formation and the desired ligand is attached to the reactive end of the PEG chain before or after liposome formation. In addition to liposome components wherein the ligand is conjugated to the distal end of a hydrophilic polymer chain, it is noted that a ligand such as a Fab antibody fragment may be directly bound to the surface of the liposomes

by attachment to surface lipid components. In one embodiment, such liposomes would further include hydrophilic polymer chains which are preferably present in the outer lipid layer of the liposomes and proximate to the Fab antibody fragments. Thus, in this instance, the Fab antibody fragment would be initially shielded by the hydrophilic surface coating from interaction with the target cells. Provided the hydrophilic polymer chains are releasable, the Fab fragment would be shielded until such time as after the removal of the hydrophilic polymers by suitable means, such as reducing agents.

A ligand, such as an antibody Fab fragment may be coupled to the polar head group of a vesicle-forming lipid and various methods have been described for attaching ligands to lipids. For example, suitable methods are described in U.S. Publication No. 2002/0172811 A1 and in the Examples below. Moreover, detailed methods of antibody modification and coupling to liposomes are described by Schwendener, et al. in Biochim, Biophys, Acta 1026: 69 (1990). In one method, the affinity moiety may be coupled to a lipid by a coupling reaction so as to form an affinity-moiety-lipid conjugate. This conjugate may then be added to a solution of lipids for formation of the liposomes. In another method, a vesicle-forming lipid which has been activated for covalent attachment of an affinity moiety, such as a Fab fragment, is employed in liposome formation. The formed liposomes may then be exposed to the affinity moiety to achieve attachment of the affinity moiety to the activated lipids.

A variety of methods are available for preparing a conjugate composed of an affinity moiety and a vesicle-forming lipid. For example, water-soluble, amine-containing affinity moieties can be covalently attached to lipids, such as phosphatidylethanolamine (PE), by reacting the amine-containing moiety with a lipid which has been derivatized to contain an activated ester of N-hydroxysuccinimide.

As another example, biomolecules, and in particular large biomolecules such as proteins (an example of which is an antibody), can be coupled to lipids according to reported methods. One method involves Schiff-base formation between an aldehyde group on a lipid, typically a phospholipid, and a primary amino acid on the affinity moiety. The aldehyde group is preferably formed by periodate oxidation of the lipid. The coupling reaction, after removal of the oxidant, is carried out in the presence of a reducing agent, such as dithiothreitol, as described by Heath et al., Biochim. Biophys. Acta 640 (1): 66-81 (1981). Typical aldehyde-lipid precursors suitable in the method include lactosylceramide,

trihexosylceramine, galacto cerebroside, phosphatidylglycerol, phosphatidylinositol and gangliosides.

A second general coupling method is applicable to thiol-containing affinity moieties, and involves formation of a disulfide or thioether bond between a lipid and the affinity moiety. In the disulfide reaction, a lipid amine, such as phosphatidyl-ethanolamine, is modified to contain a pyridylditho derivative which can react with an exposed thiol group in the affinity moiety. Reaction conditions for such a method can be found in Martin et al., Biochemistry, 20: 4229-4238 (1981). The thioether coupling method, described by Martin et al., J. Biol. Chem. 257: 286-288 (1982), is carried out by forming a sulfhydryl-reactive phospholipid, such as N-(4)P-maleimidophenyl(butyryl)phosphatidylethanolamine, and reacting the lipid with the thiol-containing affinity moiety.

Another method for reacting an affinity moiety with a lipid involves reacting the affinity moiety with a lipid which has been derivatized to contain an activated ester of N-hydroxysuccinimide. The reaction is typically carried out in the presence of a mild detergent, such as deoxycholate. Like the reactions described above, this coupling reaction is preferably performed prior to employing the lipid in liposome formation.

The above-described coupling techniques are exemplary and it will be appreciated that other suitable methods are known in the art and have been described, for example in U.S. Patent Nos. 4,605,630, 4,731,324, 4,429,008, 4,622,294 and 4,483,929.

Labels

In one embodiment of the present invention, the liposome compositions may include a label which is capable of external detection. As such, the inventive liposome compositions may be useful for monitoring the presence of the inventive composition in the endometriotic implants by detecting the label for diagnosis or treatment. In one embodiment, the label may be associated with an HSD-Type 2 polypeptide or nucleic acid form. For example, the label may be chemically or physically bound to the HSD-Type 2 polypeptide or nucleic acid form. Alternatively, the label may be separate from the HSD-Type 2 polypeptide or nucleic acid form, but proximate thereto. The label may be a fluorescent dye, contrast agent or radiopaque agent. In another embodiment, the label may be a radioactive nuclide. For example, the label can be on a radiolabeled complex that can be encapsulated along with the

HSD-type 2 form. In one embodiment of the invention, the radioactive nuclide may be a positron-emitter or γ-emitter. Preferred positron-emitters are ^{11}C, ^{18}F, ^{76}Br, ^{77}Br and ^{89}Zr. Suitable γ-emitters include but are not limited to ^{67}Ga or ^{111}In.

The monitoring may be accomplished by positron emission tomography (PET) in cases where the label is a radioactive positron-emitter. In addition, monitoring may be accomplished by Single Photon Emission Computed Tomography (SPECT, γ-camera) in cases where the label is a radioactive γ-emitter. Moreover, monitoring may be by fluorescent scanning in situations where the label is a fluorescent dye.

In embodiments where the label is separate from the HSD-Type 2 polypeptide or nucleic acid form, it is contemplated that a radioactive nuclide, such as ^{111}In may be complexed with ligands like diethylenetriaminepentaacetic acid (DTPA). Such complexes may be encapsulated along with the HSD-Type 2 form. For example, as will be described in further detail in the examples below, liposomes can be prepared that contain DTPA, for the complexation of ^{111}In. These liposomes may be prepared according to standard procedures, such as those described in U.S. Patent Publication 2002/0172711 A1, except that the dried lipid film is hydrated with an aqueous phase that includes the DPTA chelating agent and the HSD-Type 2 form. In one embodiment, the fusogenic liposomes include entrapped plasmids encoding HSD-Type 2 and the chelating agent. Following sequential extrusion through a membrane to obtain liposomes of approximately 100 nm in size, non-entrapped molecules are removed. The liposomes may then be loaded with the radioactive nuclide. For example, ^{111}In-oxine is commercially available (Amersham). This nuclide may be equilibrated with the liposome preparation so as to effect binding of the nuclide to the entrapped chelate. This is followed by removal of free radionuclide and free oxine so as to produce the desired, labeled liposome preparation.

Administration of the Inventive Liposome Composition

The liposome compositions of the present invention are designed for use in delivering an HSD-Type 2 polypeptide or nucleic acid form to a target cell, enzyme, or cytokine at their site associated with aberrant production of estradiol or testosterone, such as the endometriotic implant site. Once at this site, delivery of the therapeutic agent may be accomplished by fusion of the vesicles with the plasma membrane of cells within the endometriotic implant, releasing the agent into the cytoplasmic compartment of the cells.

In one embodiment, the liposome compositions of the present invention are administered intravenously. In another embodiment, the inventive liposome composition is administered *via* transdermal administration.

The amount and frequency of administration of the inventive composition will be regulated according to the judgment of the attending clinician (physician) considering such factors as age, condition and size of the patient, as well as severity of the endometriosis being treated. Moreover, it is well within the contemplation of the present invention that the inventive compositions may be useful for regulating the intra-conversion of estrone/estradiol in patients suffering from disorders other than endometriosis. Determination of the proper dosage regiment for a particular situation is within the scale of the art. In one embodiment, the inventive composition is administered in a therapeutically effective amount. This amount is to be determined by the attending clinician.

It is noted that in embodiments wherein a specific ligand (such as an antibody fragment) is attached to the liposome carrier component, this attachment can be either a direct or indirect attachment. For example, as described above, the ligand may be covalently attached by suitable means to a lipid in the external phospholipid layer. In an alternative embodiment, a specific ligand may be attached to the distal ends of lipid-anchored hydrophilic polymer chains. In either instance, the specific moiety would be exposed for purposes of binding and targeting the endometriotic implant or other tissues in need of regulation of the intra-conversion of estradiol/estradiol. Alternatively, the moiety can be targeted for tissue in need of regulation of the intra-conversion of testosterone/androstenedione.

In other embodiments of the present invention, targeting to selected cells or tissue of the endometriotic implant may be passive, i.e., through the normal bio-distribution of the liposomes after administration, without the requirement for ligands having a specific binding affinity for the implant. For example, it is well within the contemplation of the present invention that liposomes including hydrophilic polymer chains, such as PEG which are known to have a prolonged circulation time within the blood, can accumulate, after IV administration, at the site of the endometriotic implant by exiting the blood stream through leaky or open-ended blood vessels within the endometriotic implant.

In one embodiment, when PEG liposomes have reached a selected target site, the liposomes are contacted at the target endometriotic cells with a chemical agent effective to release the hydrophilic PEG chains on the liposome surface. For example, as described in US Publication 2002/0172711 A1, the hydrophilic polymer chains may be linked to hydrophobic chains on the liposome surface (or directly to the liposome lipids) *via* disulfide linkages. In this embodiment, after intravenous administration of the liposome composition, the subject is treated by IV administration of a reducing agent. For example, the reducing agent cysteine may be added to reduce disulfide bonds in order to release releasable PEG from the liposomes. As described above, release of the PEG chains allows exposure of the positive liposome surface charges of cationic liposomes, which in turn can enhance binding to the negative cell membranes and improve retention of the liposomes in the target tissue.

It is also noted that in embodiments where the hydrophilic polymer chains are linked *via* disulfide linkages to hydrophobic chains on the liposome surface (see US Publication 2002/0172711 A1), treatment with a reducing agent can serve to expose the hydrophobic polymers on a liposome surface to the target cells, promoting fusion of the liposomes with the target cell surface. While not wishing to be bound by any one theory, it is believed that the hydrophobic segment, now in an aqueous environment, will seek a more favorable, e.g. hydrophobic environment, both in the liposome bilayer and in the adjacent target cell membrane. This makes the liposomes more susceptible to fusion with target cells.

Gene Therapy

Gene therapy refers to therapy performed by administering to a patient an expressed or expressible nucleic acid. In one embodiment of the present invention, the liposomes contain an entrapped gene (cDNA plasmid) which is delivered to target cells, for *ex vivo* or *in vivo* gene therapy. In the latter case, a gene is directly introduced (intravenously, intraperitoneally, aerosol, etc.) into a subject. In *ex vivo* (or *in vitro*) gene transfer, the gene is introduced into cells after removal of the cells from specific tissue in an individual. The transfected cells are then introduced back into the subject or the expressed protein is isolated from the transfected host cells, which is then delivered to the subject. It is well within the contemplation of the present invention that either *ex vivo* or *in vivo* gene therapy may be useful to replace deficient HSD-Type 2 expression within the endometriotic tissue.

In one embodiment of the invention, the liposomes contain a polynucleotide designed to be incorporated into the genome of the target endometriotic cell or designed for autologous replication within the cell. In another embodiment, the compound entrapped in the lipid vesicles is an oligonucleotide segment designed for sequence-specific binding to cellular or RNA or DNA.

Polynucleotides, oligonucleotides, other nucleic acids, such as DNA plasmids, can be entrapped in the liposome by condensing the nucleic acid in single-molecule form. The nucleic acid is suspended in an aqueous medium containing spermine, spermidine, histone, lysine, mixtures thereof, or other suitable polycationic condensing agents, under conditions effective to condense the nucleic acid into small particles. This is described in U.S. Patent Publication 2002/0172711 A1. The solution of the condensed nucleic acid molecules may be used to rehydrate a dried lipid film to form liposomes with a condensed nucleic acid in entrapped form.

Suitable liposome components and methods for entrapping in the liposome an HSD-Type 2 protein or polypeptide form are described in U.S. Patent Nos. 5,567,433 and 4,241,046. This patent discloses a liposome preparation including encapsulated granulocyte-colony stimulating factor (G-CSF). In addition, U.S. Patent No. 4,241,046 describes a method for encapsulating an enzyme with a synthetic liposome, the product liposomes being useful for enzyme replacement therapy. The entire contents of these patents are herein incorporated by reference and provide suitable methods for preparing liposomes for encapsulating an HSD-Type 2 polypeptide in the present invention.

EXAMPLES

COMPARATIVE EXAMPLE 1

DETERMINING THE BIODISTRIBUTION OF PEG LIPOSOMES CONTAINING AN ENTRAPPED RADIOLABELED LIGAND

A Stealth® liposome is obtained by binding PEG on the lipid bi-layer of liposomes. PEG prevents liposomes from being quickly damaged by proteins after injection and, therefore, prolongs the circulation time in the blood. In particular, encapsulated therapeutic agents stay in the liposome until reaching target tissues. Encapsulated therapeutic agents included in such PEG liposome compositions concentrate in tissues with leaky or open-ended

blood vessels. The present example was used to assess the biodistribution of PEG liposomes containing a chelated radionuclide.

Preparation of Cationic Liposomes Containing Entrapped ^{111}In-DTPA

Cationic liposomes composed of the lipids dimethyldioxidecyl ammonium and cholesterol (DDAB:Chol) are prepared according to standard procedures by dissolving 10 μmole DDAB and 10 μmole Chol in an organic solvent containing primarily $CHCl_3$. The lipid solution is dried as a thin film by rotation under reduced pressure. The lipid film is subsequently hydrated by the addition of an aqueous phase comprised of 10 mM DTPA/0.15 M NaCl, pH 6.5, to form liposomes (at a total lipid concentration of 20 μmole/ml), which were sized by sonication or by sequential extrusion through Nucleopore polycarbonate membranes with pore sizes of 0.4 μm, 0.2 μm, 0.1 μm and 0.05 μm to obtain liposomes of approximately 100-150 nm in size. Free DTPA is removed by purification on a Sephadex G-50 column that has been pre-equilibrated with three volumes of saline (total 30 ml). Liposome fractions purified from free DTPA are collected.

The purified liposome composition is radiolabeled upon addition of ^{111}In-oxine (Amersham) to the liposome. In particular, 20 μl of indium (1 μCi/μl) per ml of liposome is added, wherein the concentration of total lipids is about 10 mM. Incubation is for one hour at room temperature, which allows the radioactive complex to diffuse inside the liposome. The nuclide then binds preferably to DTPA due to its stronger affinity therefore, allowing the release of free oxine. Free indium is removed by passage through a 10 DG column that has been pre-equilibrated with 3 volumes of saline. The radiolabeled liposome composition is loaded onto the column at a volume of not more than about 3 ml and the purified liposome fractions are collected.

Insertion of PEG

Distearyl phosphatidylethanolamine (DSPE) is derivatized with PEG, as described by Kirpotin et al., FEBS Lett. 388: 115-118 (1986); Zalipsky, Adv. Dwg. Delivery Revs., 16: 157 (1995); and Woodle et al., Biochem. Biophys. Acta. 1113:171 (1992). PEG-DSPE micelles are prepared from PEG-DSPE by dissolving 1 mM in water and sonicating.

Micelles of PEG-diothiopropionate (DTP)-DSPE, that is, PEG attached to the DSPE by a cleavable disulfide linkage, are prepared by dissolving 1 mM PEG-DTP-DSPE in water and sonicating.

Liposomes containing 2.5 mole % of PEG-DSPE are prepared by adding the PEG-DSPE micelle suspension (1 μmole lipid/ml) to 5.6 μmoles lipid of the cationic liposomes prepared as described above. The micelle-liposome suspension is incubated for 5 minutes at room temperature with gentle vortexing to achieve insertion of the PEG-DSPE into the cationic liposomes.

In Vivo Administration of PEG-Coated Cationic Liposomes Containing Entrapped ^{111}In-DTPA

The PEG-coated cationic liposomes including the entrapped, chelated radionuclide are administered to scid mice that have developed endometriosis (stage I-IV) obtained from Charles River Laboratories (Wilmington, MA). In particular, the Stealth® liposomes are used for targeting leaky vessels present at different stages of endometriosis.

The liposome composition containing the entrapped radiolabeled ligand (i.e. ^{111}In-DTPA) is administered by tail vein injection. In particular, 20 mice are each injected with 10-20 μCi per mouse (preferably, 10-100 μCi). This corresponds to about 20 nmoles lipid in 100-200 μl saline. Mice are sacrificed after 24, 48 and 72 hours and scanned as described below.

Each of the sacrificed mice is scanned by Single Photon Emitter Computed Tomography (SPECT) camera, i.e. γ-camera. Such cameras are made by manufacturers such as GE and Toshiba. Scanning is performed in order to assess biodistribution of the radiolabel within tissues. The mice are then dissected. In particular, select organs are obtained such as brain, kidneys, liver, etc. and all endometriotic implants. The amount of radiolabeled compound present in each of the organs is quantified to establish biodistribution. In particular, radioactivity can be determined in a Germanium lithium (GeLi) detector (available from EG&G Ortec or Canbera). Activity for each organ can be quantified by comparison with a phantom which is created for each organ by methods well known in the art. After activity values are obtained using the phantom, these values are normalized to an organ, usually the one that has the highest activity. The specific activity and the percentage of accumulation are also calculated by methods well known in the art. This process is repeated

for each time point and for each mouse. By establishing the biodistribution for each time point, a time point is selected which provides optimal distribution of PEG liposomes in the endometriotic implant, as a fraction of the type of implant (clear, yellow, red, etc.) and the stage of the disease (I to IV).

EXAMPLE 2

PEG LIPOSOMES WITH ENTRAPPED 17-β-HSD-TYPE 2 DNA PLASMID

In this example, DNA plasmid encoding HSD-Type 2 is condensed with spermidine in order to obtain single molecules of the plasmid, and then entrapped in liposomes as follows.

A 10 mM tris buffer solution, pH 7.5, containing 0.1 mM spermidine is prepared. To 1 ml of the buffer solution (14.52 μg spermidine; free base, Sigma Chemical Co., St. Louis, MO.), and 30 μg of the plasmid is added. The plasmid-spermidine solution, containing about 2 μg plasmid/μg spermidine, is mixed to form condensed, single molecules of DNA.

Cationic liposomes composed of the lipids dimethyldioctadecyl ammonium and cholesterol (DDAB:Chol) are prepared according to the procedure described in Example 1, except that the dried lipid film was hydrated with the plasmid-spermidine solution to form fusogenic liposomes having entrapped, condensed HSD-Type 2 plasmid molecules. These liposomes (at a total lipid concentration of 20 μmole/ml) are subsequently sized by sonication or by sequential extrusion through Nucleopore polycarbonate membranes with pore sizes of 0.4 μm, 0.2 μm, 0.1 μm and 0.05 μm to obtain liposomes of 100-150 nm in size. PEG is inserted into the external lipid layer of the liposomes by the same method as described in Example 1, except that the PEG liposomes is prepared by adding 140 μl of the PEG-DSPE micelle suspension (1 μmole lipid/ml) to 5.6 μmoles lipid of the cationic liposomes containing the entrapped HSD-Type 2 plasmid molecules.

Gene Therapy

Fusogenic PEG liposomes containing the cDNA plasmid encoding HSD-Type 2 are delivered to target cells for *in vivo* gene therapy. The gene is directly introduced intravenously by tail vein injection of the liposome composition in mice. Liposomes containing the polynucleotide are incorporated into the genome of endometriotic cells and, preferably, are suitable for autologous replication within the cell.

Mice are sacrificed at time points that were determined in Example 1 to provide optimal distribution of PEG liposomes in the endometriotic implant following liposome injection, Organs including brain, kidneys, liver, etc. and all endometriotic implants are analyzed for HSD-Type 2 activity. In particular, the PEG chains cause these liposome compositions to accumulate in endometriotic tissues, allowing for replacement of the deficient expression of HSD-Type 2 therein.

EXAMPLE 3

Fab/PEG LIPOSOMES CONTAINING ENTRAPPED HSD-TYPE 2 DNA PLASMIDS

A variety of methods are known for attaching an affinity moiety to the external phospholipid layer of the liposomes. Some of these methods have been described above. In the present example, the conjugate is first formed between an antibody Fab fragment and a vesicle-forming lipid. This conjugate is then added to PEG liposomes for formation of the Fab/PEG liposomes.

A water-soluble, amine-containing antibody fragment specific for TNF-α, (Alza, Menlo Park, CA and Centicor, Malvern, PA), is covalently attached to lipids, such as phosphatidylethanolamine (PE), by reacting the amine-containing moiety with a lipid which has been derivatized to contain an activated ester of N-hydroxy-succinimide. Micelles are prepared from this lipid conjugate by sonicating. This conjugate is then combined with the liposomes by adding 140 µl of the micelle suspension of the lipid conjugate (1 µmole lipid per ml) to 5.6 µmole lipid of cationic PEG liposomes containing entrapped DNA plasmid encoding HSD-Type 2, which are prepared according to the same procedure described in Example 2 above. The micelle-cationic liposome suspension is incubated for 5 minutes at room temperature with gentle vortexing to achieve insertion of the Fab-PE into the cationic PEG liposomes.

Immunoliposomes are separated from free antibody fragments by HPLC using methods well known in the art. Liposomal absorption is monitored and fractions of free antibody fragments and immunoliposomes are collected.

Gene therapy with the immunoliposomes in the present example is performed as described in Example 2 above. It is noted that, in addition to monitoring the restoration of HSD-Type 2 activity within the endometriotic implant, gene therapy can be monitored by either labeling the plasmid with a label capable of external detection, or alternatively,

incorporating a labeled ligand in a location proximate to the DNA plasmid. For example, as described above, ligands such as ^{111}In-DPTA that are entrapped within the liposome along with the DNA plasmids may be used to monitor the gene therapy, keeping in mind the half-life of the radionuclide. In this regard, it is noted that the half-life of indium is 3.2 days.

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WHAT IS CLAIMED IS:

1. A composition useful in the treatment of a disorder associated with aberrant production of estradiol or testosterone comprising:

(a) a liposome carrier component;

(b) a moiety targeted for a site in the body associated with said aberrant production of estradiol or testosterone, or leaky blood vessels thereat, said moiety being attached to said liposome carrier component; and

(c) an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, said polypeptide or nucleic acid form being encapsulated by said liposome carrier component.

2. The composition of claim 1, wherein said moiety is targeted for an endometriotic implant or leaky blood vessels within said implant.

3. The composition of claim 1, wherein said moiety is targeted for tissue in which intraconversion of testosterone/androstenedione *via* 17-β-hydroxysteroid dehydrogenase-type 2 occurs or is impaired.

4. The composition of claim 1, wherein said moiety is a protein or protein fragment specific for said site.

5. The composition of claim 1, wherein said moiety is a hydrophilic polymer chain.

6. The composition of claim 5, wherein said hydrophilic polymer chain is a polyethylene glycol chain.

7. The composition of claim 6, wherein a protein or protein fragment specific for said site is attached to the distal ends of said polyethylene glycol chains.

8. The composition of claim 5, wherein said liposomes with said attached hydrophilic polymer chains are capable of exiting from the blood stream to said site from said leaky vessels.

9. The composition of claim 1, wherein said 17-β-hydroxysteroid dehydrogenase-type 2 nucleic acid form encodes 17-β-hydroxysteroid dehydrogenase-type 2 (SEQ ID NO: 1), or a functional fragment or variant thereof.

10. The composition of claim 1, wherein said 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide form comprises 17-β-hydroxysteroid dehydrogenase-type 2 (SEQ ID NO: 1), or a functional fragment or variant thereof.

11. The composition of claim 2, wherein said 17-β-HSD-Type 2 polypeptide or nucleic acid form is present in amounts sufficient to restore the lack of 17-β-HSD-Type 2 within endometriotic implants.

12. The composition of claim 1, wherein said liposome carrier component comprises an external phospholipid layer and an internal phospholipid layer.

13. The composition of claim 12, wherein said moiety is chemically bound to said external phospholipid layer.

14. The composition of claim 1, wherein said attached moiety is an antibody Fab fragment.

15. The composition of claim 1, wherein said attached moiety is a peptide, polypeptide, protein, or glycoprotein.

16. The composition of claim 2, wherein said attached moiety is specific for endometriotic cells in said implant.

17. The composition of claim 2, wherein said attached moiety binds a marker produced by or associated with an endometriotic cell.

18. The composition of claim 2, wherein said attached moiety is specific for a cytokine, cell, or enzyme present in increased amounts in said implant as compared to normal tissues.

19. The composition of claim 2, wherein said attached moiety is specific for a cytokine, cell, or enzyme present in increased amounts in said implant in response to inflammation in the implant.

20. The composition of claim 19, wherein said cytokine is IL-1 β or tumor necrosis factor-α.

21. The composition of claim 19, wherein said cell is an eosinophil.

22. The composition of claim 19, wherein said enzyme is matrix metalloproteinase-7 or matrix metalloproteinase-11.

23. The composition of claim 4, wherein said liposome carrier component comprising said attached protein or protein fragment is present in suitable amounts to obtain specific binding to target cells at said site.

24. The composition of claim 1, wherein said liposome carrier component comprising said attached moiety is present in amounts of about 1 to about 1500 nM.

25. The composition of claim 1, wherein said liposome carrier component comprising said attached moiety is present in amounts of about 10 to about 150 nM.

26. The composition of claim 1, further comprising a label capable of external detection.

27. The composition of claim 26, wherein said label is chemically or physically bound to said 17-β-HSD-Type 2 polypeptide or nucleic acid form.

28. The composition of claim 26, wherein said label is separate from said 17-β-HSD-type 2 polypeptide or nucleic acid form, but is proximate thereto.

29. The composition of claim 26, wherein said label is a fluorescent dye, contrast agent or radioopaque agent.

30. The composition of claim 26, wherein said label is a radioactive nuclide.

31. The composition of claim 30, wherein said radioactive nuclide is complexed with one of the group consisting of oxine, ethylenediaminetetraacetic acid and diethylenetriaminepentaacetic acid.

32. The composition of claim 30, wherein said nuclide is a positron-emitter or γ-emitter.

33. The composition of claim 32, wherein said positron-emitter is selected from ^{11}C , ^{18}F, ^{76}Br, ^{77}Br, and ^{89}Zr.

34. The composition of claim 32, wherein said γ-emitter is ^{67}Ga or ^{111}In.

35. A pharmaceutical composition targeted for endometriotic implants comprising:

(a) a liposome carrier component having an external phospholipid layer and an internal phospholipid layer;

(b) a moiety targeted for an endometriotic implant or leaky blood vessels within said implant, said moiety being chemically bound to said external layer; and

(c) an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, said 17-β-HSD-Type 2 polypeptide or nucleic acid form being encapsulated by said liposome carrier component.

36. The composition of claim 35, wherein said moiety is an antibody Fab fragment specific for said endometriotic implant.

37. The composition of claim 35, wherein said moiety is a polyethylene glycol chain.

38. The composition of claim 37, wherein an antibody Fab fragment specific for said endometriotic implant is attached to the distal ends of said polyethylene glycol chains.

39. The composition of claim 35, wherein said 17-β-hydroxysteroid dehydrogenase-type 2 nucleic acid form encodes 17-β-hydroxysteroid dehydrogenase-type 2 (SEQ ID NO: 1), or a functional fragment or variant thereof.

40. The composition of claim 35, wherein said 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide form comprises 17-β-hydroxysteroid dehydrogenase-type 2 (SEQ ID NO: 1),

or a functional fragment or variant thereof.

41. The composition of claim 35, wherein said 17-β-HSD-Type 2 polypeptide or nucleic acid form is present in amounts sufficient to restore the lack of 17-β-HSD-Type 2 within endometriotic implants.

42. The composition of claim 36, wherein said antibody fragment is specific for endometriotic cells in said implant.

43. The composition of claim 36, wherein said antibody fragment is specific for a cytokine, cell, or enzyme present in increased amounts in said implant as compared to normal tissues.

44. The composition of claim 36, wherein said antibody fragment is specific for a cytokine, cell, or enzyme present in increased amounts in response to inflammation in the implant.

45. The composition of claim 35, further comprising a label capable of external detection.

46. The composition of claim 45, wherein said label is a fluorescent dye.

47. The composition of claim 45, wherein said label is a radioactive nuclide.

48. The composition of claim 47, wherein said nuclide is a positron-emitter or γ-emitter.

49. The composition of claim 48, wherein said positron-emitter is selected from ^{11}C, ^{18}F, ^{76}Br, ^{77}Br, and ^{89}Zr.

50. The composition of claim 48, wherein said γ-emitter is ^{67}Ga or ^{111}In.

51. A method for treating a disorder associated with aberrant production of estradiol or testosterone comprising administering to a patient a therapeutically effective amount of a composition comprising:

(i) a liposome carrier component;

(ii) a moiety targeted for a site in the body associated with said aberrant production of estradiol or testosterone, or leaky blood vessels thereat, said moiety being attached to said liposome carrier component; and

(iii) an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, said 17-β-HSD-Type 2 polypeptide or nucleic acid form being encapsulated by said liposome carrier component.

52. The method of claim 51, wherein said moiety is targeted for an endometriotic implant or leaky blood vessels within said implant.

53. The method of claim 52, wherein said disorder is endometriosis.

54. The method of claim 52, wherein said 17-β-HSD-Type 2 polypeptide or nucleic acid form is present in amounts sufficient to restore the lack of 17-β-HSD-Type 2 within endometriotic implants.

55. The method of claim 51, wherein said moiety is targeted for tissue where intraconversion of testosterone/androstenedione *via* 17-β-hydroxysteroid dehydrogenase-type 2 occurs or is impaired.

56. The method of claim 55, wherein said 17-β-HSD-Type 2 polypeptide or nucleic acid form is present in amounts sufficient to control or reduce amounts of testosterone and/or dihydrotestosterone in said targeted tissue.

57. The method of claim 55, wherein said disorder is selected from the group consisting of prostate cancer, benign prostate hyperplasia and hair loss.

58. The method of claim 51, wherein said administering comprises parenteral administration.

59. The method of claim 58, wherein said parenteral administration is by intravenous injection or transdermal administration of said composition.

60. A method for detecting an endometrial implant and/or diagnosing the presence of endometriosis in a patient, the method comprising:

(a) administering to a patient a composition comprising:

(i) a liposome carrier component;

(ii) a moiety targeted for an endometriotic implant or leaky blood vessels within said implant, said moiety being attached to said liposome carrier component;

(iii) an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, said 17-β-HSD-Type 2 polypeptide or nucleic acid form being encapsulated by said liposome carrier component;

(iv) a label capable of external detection, said label being proximate to or covalently bound to said 17-β-HSD-Type 2 polypeptide or nucleic acid form; and

(b) monitoring the presence of said composition in said endometriotic implant by detecting said label.

61. The method of claim 60, wherein said label is quantitatively measured.

62. The method of claim 60, wherein said administering comprises intravenous injection or transdermal administration of said composition.

63. The method of claim 60, wherein said composition is administered in a therapeutically effective amount.

64. The method of claim 60, wherein said monitoring comprises Positron Emission Tomography (PET) or tomography (γ-camera), and wherein said label is a radioactive positron- or γ-emitter, respectively.

65. The method of claim 60, wherein said monitoring comprises fluorescence scanning, and wherein said label is a fluorescent dye.

66. A method of preparing a composition useful in the treatment of a disorder associated with aberrant production of estradiol or testosterone comprising:

(a) providing a liposome component having an external phospholipid layer and an

internal phospholipid layer;

(b) attaching a moiety to said external layer to form a targeted liposome component, wherein the moiety is targeted for a site in the body associated with said aberrant production of estradiol or testosterone, or leaky blood vessels thereat; and

(c) combining said targeted liposome component with an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form under suitable conditions for said 17-β-HSD-Type 2 polypeptide or nucleic acid form to become encapsulated by said liposome component.

67. The method of claim 66, wherein said moiety is targeted for an endometriotic implant or leaky vessels within said implant.

68. The method of claim 66, wherein said moiety is targeted for tissue in which intraconversion of testosterone/androstenedione *via* 17-β-hydroxysteroid dehydrogenase-type 2 occurs or is impaired.

69. The method of claim 66, further comprising the step of labeling said 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form prior to said encapsulation.

70. The method of claim 66, further comprising the step of encapsulating an externally-detectable labeled complex in said liposome component.

71. The method of claim 66, further comprising the step of purifying said liposome component to remove material that is not encapsulated.

72. A method of regulating the intra-conversion of estradiol/estrone or testosterone/androstenedione in a patient comprising administering an effective amount of a composition comprising: (a) a liposome carrier component; and (b) an isolated or recombinant 17-β-hydroxysteroid dehydrogenase-type 2 polypeptide or nucleic acid form, said polypeptide or nucleic acid form being encapsulated by said liposome carrier component.

73. The method of claim 72, wherein said composition further comprises a protein or protein fragment specific for an endometriotic implant, said protein or fragment being attached to said liposome carrier component.

74. The method claim 72, wherein said composition further comprises a protein or protein fragment specific for the ovaries, said protein or fragment being attached to said liposome carrier component.

75.. The method of claim 72, further comprising a protein or protein fragment specific for tissue in which intraconversion of testosterone/androstenedione *via* 17-β-hydroxysteroid dehydrogenase-type 2 occurs or is impaired, said protein or fragment being attached to said liposome carrier component.

76. The method of claim 72, wherein said composition further comprises a protein or protein fragment specific for the prostate, said protein or fragment being attached to said liposome carrier component.

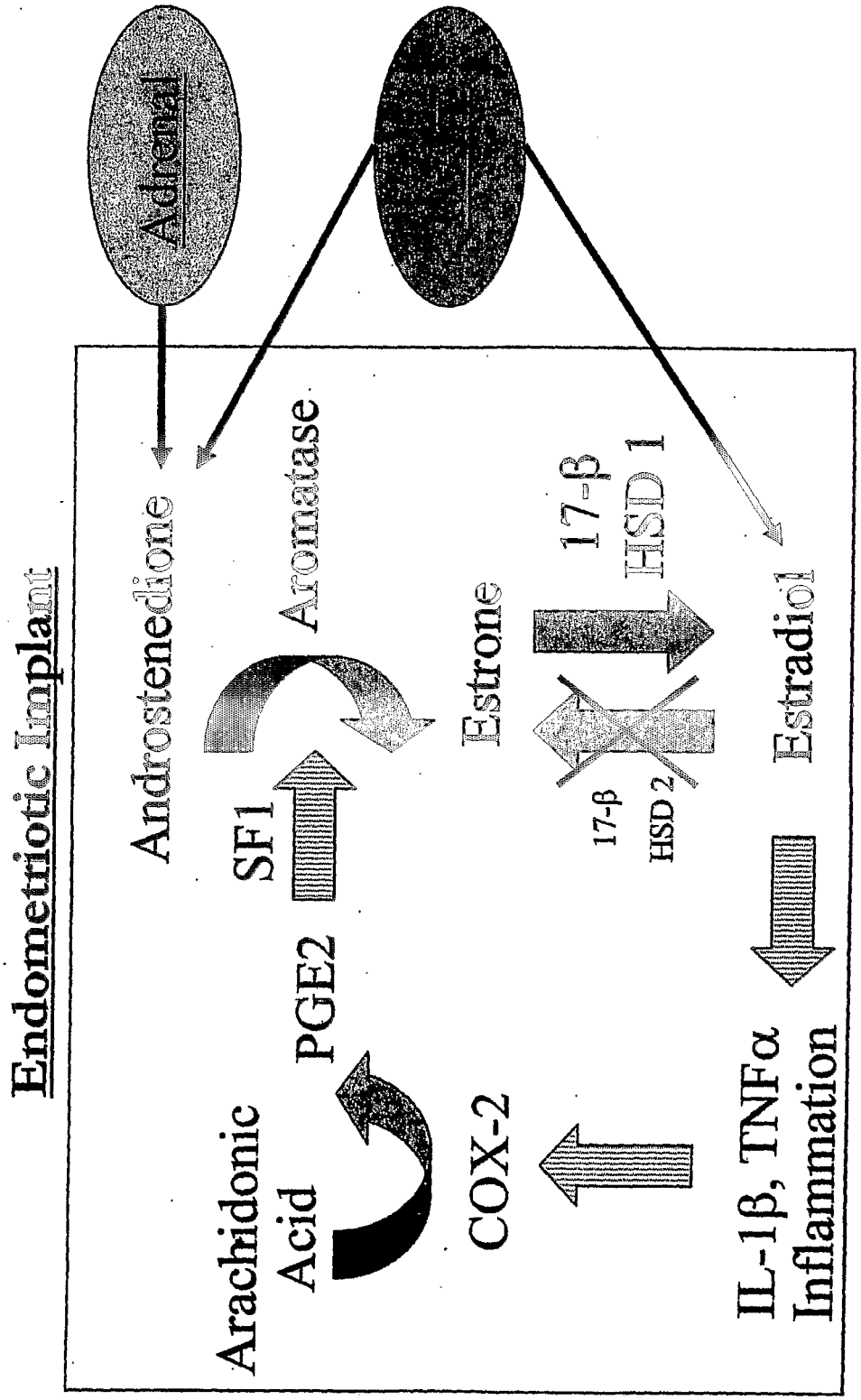
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Figure 1

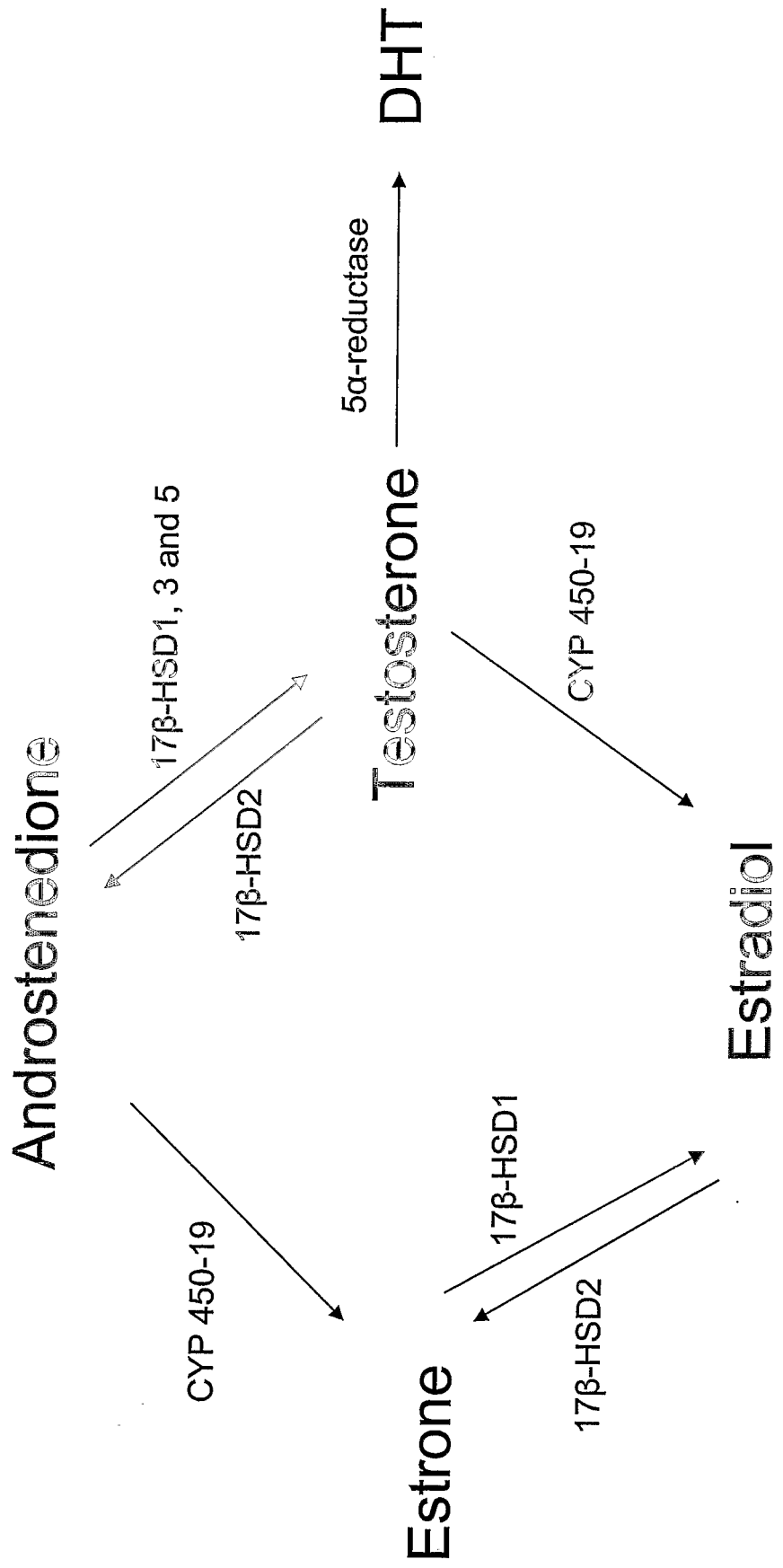

Figure. 2. Metabolism of Androstenedione to Estradiol

1264-6 PCT
SEQUENCE LISTING

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1264-6 PCT

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专利名称(译)	用17-β-羟基类固醇脱氢酶2型进行酶替代疗法		
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[标]申请(专利权)人(译)	ETHICON. INC		
申请(专利权)人(译)	ETHICON INC.		
当前申请(专利权)人(译)	ETHICON INC.		
[标]发明人	GENSINI MICHEL		
发明人	GENSINI, MICHEL		
IPC分类号	G01N33/53 A61K A61K47/48 A61K51/12 A61P5/24		
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代理机构(译)	MERCER，CHRISTOPHER PAUL		
优先权	60/455457 2003-03-18 US		
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摘要(译)

本文提供了用于调节雌二醇/雌酮或睾酮/雄烯二酮的内转化的组合物和方法。该组合物包括脂质体载体组分;外部附着于其上的部分，其靶向与雌二醇或睾酮的异常产生或其中的渗漏血管相关的体内部位。本发明的组合物还包括分离的或重组的HSD-2型多肽或由脂质体载体组分包封的核酸形式。该组合物用于克服子宫内膜异位组织中17-β-羟基类固醇脱氢酶2型的缺陷表达。 HSD-2型多肽或核酸形式可以在子宫内膜异位移植物部位递送至靶细胞，酶或细胞因子，或递送至植入物内的渗漏血管。能够外部检测的标记可以进一步包括在组合物中。