

N°d'ordre NNT : 2016LYSE1305

**THESE de DOCTORAT DE L'UNIVERSITE DE LYON**  
opérée au sein de  
**L'Université Lyon 1**

**Ecole Doctorale N° accréditation**  
**Ecole Doctorale de Chimie de Lyon**

**Spécialité de doctorat :**  
**Discipline :** Biochimie/Biotechnologie

Soutenue publiquement le 01/12/2016

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**Interaction des triterpènes avec les membranes synthétiques et  
l'albumine humaine : application aux progestatifs et corticostéroïdes  
et à deux structures pentacycliques**

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# *Remerciements*

## **Remerciements**

Tant de personnes ont rendu possible l'avènement de ce travail de thèse qu'il m'est aujourd'hui difficile de n'en oublier aucune. Au terme de ces années de doctorat, j'éprouve une sincère gratitude envers tous ceux qui ont participé à ce travail et que je tiens ici à remercier.

En premier lieu, mes plus chaleureux remerciements à *Professeur Hélène Greige*. Je la remercie d'avoir accepté de diriger ma thèse et de m'avoir transmis la passion de la recherche scientifique. Je la remercie pour ses multiples conseils et pour toutes les heures qu'elle a consacrées à diriger cette recherche. J'ai pu apprécier non seulement sa dimension scientifique, mais aussi sa dimension humaine qui est non plus de moindre importance. Je la remercie de m'avoir remotivée dans les moments de doute et d'avoir été vraiment une grande sœur pour moi. Je souhaite à tous les futurs doctorants d'avoir une encadrante de cette envergure! Ces quelques lignes sont peu de choses par rapport à tout ce qu'elle m'a apporté.

J'adresse également mes remerciements les plus sincères et les plus profonds à *Docteur Catherine Charcosset*, co-directrice de ma thèse, dont les grandes qualités professionnelles et aussi humaines m'ont aidé à aller au bout de ce travail dans la confiance et la reconnaissance. Je la remercie pour sa disponibilité, ses conseils et son soutien à tous les niveaux tout au long de la thèse.

Je tiens aussi à remercier *Professeur Hatem Fessi* de m'avoir accueillie au sein de son unité de recherche: le Laboratoire d'Automatique et de Génie des Procédés (LAGEP). Je suis très touchée par votre sympathie et votre modestie. Merci d'avoir accepté de présider le jury de ce travail.

Je remercie *Docteur Gérard Lizard* et *Docteur David Landy* pour l'intérêt que vous portez à ce travail de thèse en acceptant de le rapporter.

Je remercie également *Professeur Roland Habchi* pour votre aide à la réalisation des analyses infrarouges.

Je remercie *Catherine Bosser* pour votre aide à la réalisation des mesures de spectroscopie Raman à l'École Centrale de Lyon.

A tout le personnel du Laboratoire d'Automatique et de Génie des Procédés (Lyon, France) et de la Plateforme de l'École Doctorale des Sciences et de Technologie (EDST), (Hadath, Liban), en particulier *Géraldine Agusti* et *Sahar Rihan* : en signe de reconnaissance pour votre aide précieuse lors de la réalisation de ce travail.

Je remercie infiniment l'Agence Universitaire de la Francophonie (AUF) (projet de coopération scientifique inter-universitaire) pour l'aide financière qu'ils m'ont accordée durant ma thèse.

En dehors des laboratoires, il reste du temps, trop peu parfois, pour les amis. Tous, vous m'avez aidée et soutenue. Tout d'abord, *Emilie* et *Chirine* pour votre soutien moral et pour être toujours près de moi. Je remercie de tout mon cœur *Hanine*, *Joyce* et plus particulièrement *Riham* avec lesquelles j'ai passé des moments inoubliables. Merci de m'avoir aidée et encouragée.

Mes remerciements s'adressent à ma famille et à ma belle famille pour leur soutien constant. Ils ont toujours cru en moi et j'espère être toujours à leur hauteur. C'est une chance exceptionnelle de vous avoir dans ma vie. Un grand merci à mes parents, mon frère et ma sœur.

Pour conclure, je souhaite bien évidemment remercier mon mari *Maroun* qui m'épaule, m'accompagne et me soutient tout le temps par la force et l'énergie qui lui sont disponibles. Dans tout ce que j'ai pu souffrir et obtenir, il a été l'autre moi. Je le remercie infiniment.

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# *Résumé*

Les triterpènes sont un groupe important et structurellement diversifié de produits naturels issus du squalène. Les progestatifs et les glucocorticoïdes sont des triterpènes oxygénés ayant un squelette tétracyclique et reconnus pour leurs diverses propriétés thérapeutiques. Par ailleurs, l'érythrodiol et l'uvaol sont des triterpènes pentacycliques reconnus pour leurs effets bénéfiques dans l'alimentation humaine. Dans ce travail de thèse, l'interaction avec les membranes des vésicules lipidiques et la liaison à la sérum albumine humaine de ces molécules sont étudiées dans le but de mieux comprendre leurs propriétés pharmacologiques. Nous avons préparé des liposomes en absence et en présence des molécules triterpéniques par la méthode d'hydratation du film lipidique. Les vésicules lipidiques ont été caractérisées par calorimétrie différentielle à balayage (DSC), spectroscopie Raman, spectroscopie infrarouge à transformée de Fourier (FTIR) et polarisation de fluorescence du 1,6-diphényl-1,3,5-hexatriene (DPH) pour comprendre l'effet des molécules sélectionnées sur la fluidité membranaire.

Également, nous avons étudié la liaison d'une série de progestatifs et d'une autre série de glucocorticoïdes à l'albumine humaine par la spectroscopie de fluorescence. Par ailleurs, la liaison de la bilirubine (ligand du Site I de l'albumine) a été étudiée en absence et en présence des molécules triterpéniques. La spectroscopie infrarouge est utilisée pour évaluer l'effet d'un triterpène sur la structure secondaire de l'albumine.

Les résultats ont démontré que les progestatifs, les glucocorticoïdes, l'érythrodiol et l'uvaol altèrent les propriétés physiques de la bicoche lipidique. Tous les triterpènes interagissent avec les têtes polaires et les chaînes acyles des phospholipides. Par ailleurs, les caractéristiques structurales contrôlent l'interaction des molécules avec la membrane et par conséquent modulent leurs effets sur la fluidité membranaire.

Les progestatifs et les glucocorticoïdes démontrent un attachement modéré à l'albumine. La liaison des triterpènes à l'albumine au site I est favorisée par des interactions hydrophobes et des liaisons hydrogènes.

Notre étude a conduit à une connaissance approfondie des mécanismes moléculaires impliqués dans l'interaction des triterpènes avec les protéines et les membranes synthétiques et les caractéristiques structurales contrôlant ces interactions.

Mots clés: Calorimétrie différentielle à balayage, érythrodiol, glucocorticoïdes, liposomes, polarisation de fluorescence, progestatifs, spectroscopie infrarouge à transformée de fourier, spectroscopie Raman, spectroscopie de fluorescence, uvaol.

# *Abstract*

The triterpenoids are a large and structurally diverse group of natural products derived from squalene. Progesterone derivatives and glucocorticoids are a group of oxygenated triterpenes having a tetracyclic skeleton and identified for their therapeutic properties. Whereas, erythrodiol and uvaol are pentacyclic triterpenes, known for their beneficial effects on human diet. In this thesis, we studied their interaction with the membranes of lipid vesicles and with human serum albumin to better understand their pharmacological properties. We have prepared liposomes in the absence and presence of the mentioned molecules using the thin film hydration method. Differential scanning calorimetry (DSC), Raman spectroscopy, Fourier transform infrared spectroscopy (FTIR) and fluorescence polarization of 1,6-diphenyl- 1,3,5-hexatriene (DPH) were used to investigate the effect of triterpenes on the membrane fluidity.

Besides, we used fluorescence spectroscopy to study the binding of a series of progesterone derivatives and another series of glucocorticoids to albumin. Also, the binding of bilirubin (site I ligand) to albumin was studied in the absence and presence of the mentioned molecules. FTIR spectra of HSA and HSA-complexes were analyzed to evaluate the effect of a triterpene on the albumin secondary structure.

The results revealed that progesterone derivatives, glucocorticoids, erythrodiol and uvaol changed the physical properties of the bilayers. All the studied triterpenes interacted with the choline head group and the alkyl chains of phospholipids. Furthermore, the structural features control the interaction of triterpenes with the membrane and therefore modulate their effects on membrane fluidity.

Progesterone derivatives and glucocorticoids have been proven to bind moderately to albumin. Hydrophobic interactions and hydrogen bonds played a major role in stabilizing the complexes. Our work has led to a better understanding of triterpenes molecular mechanisms of their interaction with proteins and biological membranes and structural features controlling these interactions.

**Keywords:** Differential scanning calorimetry, erythrodiol, Fourier transform infrared spectroscopy, fluorescence polarization, fluorescence spectroscopy, glucocorticoids, liposomes, progesterone derivatives, Raman spectroscopy, uvaol.

**Liste des abréviations:**

Co: cortisol

Cn: cortisone

DSC: differential scanning calorimetry; calorimétrie différentielle à balayage

DPH: 1,6-diphényl-1,3,5-hexatriène

ER: érythrodiol

9-FA: 9-fluorocortisol acétate

FTIR: fourier transform infrared; spectroscopie infrarouge à transformée de fourrier

GC: glucocorticoïde

17-OHPG: 17-hydroxyprogesterone

21-OHPG: 21-hydroxyprogesterone

6-MPd: 6-méthyl-prednisolone

MP: medroxyprogesterone

MPA: medroxyprogesterone acétate

MLV: multilamellar large vesicles

Pd: prednisolone

PG: progesterone

Pn: prednisone

SAH: sérum albumine humaine

Trp: tryptophane

TTPs: triterpènes

UV: uvaol

## *Introduction générale*

Les triterpènes (TTPs) forment un groupe important et structurellement diversifié de produits naturels issus du squalène (Xu et al., 2004), auxquels nous sommes exposés par la nourriture et les médicaments. Presque tous les TTPs démontrent des activités biologiques chez l'animal et jouent un rôle important dans la médecine humaine.

Différents mécanismes moléculaires sont impliqués dans l'action des TTPs: une inhibition des enzymes, une activation ou inhibition des voies de signalisation (Falkenstein et al., 2000) et une activation de la transcription de certains gènes (Falkenstein et al., 2000). Ces mécanismes moléculaires peuvent être soit des mécanismes génomiques ou non-génomiques. Ces derniers sont en partie liés à leur interaction avec les membranes. De ce fait, il est probable que de telles interactions puissent moduler les propriétés structurales, physicochimiques et biologiques des membranes. Dans ce contexte, une partie de ce travail vise à comprendre l'effet des structures tétra et penta triterpéniques sur les membranes biomimétiques, avec comme objectif une compréhension plus approfondie des propriétés pharmacologiques des molécules d'intérêts. De plus, l'affinité de liaison entre une molécule bioactive donnée et une protéine plasmatique peut gouverner sa distribution dans les tissus en diminuant sa concentration libre diffusible, affecte son élimination du corps, et donc influence ses effets thérapeutiques ou toxiques (Lee and Wu 2015). Habituellement, la forme non liée de la molécule bioactive interagit avec son récepteur pour produire un effet pharmacologique (Subramanyam et al., 2009). De ce fait, la deuxième partie de ce travail porte sur l'interaction des structures triterpéniques avec la sérum albumine humaine. L'étude permettra de saisir les motifs structuraux qui gouvernent l'interaction des triterpènes avec la protéine.

Dans notre étude une série de progestatifs et une autre de glucocorticoïdes sont choisies pour représenter les structures tétra triterpéniques alors que l'uvaol (UV) et l'érythrodiol (ER) sont utilisés comme modèles des structures penta triterpéniques.

Les progestatifs et les glucocorticoïdes comptent parmi les cinq principales classes d'hormones stéroïdiennes (Ruiz Cortes 2012), réglant différents processus tissulaires. Ces molécules ressemblent structurellement au cholestérol (CHO) qui est un constituant des membranes biologiques chez les mammifères et un modulateur des propriétés membranaires (Pucadyil and Chatopadhyay 2006), d'où l'importance d'étudier leur rôle au niveau membranaire.

Les progestatifs sont caractérisés par leur squelette de base de 21 atomes de carbone, appelée prégnane. Ils sont connus pour leur fonction dans le maintien de la grossesse mais ils sont également présents dans différentes phases du cycle menstruel (Ruiz Cortes, 2012). Ils sont utilisés à la fois dans les contraceptifs oraux (Sarfati et De Vernejoul, 2009; Lidegaard et al., 2009) ainsi que dans les traitements substitutifs hormonaux (Binkowska et Worron, 2015). Les progestatifs peuvent affecter de nombreux systèmes comme le système cardiovasculaire et le système nerveux central (Olive, 2002; Sun et al., 2012). Les progestatifs naturels sont la progesterone (PG), la 17-hydroxyprogesterone (17-OHPG) et la 21-hydroxyprogesterone (21-OHPG). La PG est principalement sécrétée par les cellules granuleuses du corps jaune (Ruiz Cortes, 2012; Al Asmakh, 2007). Ces dernières présentent les enzymes nécessaires à la conversion du cholestérol en pregnénolone, qui est ensuite convertie en PG (Ruiz Cortes, 2012). La 17-OHPG est dérivée de la PG via la 17-hydroxylase, ou à partir du 17-hydroxypregnénolone via la  $3\beta$ -hydroxy déshydrogénase/ $\Delta^{5-4}$  isomérase (Dunn et al., 1981). La 21-OHPG, également connue sous le nom de désoxcorticostérone, elle est produite à partir de la PG via la  $21\beta$ -hydroxylase (Wood, 2006).

Après administration par voie orale, les progestatifs naturels sont rapidement dégradés dans le foie (Shindler et al., 2003), ce qui leur confère des taux plasmatiques faibles (Al Asmakh 2007; De Ziegler 2000). Pour cette raison, les progestatifs de synthèse ont été développés. On cite, la medroxyprogesterone (MP), la medroxyprogesterone acétate (MPA), la dydrogéstérone (DYG) et d'autres. La MP est la forme méthylée de la 17-OHPG, alors que la MPA est la forme acétylée de la MP. La DYG est un stéréo-isomère de la PG avec une double liaison supplémentaire en position C6 = C7. Ils se diffèrent par la configuration du groupement méthyl en C10 et de l'atome d'hydrogène en C9. Ces différences font de la PG une molécule presque plate, alors que la DYG est pliée (Schindler et al., 2003). Par conséquent, ces changements structuraux rendent les progestatifs de synthèse lentement métabolisables par le foie et donc effectifs par voie orale.

Les glucocorticoïdes (GCs) affectent de nombreux tissus dans le corps humain. Ils influencent le métabolisme protidique, glucidique et lipidique (Patel et al., 2014; Gupta et al., 2008; Christiansen et al., 2007) et montrent un effet anti-inflammatoire et immunosuppressif (Gupta et al., 2008). Les principaux GCs naturels humains sont la cortisone (Cn) et le cortisol (Co). Leur structure est basée sur le noyau prégnane, sur lequel s'ajoutent des fonctions indispensables à l'activité biologique ou modulant cette activité. Les corticoïdes naturels sont bio-synthétisés par

les glandes surrénales (Kumar et al., 2007). La présence d'un groupement hydroxyle sur le carbone 11 de la molécule stéroïdienne est cruciale pour l'activité des GCs (Gupta et al., 2008). La biodisponibilité du Co est réduite par les acides gastriques et par le premier passage hépatique (Gupta et al., 2008). C'est ainsi que de nombreux GCs synthétiques ont été développés, comme la prednisolone (Pd), la prednisone (Pn), la méthyl-prednisolone (MPd), le fluorocortisol acétate (FA) et d'autres.

Le Co, sous l'action d'une déshydrogénase aboutit à la synthèse de la Cn. La Pd diffère du Co par la présence d'une double liaison supplémentaire entre C<sub>1</sub> et C<sub>2</sub>. La Pn et la Pd sont obtenues par déshydrogénération de la Cn et du Co en C<sub>1</sub>-C<sub>2</sub>, respectivement. Comme la Cn, qui doit être convertie en Co par la 11 $\beta$ -hydroxystéroïde déshydrogénase type 1 (11 $\beta$ -HSD1), la Pn doit être convertie en Pd afin d'exercer une action glucocorticoïde (Miller and Auchus, 2011). La MPd se caractérise par la présence d'une fonction méthyle sur le carbone C6, par rapport à la Pd dont elle dérive. Le 9-FA est la fluoro-hydrocortisone acétate caractérisée par la présence d'un atome de fluor en position  $\alpha$  sur C9 et un groupement acétate en C21. C'est un corticostéroïde synthétique avec une action minéralo-corticoïde plus prononcée que son action glucocorticoïde (Gupta et al., 2008; Cisternino et al., 2003). Co, Cn, Pd, Pn et 6-MPd sont classés comme médicaments à action rapide ayant des demi-vies biologiques comprises entre 8 et 12 h (Gupta et al., 2008). Relativement au Co, les GCs synthétiques présentent une activité anti-inflammatoire plus importante qui est 4; 5 et 10 fois supérieure pour la Pd et Pn, le MPd et la fludrocortisone (Gupta et al., 2008).

Appartenant au groupe des TTPs pentacycliques, l'uvaol (UV) et l'érythrodiol (ER) sont des constituants de l'olive et de l'huile d'olive vierge. Cette dernière est la principale source de lipide dans le régime méditerranéen, et sa consommation est associée à la réduction des maladies chroniques (notamment cardiovasculaires) (Lopez et al., 2014). UV et ER sont des isomères et diffèrent par la position d'un groupe méthyl au niveau de leur structure chimique. L'ER et l'UV sont les précurseurs de l'acide oléanolique et de l'acide ursolique, respectivement. L'UV et l'ER possèdent une activité inhibitrice envers divers types de lignées cellulaires cancéreuses (Juan et al., 2008; Yang et al., 2009; Allouche et al., 2011; Quesada et al., 2015). L'ER est capable de produire une relaxation des anneaux aortiques des rats (Rodriguez et al., 2004), et l'UV exerce une inhibition de la protéase du virus de l'immunodéficience humaine (VIH) (Dzubak et al., 2006).

Les membranes biologiques représentent un élément fondamental de l'organisation cellulaire et constituent une barrière d'importance cruciale pour la vie. Elles permettent la reconnaissance

entre les cellules, le fusionnement avec d'autres membranes et sont le premier site de réception de tous les signaux extracellulaires qui déclenchent les activités cellulaires. Le modèle de « mosaïque fluide » a été introduit dès 1972 par Singer et Nicholson. Ce modèle décrit les membranes biologiques, qui sont organisées en une double couche de phospholipides dans laquelle les chaînes hydrophobes se font face, traversée par des protéines membranaires. Les proportions des constituants membranaires varient selon les membranes. Les lipides y sont en perpétuel mouvement de diffusion latérale et les protéines membranaires se déplacent également, mais plus lentement que les lipides qui les entourent (Eeman and Deleu, 2010; Pietzsch et al., 2004; Ritchie et al., 2003). La membrane plasmique est principalement constituée de lipides (phospholipides, sphingolipides et stérols), de protéines (intrinsèques et extrinsèques) et de glucides (glycophospholipides et glycoprotéines) (Eeman and Deleu, 2010). En raison de la complexité de composition des membranes biologiques, des membranes synthétiques ont été élaborées dont la taille, la géométrie et la composition peuvent être ajustées avec précision. Plusieurs modèles membranaires ont été établis pour étudier le rôle de molécules bioactives au niveau membranaire tel que les monocouches lipidiques (Laing et al., 2009; Micieli et al., 2011) et les bicouches lipidiques (Prades et al., 2011; Castangia et al., 2013). Pour ce même objectif, des membranes biologiques sont également utilisées comme les membranes des spermatozoïdes (sharma et al., 2013; Soares et al., 2013), les membranes hépatiques (Kapitulnik et al., 1986) et intestinales (Brasitus et al., 1987), ainsi que des modèles cellulaires comme les érythrocytes (Tsuda et al., 2002) et les granulocytes (Lamche et al., 1990). Les liposomes constituent un des modèles principaux des membranes biologiques.

La première partie de notre travail de thèse porte sur l'étude de l'interaction des progestatifs, des glucocorticoïdes, de l'uvaol et de l'érythrodiol avec les membranes des liposomes. Au cours de ce travail, nous avons préparé des liposomes en absence et en présence des molécules d'intérêts. Des techniques calorimétriques et spectroscopiques ont été utilisées afin d'étudier leur effet sur la fluidité membranaire. Ces techniques ont aussi fourni des informations valables concernant la localisation des triterpènes au niveau membranaire.

Tout médicament, qu'il soit administré par voie orale, intraveineuse, sublinguale, sous-cutanée ou intramusculaire, est transporté par le sang (Kratz and Elsadek, 2012). Dans le sang, le médicament se fixe aux protéines plasmatiques avec une affinité de fixation plus ou moins importante. L'affinité de liaison des médicaments aux protéines plasmatiques est un facteur

important à prendre en considération lors de la conception et du développement de nouveaux médicaments, car cela peut avoir une influence sur l'efficacité du médicament. Ainsi, une telle interaction affecte la distribution et la biodisponibilité du médicament (Naik et al., 2009; Kamat et al., 2005). En d'autres termes, elle affecte la pharmacocinétique, y compris la demi-vie dans la circulation sanguine, la stabilité et la toxicité du principe actif (Yang F et al., 2014; Naik et al., 2009). Les médicaments sont fixés à l'albumine, à la glycoprotéine acide  $\alpha 1$ -, aux lipoprotéines et aux immunoglobulines. L'albumine étant la protéine la plus impliquée, en raison de son abondance et de sa petite taille (Kratz and Elsadek, 2012). Elle assure le transport de nombreux composés endogènes et exogènes de structures très variées parmi lesquelles figure les acides gras, les hormones, les acides biliaires, les acides aminés, les métaux et les métabolites toxiques. La sérum albumine humaine (SAH) possède six sites (site I à VI) de fixation de ligands distribués le long de la molécule. Sudlow et ses collaborateurs ont montré que les ligands se fixent principalement sur la SAH au niveau de deux sites, le site I qui se trouve dans le sous-domaine IIA et le site II localisé dans le sous-domaine IIIA (Sudlow et al., 1975; Ghuman et al., 2005).

Aucune étude n'a porté sur l'interaction d'une série de progestatifs ou de glucocorticoïdes à l'albumine ni cherché l'effet des motifs structuraux sur l'affinité de cette liaison.

Ainsi, la deuxième partie de ce travail de thèse a essentiellement porté sur l'interaction des progestatifs et des glucocorticoïdes avec la sérum albumine humaine. La spectroscopie de fluorescence et infrarouge sont utilisées afin d'étudier leur interaction avec l'albumine et leur effet sur la structure secondaire de la protéine.

Le manuscrit s'organise en deux chapitres:

Le chapitre 1 comprend:

- i. une revue bibliographique portant sur l'effet des hormones stéroïdiennes sur les caractéristiques des membranes synthétiques et biologiques. Les techniques de caractérisation utilisées dans la littérature pour étudier l'interaction hormone-membrane sont décrites.
- ii. les résultats des travaux de thèse qui concernent les effets des progestatifs, des glucocorticoïdes, de l'érythrodiol et de l'uvaol sur les propriétés des membranes lipidiques sont présentés et discutés dans deux articles.

Le chapitre 2 comprend:

- i. une revue bibliographique sur l'interaction des TTPs avec la sérum albumine humaine.  
Cette revue bibliographique est préparée pour saisir l'effet de la structure du ligand sur la constante de liaison entre le ligand et la protéine.
- ii. les résultats des travaux de thèse qui concernent la determination des constantes de liaison TTP–albumine, l'identification du site de liaison des progestatifs et des glucocorticoïdes avec la sérum albumine humaine et la discussion des motifs structuraux qui peuvent moduler l'affinité de liaison. Ces études sont présentées dans un article.

La dernière partie comprend la conclusion générale et les perspectives.

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*Chapitre 1:*

*Interaction des molécules triterpéniques avec les  
membranes lipidiques*

## Introduction

Les liposomes, définis comme des vecteurs vésiculaires, ont été découverts dans les années 60 par l'hématologue britanique Dr. Alec Bangham, à l'institut Babraham de Cambridge (Bangham et al. 1965). Leur taille varie d'une vingtaine de nanomètres à quelques micromètres (Anwekar et al., 2011). Ce sont des vésicules sphériques formées par une ou plusieurs bicouches lipidiques concentriques, entourant un compartiment aqueux interne, où les groupes de têtes polaires sont orientés vers les phases aqueuses intérieures et extérieures (Wagner et al., 2011). Ils peuvent encapsuler des principes actifs lipophiles au sein de leur bicouche lipidique, des principes actifs hydrophiles au niveau de leur cavité aqueuse, ainsi que des molécules amphiphiles à l'interface eau-lipide (Laouini et al., 2012). Les liposomes sont constitués généralement d'un ou de plusieurs types de phospholipides. Ils sont non-toxiques, non-immunogènes, biocompatibles et biodégradables (Laouini et al., 2012). Le cholestérol entre souvent dans la composition des liposomes.

Les liposomes sont classés suivants différentes catégories, en fonction de leur taille et leur nombre de bicouches (Lorin et al., 2004). On distingue: les vésicules multilamellaires, appelés les MLV pour « multilamellar large vesicles » (1 $\mu$ m à 10 $\mu$ m de diamètre) et les vésicules multivésiculaires MVV pour « multivesicular vesicles ». Pour les unilamellaires, on distingue les vésicules de petite taille, appelés SUV pour « small unilamellar vesicles » (20 à 100 nm de diamètre), les vésicules de grande tailles, appelées LUV pour « large unilamellar vesicles » (100 à 1000 nm de diamètre) et les vésicules géantes, appelées GUV pour « giant unilamellar vesicles » (> à 1000 nm de diamètre) (Laouini et al., 2012).

Les méthodes de préparation des liposomes sont nombreuses et se basent généralement sur la dispersion des lipides dans un milieu aqueux après élimination du solvant organique (Akbarzadeh et al., 2013). Elles se divisent en quatre groupes (Rongen et al., 1997; Akbarzadeh et al., 2013): les méthodes mécaniques de dispersion de phospholipides, les méthodes basées sur l'élimination du détergent, les méthodes basées sur la transformation de liposomes préformés et les méthodes basées sur l'élimination du solvant organique.

Il est devenu de plus en plus clair que les interactions des molécules bioactives avec les groupes polaires et les chaînes acyles des phospholipides peuvent induire des changements au niveau des

fonctions membranaires comme la fluidité (Kazanci et al., 2001; Korkmaz and Severcan 2005; Ghosh et al., 1996). Ainsi, elles peuvent fournir une base moléculaire qui peut expliquer les mécanismes d'action de ces molécules. De plus, les propriétés membranaires peuvent moduler le comportement de la molécule bioactive, en particulier sa diffusion et son accumulation (Seydel and Wiese, 2002).

Pour mieux comprendre le mécanisme d'action non-génomique des TTPs médié par les lipides membranaires et identifier les TTPs qui peuvent affecter la fluidité membranaire. Nous avons étudié l'interaction des tétra- et penta TTPs avec les membranes biomimétiques. Les liposomes, vésicules phospholipidiques, sont utilisés comme modèles membranaires. Ils constituent de bons modèles pour étudier ce qui se déroule au niveau membranaire lors de différents processus tels que l'exocytose (Cans et al., 2003), l'endocytose (Duzgunes and Nir, 1999), la fusion des membranes (Martin and Ruysschaert, 2000) et l'interaction des protéines avec la membrane qui est cruciale pour la régulation des fonctions biologiques (Hofer et al., 2010).

Au cours de ce travail, nous avons préparé nos liposomes de type MLV en absence et en présence des molécules triterpéniques à différents rapports molaires par la méthode d'hydratation du film lipidique. C'est une méthode simple et rapide et est la plus utilisée pour la préparation des MLVs. Elle implique tout d'abord la dissolution des phospholipides dans un solvant organique ou dans un mélange de solvants organiques. Le solvant est ensuite évaporé ce qui aboutit à la formation d'un film lipidique mince sur la paroi du ballon. Ce film lipidique est hydraté par l'ajout d'un tampon aqueux; la dispersion ainsi formée est mélangée par un vortex pour une durée de quelques minutes. L'étape d'hydratation est réalisée à une température supérieure à la température de transition ( $T_m$ ) du phospholipide utilisé ou à la  $T_m$  la plus élevée si un mélange de phospholipides est utilisé. Le composé à incorporer est ajouté selon sa solubilité, soit dans la phase aqueuse soit dans le solvant organique avec le mélange de phospholipides. Cette méthode possède certains inconvénients. En effet le volume aqueux interne des liposomes formés est petit, la distribution de taille des vésicules est hétérogène et l'efficacité d'encapsulation est faible (Bangham et al., 1965).

Différentes techniques sont utilisées pour étudier l'effet d'une molécule bioactive sur les propriétés physicochimiques des membranes lipidiques. Dans notre thèse, l'effet des TTPs sur le comportement thermodynamique et la fluidité des membranes des vésicules lipidiques formées à

partir de dipalmitoyl phosphatidylcholine a été étudié par DSC (calorimétrie différentielle à balayage), spectroscopie Raman, FTIR (spectroscopie infrarouge à transformée de fourrier) et la polarisation de fluorescence du 1,6-diphényl-1,3,5-hexatriene (DPH). Chaque méthode fournie des informations uniques et ou complémentaires. Les effets induits par les TTPs sont discutés en terme de structure chimique et d'hydrophobicité.

La calorimétrie différentielle à balayage (DSC) permet la mesure des paramètres thermodynamiques de la pré-transition et de la transition de phase des phospholipides. Ces paramètres sont les températures de pré-transition  $T_p$  et de transition  $T_m$ , les quantités d'énergie absorbées au cours de la pré-transition  $\Delta H_p$  et de la transition  $\Delta H_m$  et  $\Delta T_{1/2}$  qui est la largeur de la transition à mi-hauteur du pic principal (Demetzos 2008), c'est une mesure de la coopérativité de la transition (El-Maghraby et al., 2005).

Toute molécule peut interagir avec la partie lipophile de la bicouche (chaînes hydrocarbonées) aboutissant à un changement au niveau de  $T_m$  et  $\Delta H_m$ , et/ou avec la partie polaire des phospholipides conduisant à la disparition et/ou au déplacement du pic de pré-transition (Demetzos, 2008). C'est une technique simple à réaliser et dont les paramètres thermodynamiques sont bien discutés dans la littérature.

La spectroscopie infrarouge à transformée de fourrier (FTIR) permet via la détection des vibrations caractéristiques des liaisons chimiques, d'effectuer l'analyse des fonctions chimiques présentes dans le matériau (Lewis and McElhaney, 2013). Elle permet de suivre les changements de conformation ou l'état d'hydratation des différentes régions du phospholipide, en fonction de la température (Lee and Chapman 1986). Par exemple, dans la région spectrale des vibrations des groupements fonctionnels lipidiques  $C=O$  ( $1800\text{--}1650\text{ cm}^{-1}$ ) et  $PO_2^-$  ( $1230\text{ cm}^{-1}$ ) de la tête polaire des phospholipides, les variations des fréquences des bandes d'étirement  $C=O$  et  $PO_2^-$  permettent d'analyser le comportement des molécules bioactives au niveau de la région polaire des phospholipides (Lewis et al., 1998, Arrondo et al., 1998). Ainsi, dans la région spectrale des vibrations des méthylènes  $C-H$  ( $3000\text{--}2800\text{ cm}^{-1}$ ), les variations de la fréquence ou de la largeur des bandes caractéristiques provenant de l'étirement vibrationnels des  $CH$  symétriques ou antisymétriques nous donnent des informations qualitatives sur la proportion de conformères gauche ou trans dans les chaînes hydrocarbonées (Lewis and McElhaney 1998, Arrondo et al., 1998). Par ailleurs, la largeur de la bande d'étirement  $CH_2$  antisymétrique mesurée à 50% de la hauteur des pics donne des informations sur la dynamique du système. Une

augmentation de la bande indique une augmentation de la dynamique (Korkmaz et al., 2005). Il s'agit d'une technique facile et rapide. Elle ne requiert qu'une faible quantité d'échantillon.

La spectroscopie Raman est une technique bien adaptée pour étudier les interactions des molécules exogènes avec les bicouches lipidiques ainsi que la structure des phospholipides (Fox et al., 2007). L'information obtenue est principalement qualitative. En surveillant les changements d'intensités des pics de diffraction Raman des vésicules phospholipidiques, la conformation des chaînes acyles, ainsi que l'ordre intra et intermoléculaire de la membrane sont déterminés (Fox et al., 2007). Par exemple, les rapports d'intensité de la hauteur des pics  $I_{2935}/I_{2880}$ ,  $I_{2844}/I_{2880}$  et  $I_{1090}/I_{1130}$  attribués aux bandes d'étirements des liaisons C-H et C-C respectivement, sont des indicateurs globaux des changements intra- (rapport gauche/trans) et intermoléculaires (ordre des chaînes lipidiques) (Potamitis et al., 2011; Gardikis et al., 2006). Le changement d'intensité du pic à  $715 \text{ cm}^{-1}$  attribué à la bande d'étirement C-N indique l'interaction des molécules exogènes avec le groupement choline de la tête polaire des phospholipides (Gardikis et al, 2006 ; Potamitis et al., 2011). Par rapport à FTIR, les avantages de la spectroscopie Raman résident principalement dans la possibilité d'analyser des échantillons aqueux. L'absorption IR intense de l'eau étant évitée. Par ailleurs, les bandes spectrales sont plus pointues (nettes), plus étroites et mieux différencierées. Les spectroscopies infrarouge et Raman sont souvent dites complémentaires.

La polarisation de la fluorescence du 1,6-diphényl-1,3,5-hexatriène (DPH), molécule hydrophobe qui s'intègre au sein de la bicoche lipidique est couramment utilisée comme une sonde dans les applications biochimiques, pour mesurer les associations protéiques, la fluidité et l'ordre des lipides. Le DPH est une molécule qui présente une forte augmentation de sa fluorescence après intercalation dans la membrane. L'anisotropie de fluorescence est définie par:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

où  $I_{\parallel}$  et  $I_{\perp}$  sont les composantes polarisées respectivement parallèle et perpendiculaire à la direction de polarisation de la lumière incidente (Lakowicz 2006). L'anisotropie est inversement proportionnelle à la fluidité (Park et al., 2006). Il s'agit d'une technique sensible et trop utilisée en littérature.

En comparant avec les vésicules témoins, un changement d'intensité du pic à  $715 \text{ cm}^{-1}$ , une

disparition du pic de la pré-transition et une variation de la fréquence des groupements fonctionnels lipidiques C=O et PO<sub>2</sub><sup>-</sup>, ont montré que tous les TTPs interagissent avec le groupement choline de la tête polaire des phospholipides.

Les changements induits par les TTPs au niveau des paramètres thermodynamiques ( $T_m$  et  $\Delta H_m$ ), des rapports d'intensité de la hauteur des pics Raman  $I_{2935/2880}$  et  $I_{2844/2880}$  et des fréquences des CH symétriques des chaînes acyles des phospholipides, impliquent leur localisation au niveau membranaire. Le Co, la Pd et le 9-FA sont localisés à proximité des têtes polaires des phospholipides. Alors que les molécules de PG, de 21-OHPG, d'ER et d'UV interagissent avec le cœur lipophile de la bicouche. Elles s'insèrent au sein de la bicouche perturbant ainsi les fortes interactions hydrophobes entre les molécules de lipides. La 17-OHPG, la MPA, la MP et la DYG, s'incorporent aussi dans la bicouche lipidique.

En outre, l'aspect du pic de transition en présence d'une molécule peut être modifié. En effet, il est élargi en présence de tous les TTPs étudiés. De plus, à des rapports molaires élevés (10%) seuls les liposomes encapsulant la 21-OHPG, la Pd et le 9-FA ont démontré une décomposition du pic de transition suggérant une séparation de phase au niveau de la bicouche et une formation des domaines riches et d'autres pauvres en molécules triterpéniques.

Ainsi, le Co, la Pd, le 9-FA, l'UV et l'ER fluidifient la membrane des liposomes à l'état de gel (28°C), lors de la transition (41°C) et à l'état liquide (50°C) comme ils diminuent l'anisotropie de la DPH et augmentent le rapport d'intensité  $I_{1090/1130}$  déterminé par la technique Raman. À l'état liquide, les penta TTPs ont montré un effet fluidifiant plus prononcé que les tétra TTPs.

On a suggéré qu'en fonction de la présence des motifs structuraux, les TTPs se positionnent différemment au sein de la membrane induisant ainsi des effets distincts sur l'ordre et la fluidité.

Ce chapitre est présenté sous forme d'une revue bibliographique et des deux articles.

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## **1.1 Effect of human steroid hormones on the liposome membrane fluidity: a review**

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**“Revised journal of membrane biology”**

## Abstract

Human steroid hormones are involved in many aspects of physiology and have long been known to exert rapid and delayed effects. They are lipophilic molecules which can be incorporated into the lipid membranes. Through non-covalent interactions they can alter the properties of the membrane, including fluidity, lipid raft formation and others. Biophysical techniques were used to study the interaction of human steroid hormones with biological and biomimetic membranes such as differential scanning calorimetry, electron paramagnetic resonance, fluorescence spectroscopy and Fourier transform infrared spectroscopy. The aim of this review is to summarize the effects of these hormones on thermotropic and dynamic membrane properties. Meanwhile, the disorder induced by human steroid hormones is discussed in terms of hydrophobicity and chemical structure.

**Keywords:** differential scanning calorimetry, electron paramagnetic resonance, fluidity, fluorescence spectroscopy, Fourier transform infrared spectroscopy, membrane, steroid hormones.

## Abbreviations

ANS: 1-anilino-8-naphthalene sulfonate; 12-AS: 12-(9-antroiloxy)-stearic acid; BCM: beclomethasone; BDP: beclomethasone dipropionate; BM: betamethasone; Co: cortisol; Cpa: cyproterone acetate; DEX: dexamethasone; DSC: differential scanning calorimetry; DHT: dihydrotestosterone; DMPC: 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC: dipalmitoylphosphatidylcholine; DPH: 1,6-diphenyl-1,3,5-hexatriene; DSPC: 1,2-distearoyl-*sn*-glycero-3-phosphocholine; DYG: dydrogesterone; EPR: electron paramagnetic resonance; E<sub>2</sub>: 17 $\beta$ -estradiol; E<sub>3</sub>: estriol; 9-FA: 9-fluorocortisol acetate; FTIR: Fourier transform infrared spectroscopy; 17-OHPG: 17-hydroxyprogesterone; 21-OHPG: 21-hydroxyprogesterone; MP: medroxyprogesterone; MPA: medroxyprogesterone acetate; MPd: methylprednisolone; PC: phosphatidylcholine; Pd: prednisolone; Pdn: pregnanedione; PG: progesterone; SR: sarcoplasmic reticulum; SAM: sperm acrosomal membrane; SPM: sperm plasma membrane; SyPM: synaptosomal plasma membrane; TMA-DPH: 4-trimethyl-ammonio-1,6- diphenyl-1,3,5-hexatriene.

## Introduction

Human steroid hormones represent a large class of biologically active molecules. Their classical genomic mechanism of action involves their binding to specific intracellular or nuclear receptors, which act as ligand-dependent transcription factors exerting positive or negative effects on the expression of target genes. In addition to the delayed genomic action, increasing evidence for rapid, non-genomic steroids effect has been demonstrated for virtually all groups of steroids (Liu et al., 1995; Benten et al., 1997; Blackmore 1998; Burger et al., 1999; Kim et al., 1999). Three non-genomic mechanisms for steroids action have been proposed: i- membrane-bound receptors for steroids have been identified and are associated with adenylyl cyclase and second messenger cascades (Kelly et al., 1999; Estrada et al., 2000; Kelly et al., 2003; Dressing et al., 2011); ii- steroids also have a specific binding site on neurotransmitter receptors (Gee 1988; Vincens et al., 1989); iii- or they can intercalate into the lipid membranes resulting in the perturbation of lipid-lipid interactions thus modulating cell functions (Mendoza et al., 1995; Korkmaz and Severcan, 2005).

There are five major classes of human steroid hormones: androgens, estrogens, progestogens, glucocorticoids and mineralocorticoids (Miller and Auchus 2011). They are all synthesized from cholesterol and hence have closely related structures based on the classic cyclopentanophenanthrene 4-ring structure (Miller and Auchus 2011).

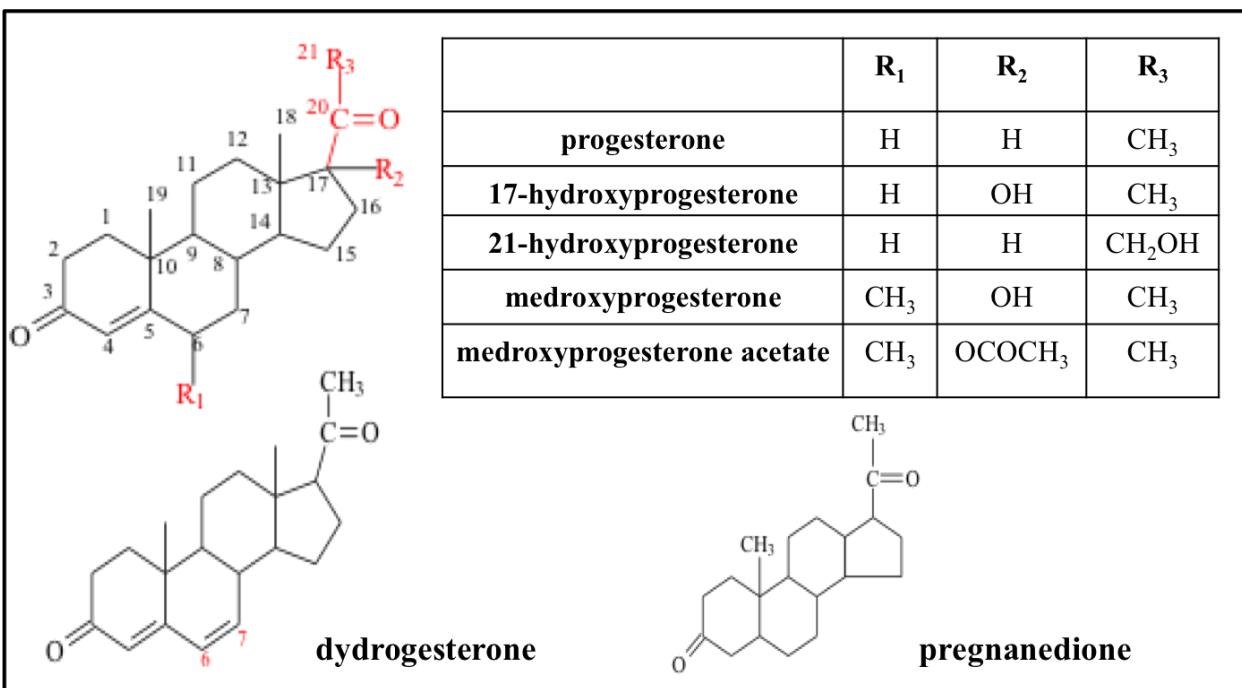
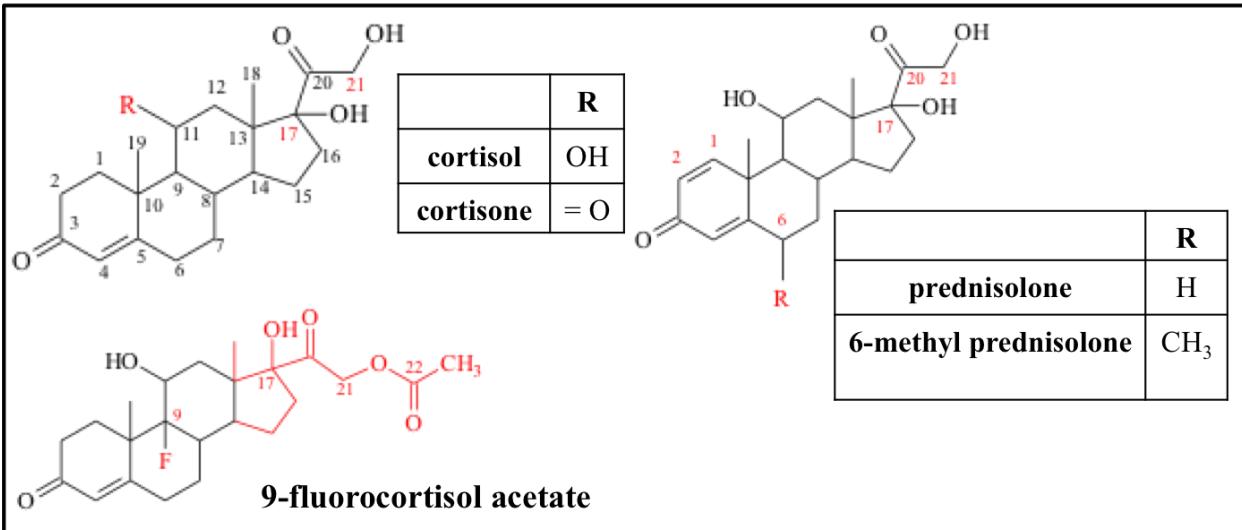
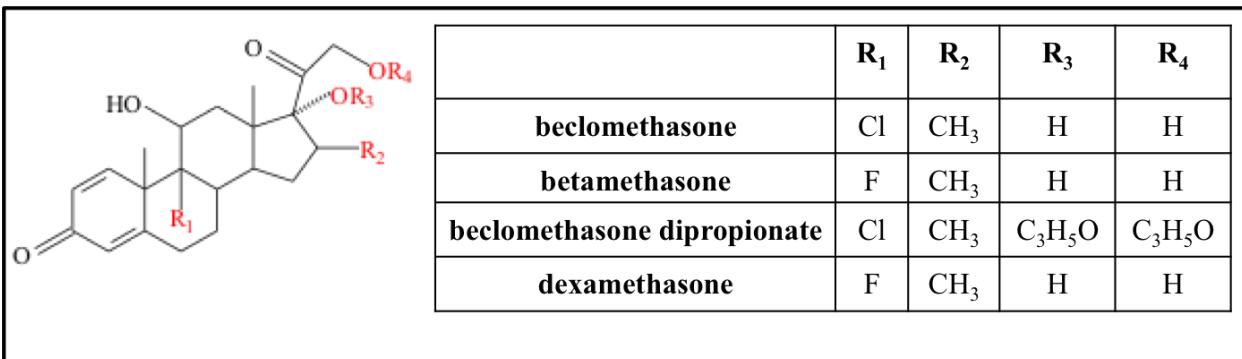
Biological membranes play an essential role in the cellular protection as well as in the control and transport of nutrients. They display a very complex composition in terms of lipids and proteins. The lipid part contains a variety of head group and acyl chain structures, sphingolipids and sterols (Brown and London 1998b). The presence of long and saturated acyl chains in lipids allows cholesterol to become tightly intercalated in the lipid bilayer, resulting in the organization of liquid-ordered ( $l_o$ ) phases (Simons and Ikonen 1997). Sphingolipids, sterols and saturated acyl chains can cluster to form microdomains called rafts (Brown 2002) that exist in the liquid ordered phase ( $l_o$ ) and are detergent-insoluble membrane domains (Xu and London 2000; Megha et al., 2006). Raft formation is coupled to the packing and ordering of the molecules in the bilayer (Shahidi et al., 2006). The functions of rafts are reported in literature (Brown and Rose 1992; Brown and London 1998a; Brown and London 2000; Brown 2002). The complexity of biological membranes has encouraged the development of a wide variety of simpler biomimetic model membranes whose size, geometry, and composition can be tailored with precision. The most common cell membrane models range from vesicles to different types of supported lipid bilayers,

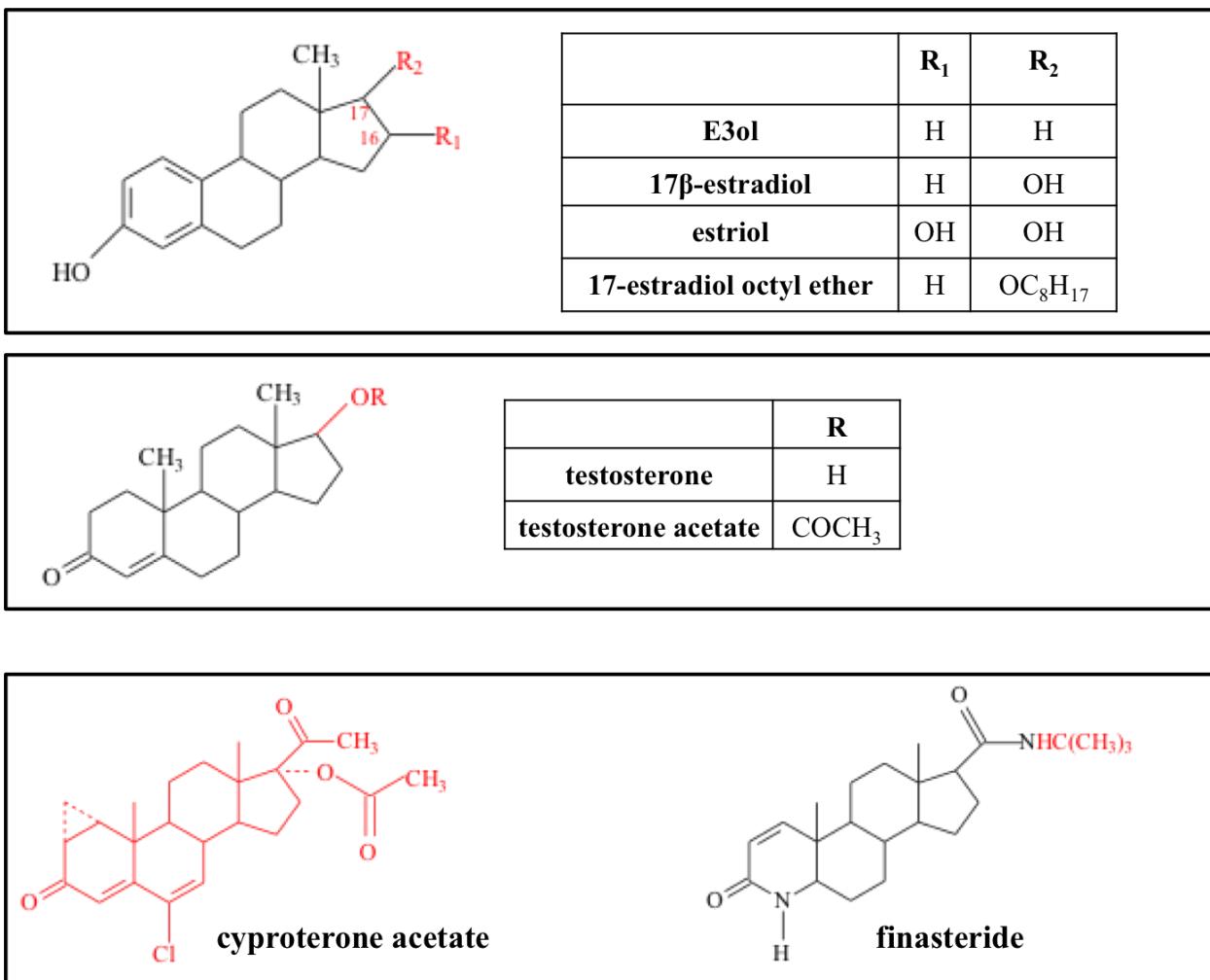
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although lipid monolayers have also been studied (Chan and Boxer 2007).

The interaction of steroids with polar head groups and non-polar hydrocarbons chains of lipid membranes, or both, can induce several changes in the membranes functions such permeability, lipid rafts formation and fluidity. The steroids effect on domain formation is studied extensively in literature (Xu and London 2000; Xu et al., 2001; Wenz and Barrantes 2003). The documented activities of steroids was translated into quantitative data by Wenz (2015) where steroids were divided into two categories. Steroids that present rigidifying, molecular ordering, condensing effect, and/or raft promoting/stabilizing ability on membranes, relative to that of steroid-free membranes were designated as “promoters”. However, steroids that present fluidifying, disordering, and/or raft disrupting/destabilizing effect on membranes were designated as “disrupters” (Wenz 2015).

Among steroids, human steroid hormones were selected (Figure 1) in this review to summarize their effect on liposome membrane fluidity, since liposomes are the most common biomimicking models largely used for investigating drug-membrane interactions. The main techniques used to study human steroid membrane interaction are introduced, and literature data are resumed into conclusive tables. Finally, a conclusion is prepared to give an overview on the effect of human steroid hormones on membrane fluidity and the role of their hydrophobicity and functional groups in the steroid membrane interaction.





**Figure 1** Chemical structures of steroids hormones.

### Effect of human steroid hormones on liposome membranes fluidity measured by DSC

Differential Scanning Calorimetry (DSC) is a suitable thermal analysis technique for determining the purity, the polymorphic forms and the melting point of a sample. It is used to study the thermal behavior of lipid bilayers by measuring the thermodynamic parameters that reflect the stability of the liposomal suspension under given storage conditions (Demetzos 2008).

Lipid bilayers demonstrated structural changes with temperature named thermotropic transitions. The pre-transition represents the transition of lipid bilayer from a planar gel phase (lamellar gel phase) to a rippled gel phase. It is mainly related to the polar region of bilayers (Demetzos 2008). The main transition represents the passage from the rippled gel phase to the liquid crystalline phase and is closely related to the acyl chains of the bilayer. The liquid crystalline phase has an increased number of acyl chains with gauche conformers and offers a large increase in membrane fluidity and disorder compared to planar and rippled gel phase (Kambara and Sasaki 1984; Demetzos 2008). In general, the lipid tails in the gel state are predominantly as trans conformers,

whereas both trans and gauche conformers are present in a liquid-crystalline state. At low temperatures, the trans conformations exist because they are energetically more favorable for the lipids in the bilayer arrangement. As the temperature increases to above the phase transition point, the carbon atoms in the lipid tails are less restricted to move, thus creating defects in the hydrocarbon tails (Leekumjorn and Sum 2007).

The pre-transition is a flat endothermic peak. In contrast, the main-transition temperature represents a sharp, intense endotherm. The main transition temperature,  $T_m$ , is the temperature represented by the apex of the peak and generally used to monitor the passage from the gel to the liquid crystalline phase. It depends on the phospholipid type, the length and the saturation of the acyl chains, the presence of exogenous molecules. Many drugs can strongly interact with the lipophilic part of a phospholipid leading to greater thermotropic changes in  $T_m$ , or with the polar group resulting in an abolition of the pre-transition ( $T_p$ ) (Demetzos 2008; Cong et al., 2009).

The temperature that corresponds to the half of the enthalpy change during the transition is  $T_{1/2}$ , while  $\Delta T_{1/2}$ , is the width of the transition at half peak height (Demetzos 2008). It will be taken as a measure for the co-operativity of the transition.  $\Delta T_{1/2}$  is inversely proportional to the co-operativity. Co-operative units measure the number of phospholipids undergoing simultaneous transition (El-Maghraby et al., 2005). The transition enthalpy,  $\Delta H_m$ , is the actual heat required for the entire transition, normalized per mol. This is calculated from the area under the transition peak (El-Maghraby et al., 2005). The decrease in  $\Delta H_m$  suggests an increase in the number of gauche conformers of fatty acyl chains and consequently in the membrane fluidity and disorder.

The interaction of human steroid hormones with lipid bilayers causes alteration in the calorimetric parameters of the membrane. The changes of the calorimetric parameters of the main phase transition ( $T_m$ ,  $\Delta H_m$  and  $\Delta T_{1/2}$ ) can be attributed to the packing of exogenous molecules within the hydrophobic interior of phospholipid array. The DSC results for various steroids loaded liposomes are summarized in Table 1. DSC thermograms of dipalmitoylphosphatidylcholine (DPPC), 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) liposomes demonstrated two transitions: the pre- and the main phase- transition. A large series of steroids abolished the pre-transition peak of lipid bilayer and includes beclomethasone dipropionate (Elhissi et al., 2006), cortisone hexadecanoate (Arrowsmith et al., 1983), cortisol (Ganesan et al., 1984; Abboud et al., 2016), dexamethasone (Bhardwaj and Burgess 2010), dydrogesterone (Abboud et al., 2015), 9-

fluorocortisol acetate (Abboud et al., 2016), 17-hydroxyprogesterone (Abboud et al., 2015), 21-hydroxyprogesterone (Abboud et al., 2015), medroxyprogesterone (Abboud et al., 2015), medroxyprogesterone acetate (Abboud et al., 2015), prednisolone (Abboud et al., 2016) and progesterone (Carlson et al., 1982; Ganesan et al., 1984; Korkmaz and Severcan 2005; Abboud et al., 2015). The pre-transition peak is broadened in the presence of pregnanediol (O'leary et al., 1984), progesterone (O'leary et al., 1984), testosterone (O'leary et al., 1984) and testosterone acetate (O'leary et al., 1984) and shifted to lower values in the presence of androsterol (Gao et al., 2008), cyproterone acetate (Biruss et al., 2007), 17- $\beta$ -estradiol (El Mghraby et al., 2005; Biruss et al., 2007) and finasteride (Biruss et al., 2007) (Table 1). Results indicate that these molecules interact with the polar heads of phospholipids and/or the surrounding water molecules via their hydroxyl and ketone groups.

Human steroid hormones are grouped below according to their effect on the calorimetric parameters of the main transition peak relative to steroid-free liposomes: i) Steroids that decreased significantly the  $T_m$  and  $\Delta H_m$  values, associated with a broadening of the peak. ii) Steroids that decreased in a non-significant way the  $T_m$  and  $\Delta H_m$  values, associated with a broadening of the peak. iii) Steroids that decreased the  $T_m$  and  $\Delta H_m$  values without inducing a broadening of the peak. iv) Steroids that decreased the  $T_m$  values and increased those of  $\Delta H_m$ .

The transition temperature ( $T_m$ ) and the enthalpy of the main transition ( $\Delta H_m$ ) decreased significantly in the presence of 21-hydroxyprogesterone, progesterone (Abboud et al., 2015; O'leary et al., 1984), pregnanediol, testosterone and testosterone acetate (O'leary et al., 1984) and this was associated with a broadening of the peak, suggesting a deep incorporation of these steroids into lipid bilayers and an increase in fluidity. Same results were reported for androsterol except the effect on  $\Delta H_m$  value which was not determined (Gao et al., 2008). The presence of a conjugated 3-Keto group in the steroid molecule promotes a destabilization of the bilayers (Gallay and De Kruijff 1984; Wenz 2012). 21-hydroxyprogesterone, progesterone, pregnanediol, testosterone and testosterone acetate have this structural feature. Moreover, the free polar groups, as substituents of the ring D, seem to favor the destabilization of lipid membranes (progesterone, pregnanediol, testosterone and testosterone acetate). It is worth noting that testosterone and testosterone acetate, which differ by the acetyl function on ring D, present the same effect on the membrane fluidity.

The same results (decrease in  $T_m$  and  $\Delta H_m$  values and broadening of the main transition peak), but in a non-significant way, were obtained for cortisone hexadecanoate (Arrowsmith et al., 1983), cyproterone acetate, finasteride and  $17\beta$ -estradiol (Biruss et al., 2007) supposing also their ability to increase the membrane fluidity. The long acyl chain of hexadecanoate seems to favor the insertion of cortisone in the membrane since cortisol has a less effect on DSC parameters (Table 1). Finasteride, which is a 4-azasteroid, contains a bulkier t-butyl group at C17. Cyproterone acetate, has also a distinct bulkier structure (rings A and D). Despite this bulkier feature, cyproterone acetate and finasteride may incorporate deeply the bilayer.

Compared to steroid-free liposomes, the main transition temperature shifted significantly to lower values for medroxyprogesterone acetate, medroxyprogesterone, dydrogesterone and 17-hydroxyprogesterone loaded liposomes (Abboud et al., 2015). Decreases of the main transition enthalpy values were also obtained at various extents while no broadening of the peak was observed (Abboud et al., 2015). These molecules present a lower membrane disrupting effect when compared to the first two groups. An intramolecular hydrogen bond in medroxyprogesterone and 17-hydroxyprogesterone may explain their lower fluidifying effect when compared to the series of molecules listed above. Moreover, with respect to testosterone acetate structure, the presence of  $\text{CH}_3\text{CO}$  group on C17 (medroxyprogesterone acetate) may produce a steric effect that prohibits the incorporation of medroxyprogesterone acetate inside the bilayers. Dydrogesterone, having an additional C6-C7 double bond and presenting a bent shape showed a weaker effect on the membrane fluidity (Abboud et al., 2015).

For the following series of steroids (beclomethasone dipropionate (Elhissi et al., 2006), cortisol, 9-fluorocortisol acetate, prednisolone (Abboud et al., 2016)), the  $T_m$  values decreased and the  $\Delta H_m$  increased with respect to the steroid-free liposomes in significant manners (Elhissi et al., 2006; Abboud et al., 2016). The presence of many hydroxyl group(s) (cortisol, 9-fluorocortisol acetate and prednisolone), the crowded D ring of beclomethasone dipropionate due to the presence of two propionate groups, the presence of the fluorine (9-fluorocortisol acetate) and chlorine atoms (beclomethasone dipropionate) at C9 may explain their low effect on the membrane fluidity. Cortisol, 9-fluorocortisol acetate and prednisolone may display an intramolecular hydrogen bond rendering the molecules less potent to affect the fluidity. Among the studied corticosteroids, only dexamethasone showed a different behavior but this may be due to the higher molar ratio used in the study of Bhardwaj and Burgess 2010.

At high concentrations, some steroids were reported to produce a splitting of the main peak (cortisone hexadecanoate (12.5%) (Arrowsmith et al., 1983), dexamethasone (20%) (Bhardwaj and Burgess 2010), 9-fluorocortisol acetate (10%) (Abboud et al., 2016), (21-hydroxyprogesterone) (10%) (Abboud et al., 2015) progesterone (6 and 12%) (Korkmaz and Sevrcan 2005), prednisolone (10%) (Abboud et al., 2016) and androsterol (10%) (Gao et al., 2008)) (Table 1). This is generally attributed to a phase separation and a formation of steroid-rich and -poor domains. Hence, new structures can be formed in the membrane and need to be confirmed by other techniques such as RMN and X-ray diffraction.

**Table 1** Thermodynamics parameters obtained by differential scanning calorimetry (DSC) for human steroid hormones loaded liposomes.

Drug	Phospholipid/Steroid molar ratio	T <sub>p</sub>	T <sub>m</sub>	ΔH <sub>m</sub>	Shape of the main transition peak	Conclusions
Androsterol <sup>14</sup>	DPPC/Androsterol : 100/2.5, 100/5, 100/10, 100/20, 100/25, 100/30, 100/35 and 100/40	• Decreased at 100/2.5 and 100/5 • Abolished from 100/10	Decreased significantly	not determined	Broadening and splitting of the peak above 100/20	[iii]
BDP <sup>11</sup>	DMPC/BDP: 100/1, 100/2.5, 100/5	Abolished from 100/2.5	Decreased at 100/1 and 100/2.5 while at 100/5 a significant decrease	Increased at 100/1 and 100/2.5	Broadening	[i]
Co <sup>2, 13, 15</sup>	DPPC/Co: • 100/0.1 <sup>15</sup> • 100/1, 100/2.5 and 100/10 <sup>2</sup> • 100/0.01, 100/0.05, 100/0.14 <sup>13</sup>	Abolished <sup>2, 15</sup>	• Decreased significantly at 100/1, 100/2.5 and 100/10 • Decreased <sup>13</sup>	• Increased significantly at 100/1, 100/2.5 and 100/10 • Decreased <sup>13</sup>	Broadening at 100/10 <sup>2</sup>	[i]
Cortisone hexadecanoate <sup>3</sup>	DPPC/Drug: 100/2.5, 100/5, 100/7.5, 100/10, 100/12	Abolished	Decreased	Decreased	Broadening and splitting of the peak at 100/12.5	[iii]
Cpa <sup>5</sup>	DPPC/Cpa: 100/0.5	Decreased	Decreased	Decreased	Broadening	[iii]

DEX <sup>4</sup>	DMPC/DEX, DPPC/DEX and DSPC/DEX: 100/20	Abolished	Decreased	Decreased	Broadening and a shoulder appeared with DMPC, DPPC and DSPC	[ii]
DYG <sup>1</sup>	DPPC/DYG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Abolished	Decreased significantly at 100/1, 100/5, 100/10 and 100/25	Decreased	Not affected	[i]
E <sub>2</sub> <sup>5, 12</sup>	DPPC/E <sub>2</sub> : • 100/0.5 <sup>5</sup> • 100/0.1 <sup>12</sup>	Decreased	Decreased	Decreased <sup>5</sup>	Broadening	[iii]
9-FA <sup>2</sup>	DPPC/9-FA: 100/1, 100/2.5 and 100/10	Abolished	Decreased significantly at 100/1, 100/2.5 and 100/10	Increased significantly at 100/1, 100/2.5 and 100/10	Broadening and splitting of the peak at 100/10	[i]
Finasteride <sup>5</sup>	DPPC/finasteride: 100/0.5	Decreased	Decreased	Decreased	Broadening	[iii]
17-OHPG <sup>1</sup>	DPPC/17-OHPG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Abolished	Decreased significantly at 100/1, 100/2.5, 100/5 and 100/10	Decreased significantly at 100/5 and 100/25	Not affected	[ii]
21-OHPG <sup>1, 13</sup>	DPPC/21-OHPG: • 100/1, 100/2.5, 100/5, 100/10 and 100/25 <sup>1</sup>	Abolished	• Decreased significantly at all molar ratios <sup>1</sup>	• Decreased significantly at 100/10 <sup>1</sup>	Broadening and splitting of the peak at 100/10	[iii]

	• 100/0.07, 100/0.18 <sup>13</sup>		• Decreased <sup>13</sup>	• Decreased <sup>13</sup>		
MP <sup>1</sup>	DPPC/MP: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Abolished	Decreased significantly at 100/2.5 and 100/25	Decreased	Not affected	[ii]
MPA <sup>1</sup>	DPPC/MPA: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Abolished	Decreased significantly at 100/1, 100/2.5, 100/5 and 100/25	Decreased significantly at 100/5, 100/10 and 100/25	Not affected	[ii]
Pdn <sup>23</sup>	DPPC/Pdn: 9/1	Significant broadening	Decreased significantly	Decreased significantly	Significant broadening	[iii]
Pd <sup>2</sup>	DPPC/Pd: 100/1, 100/2.5 and 100/10	Abolished	Decreased significantly at 100/10	Increased significantly at 100/1	Broadening and splitting of the peak at 100/10	[i]
PG <sup>1, 9, 13, 15, 20, 23</sup>	DPPC/PG: • 100/1, 100/2.5, 100/5, 100/10 and 100/25 <sup>1</sup> • 100/5, 100/10, 100/20, 100/30 <sup>9</sup> • 100/0.1 <sup>15</sup> • 100/3, 100/6, 100/12, 100/24 <sup>20</sup> • 9/1 <sup>23</sup>	• Abolished <sup>1, 9, 15, 20</sup> • Significant broadening <sup>23</sup>	• Decreased significantly at all molar ratios <sup>1, 23</sup> • Decreased <sup>20, 13</sup>	• Decreased significantly at 100/5, 100/10 and 100/25 <sup>1</sup> and 9/1 <sup>23</sup> • Decreased <sup>13</sup>	• Broadening at all molar ratios <sup>1</sup> • Significant broadening <sup>23</sup> • Broadening and splitting of the peak at 100/6 and 100/12 <sup>20</sup>	[iii]

	• $100/0.27^{13}$					
T <sup>23</sup>	DPPC/T: 9/1	Significant broadening	Decreased significantly	Decreased significantly	Significant broadening	[iii]
TA <sup>23</sup>	DPPC/TA: 9/1	Significant broadening	Decreased significantly	Decreased significantly	Significant broadening	[iii]

[i]: Interacts with polar head group and acyl chains. It is localized at the upper chain/glycerol/head group region of the lipid bilayer.

[ii]: Interacts with polar head group and acyl chains. It may have a moderate insertion in the bilayer.

[iii]: Interacts with polar head group and acyl chains. It may incorporate deeply the bilayer.

[1] Abboud et al., 2015; [2] Abboud et al., 2016; [3] Arrowsmith et al., 1983; [4] Bhardwaj and Burgess 2010; [5] Biruss et al., 2007; [9] Carlson et al., 1982; [11] Elhissi et al., 2006; [12] El Maghraby et al., 2005; [13] Gallay and De Kruijff 1984; [14] Gao et al., 2008; [15] Ganesan et al., 1984; [20] Korkmaz and Sevencan 2005; [23] O'leary et al., 1984.

**Effect of human steroid hormones on FTIR spectral characteristics of liposome membranes**

Fourier transform infrared spectroscopy (FTIR) is a powerful yet relatively inexpensive and convenient technique for studying the structure and organization of membrane lipids in their various polymorphic phases. This spectroscopic technique gives information about the order/disorder of acyl chains of lipids without the necessity of introducing extrinsic probes (Lewis and McElhaney 2013). Vibrational spectroscopy has several advantages for membrane studies. Firstly, variations in frequency, line width, and intensity are sensitive to structural transitions of both lipids and protein components. Secondly, the vibrations of individual groups provide structural information on highly localized regions of the bilayer. Thus, C-H stretching absorptions of the lipid acyl chains are readily distinguished from the carbonyl stretching of the interfacial region and the phosphate stretching of the polar head group (Lee and Chapman 1986). The C=O stretching absorption band provides important clues to the structure and organization of head group and polar/apolar interfacial regions of liquid-crystalline lipids. With most of the common diacyl-phospholipids, the observed C=O stretching absorption band has been shown to be a summation of at least two major components that are usually centered near 1738-1742 cm<sup>-1</sup> and near 1724-1729 cm<sup>-1</sup>. According to the empirical rules, a decrease in the frequency implies either the strengthening of existing hydrogen bonding or formation of new hydrogen bonding between the components (Korkmaz et al., 2005). The absorption band attributable to phosphate O-P-O asymmetric stretching vibrations can also provide important clues about hydration and hydrogen bonding interactions at the surfaces of hydrated phospholipid assemblies. The antisymmetric PO<sub>2</sub><sup>-</sup> stretching mode appears around 1240 cm<sup>-1</sup> in dry phosphate and shifts to around 1220 cm<sup>-1</sup> in fully hydrated DPPC (Arrondo et al., 1998; Lewis and McElhaney 1998). Dehydration results in band shifts of PO<sub>2</sub><sup>-</sup> antisymmetric towards higher wavenumbers (Kazanci et al., 2001), while a strengthening of the hydrogen bonding interactions with water results in band shifts towards lower wavenumbers (Lewis and McElhaney 1998).

Moreover, it is well known that in the so-called methylene-stretching region of the infrared spectrum (2800-3000 cm<sup>-1</sup>), polymethylene chains which contain gauche conformers absorb infrared radiation at higher frequencies than do those which contain all-trans conformers. Thus, increases in hydrocarbon chain conformational disorder are accompanied by increases in the frequencies of infrared absorption bands arising from the symmetric stretching vibrations of the methylene groups on lipid hydrocarbon chains. This has frequently been used for the detection and/or monitoring of lipid hydrocarbon chain-melting phase transitions or for monitoring of lipid hydrocarbon chain disorder (Arrondo et al., 1998; Lewis and McElhaney 1998). Furthermore, the

CH<sub>2</sub> antisymmetric stretching bandwidth of the methylene groups measured at 50% of height of the peaks gives information about the disorder of the lipid membrane. An increase in bandwidth is the indication of an increase in the membrane disorder (Korkmaz et al., 2005).

Considering the following data resulting from FTIR spectra analysis, we examine how human steroid hormones influence the structure of the host lipid bilayer. Tables 2 and 3 resume the literature results obtained by FTIR. Table 2 showed the frequency of the polar phospholipid groups (C=O and PO<sub>2</sub><sup>-</sup>). Compared to steroid-free liposomes, a shifting of the frequency values of PO<sub>2</sub><sup>-</sup> antisymmetric double stretching band was obtained in the presence of cyproterone acetate, 17 $\beta$ -estradiol, finasteride (Biruss et al., 2007), dydrogesterone, 17-hydroxyprogesterone, 21-hydroxyprogesterone, medroxyprogesterone, medroxyprogesterone acetate and progesterone (Abboud et al., 2015) in the gel, transition and liquid crystalline phases (Table 2).

**Table 2** Frequency of the polar phospholipid groups obtained by FTIR for human steroid hormones loaded liposomes at different phases.

<b>Drug</b>	<b>Phospholipid/molecule</b>	<b>C=O stretching modes relative to steroid-free liposomes</b>	<b>PO<sub>2</sub><sup>-</sup> stretching modes relative to steroid-free liposomes</b>	<b>Conclusions</b>
Cpa <sup>5</sup>	DPPC/Cpa: 100/0.5	Frequency shifted to lower values in the gel and the liquid crystalline phases.	Frequency shifted to higher values in the gel and the liquid crystalline phases.	[i]
DYG <sup>1</sup>	DPPC/DYG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Frequency shifted to lower values at the transition phase and to higher values in the gel and liquid crystalline phases.	Frequency shifted to lower and higher values in the gel, transition and liquid phases.	[i]
E <sub>2</sub> <sup>5</sup>	DPPC/E <sub>2</sub> : 100/0.5	Frequency shifted to lower values in the gel and the liquid crystalline phases.	Frequency shifted to lower values in the gel and the liquid crystalline phases.	[i]
Finasteride <sup>5</sup>	DPPC/Finasteride: 100/0.5	Frequency shifted to lower values in the gel and the liquid crystalline phases.	Frequency shifted to lower values in the gel and the liquid crystalline phases.	[i]
17-OHPG <sup>1</sup>	DPPC/17-OHPG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Frequency shifted to lower values at the transition phase and to higher values in the gel and liquid crystalline phases.	Frequency shifted to lower and higher values in the gel, transition and liquid phases.	[i]
21-OHPG <sup>1</sup>	DPPC/21-OHPG: 100/1, 100/2.5, 100/5, 100/10 and	Frequency shifted to lower values at the transition phase	Frequency shifted to lower and higher values in the gel, transition and liquid	[i]

	100/25	and to higher values in the gel and liquid crystalline phases.	phases.	
MP <sup>1</sup>	DPPC/MP: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Frequency shifted to lower values at the transition phase and to higher values in the gel and liquid crystalline phases.	Frequency shifted to lower and higher values in the gel, transition and liquid phases.	[i]
MPA <sup>1</sup>	DPPC/MPA: 100/1, 100/2.5, 100/5, 100/10 and 100/25	Frequency shifted to lower values at the transition phase and to higher values in the gel and liquid crystalline phases.	Frequency shifted to lower and higher values in the gel, transition and liquid phases.	[i]
PG <sup>1, 19, 20</sup>	DPPC/PG: • 100/1, 100/3, 100/6, 100/9, 100/12, 100/18, 100/24 <sup>19, 20</sup> • 100/1, 100/2.5, 100/5, 100/10 and 100/25 <sup>1</sup>	<ul style="list-style-type: none"> <li>• Frequency shifted to lower and higher values in the gel, transition and liquid phases<sup>1</sup>.</li> <li>• Frequency shifted to higher values in the gel, transition, and liquid phases<sup>19, 20</sup>.</li> </ul>	Frequency shifted to lower and higher values in the gel, transition and liquid phases <sup>1</sup> .	[i]

[i]: Interacts with polar head group and disturbs the glycerol backbone.

[1] Abboud et al., 2015; [5] Biruss et al., 2007; [19] Korkmaz et al., 2005; [20] Korkmaz and Severcan 2005.

Moreover, compared to steroid-free liposomes, cyproterone acetate, 17 $\beta$ -estradiol, and finasteride (Biruss et al., 2007) shifted the frequency of the characteristic lipid peak C=O of DPPC glycerol backbone to lower values in the gel and the liquid crystalline phases. Similar results were obtained for dydrogesterone, 17-hydroxyprogesterone, 21-hydroxyprogesterone, medroxyprogesterone, medroxyprogesterone acetate and progesterone at the transition phase (Abboud et al., 2015), indicating a strengthening of the hydrogen bonds or even a formation of new hydrogen bonds between the components. However, in the gel and the liquid crystalline phases, dydrogesterone, 17-hydroxyprogesterone, 21-hydroxyprogesterone, medroxyprogesterone, medroxyprogesterone acetate (Abboud et al., 2015) and progesterone (Korkmaz et al., 2005; Abboud et al., 2015) shifted the peak position to higher values suggesting a decrease in the hydrogen bond between the components of the system. Changes of the peak position of CO prove that the steroid hormones interacted with the glycerol backbone. The OH and CO groups of the steroid molecules and the NH group of finasteride may display hydrogen bonding with the phospholipids and/or the surrounding water molecules.

Table 3 shows the frequency of CH<sub>2</sub> symmetric and the bandwidth of the CH<sub>2</sub> antisymmetric stretching bands at different temperatures. CH<sub>2</sub> symmetric represents CH bonds that stretch in-phase while CH<sub>2</sub> antisymmetric represents those stretching out-of-phase. The steroid hormones (dydrogesterone, 17-hydroxyprogesterone, 21-hydroxyprogesterone, medroxyprogesterone and medroxyprogesterone acetate (Abboud et al., 2015)) did not affect significantly the C–H stretching modes at 2800–3000 cm<sup>-1</sup> in the gel, transition and liquid crystalline phases, leading to a small effect on lipid hydrocarbon chain disorder (Abboud et al., 2015). Whereas, progesterone induced a shift leading to an increase in hydrocarbon chain conformational disorder (Korkmaz et al., 2005; Korkmaz and Severcan 2005) (Table 3).

Compared to steroid-free liposomes, cyproterone acetate and finasteride (Biruss et al., 2007) induced a slight shift in the peak position of C–H to lower frequency values in the gel phase. The same effect was detected in the liquid crystalline phase but in a more pronounced manner (Table 3). This might indicate an increase in the number of trans conformers, suggesting an increase in the order of the bilayer.

Compared to steroid-free liposomes, the frequency of C–H stretching mode of 17 $\beta$ -estradiol loaded liposomes depends on its concentration (Boyar and Severcan 1997; Dicko et al., 1999; Cakmak 2006; Biruss et al., 2007), the  $\alpha$ /trans or  $\beta$ /cis configuration at C-17 and the membrane composition. 17 $\beta$ -estradiol promotes an increase in the order of the membrane at low

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phospholipid/17 $\beta$ -estradiol molar ratio and enhanced membrane disorder at high molar ratio (Boyar and Severcan 1997; Dicko et al., 1999; Cakmak 2006; Biruss et al., 2007).

With respect to the bandwidth of the CH<sub>2</sub> antisymmetric, the data presented in Table 3 proved that each molecule modulates in a specific manner this parameter and that depends on the bilayer phase as well as on the steroid concentration used. Compared to DSC results, the data obtained by FTIR did not give additional deeper information regarding the interaction of steroids with membranes.

**Table 3** Wavenumber and bandwidth of the CH<sub>2</sub> symmetric and antisymmetric stretching bands, respectively for human steroid hormones loaded liposomes at different temperatures, obtained by FTIR.

Steroids	Phospholipid/Steroid molar ratio	Wavenumber CH <sub>2</sub> symmetric relative to steroid-free liposomes	Bandwidth of CH <sub>2</sub> antisymmetric relative to steroid-free liposomes	Conclusion
Cpa <sup>5</sup>	DPPC/Cpa: 100/0.5	<ul style="list-style-type: none"> <li>At 32°C: slight shift to lower frequency.</li> <li>At 50°C: significant shift to lower frequency.</li> </ul>	At 32°C and 50°C: decrease of the bandwidth.	Cpa increased the order of the system in the gel and liquid crystalline phases.
DYG <sup>1</sup>	DPPC/DYG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	<ul style="list-style-type: none"> <li>At 28°C: significant shift to higher frequency at 100/10 and 100/25.</li> <li>At 41°C: significant shift to higher frequency at 100/25.</li> <li>At 50°C: negligible variation.</li> </ul>	<ul style="list-style-type: none"> <li>At 28°C: significant decrease of the bandwidth at 100/1.</li> <li>At 41°C: significant decrease of the bandwidth at 100/1, 100/2.5, 100/5 and 100/25.</li> <li>At 50°C: negligible variation.</li> </ul>	DYG increased the order of the system at the gel and transition phases.
E <sub>2</sub> <sup>5, 6, 8, 10</sup>	<ul style="list-style-type: none"> <li>DPPC/E<sub>2</sub>: 100/0.5<sup>5</sup></li> <li>DPPC/E<sub>2</sub>: 100/20<sup>6</sup></li> <li>DMPC<sub>d54</sub>/E<sub>2</sub>: 100/25<sup>10</sup></li> <li>Liver tissue of a rainbow trout (22µg/L of E<sub>2</sub>)<sup>8</sup></li> </ul>	<ul style="list-style-type: none"> <li>For (100/0.5), at 32°C, slight shift to lower frequency and at 50°C, significant shift to lower frequency.</li> <li>For (100/20 and 100/25), at gel and liquid phases, slight shift to higher frequency.</li> <li>In liver tissue, a shift to lower frequency.</li> </ul>	<ul style="list-style-type: none"> <li>For (100/0.5), at 32°C and 50°C: decrease of the bandwidth.</li> <li>For (100/20), at gel and liquid phases: increase of the bandwidth.</li> <li>In liver tissue, decrease of the bandwidth.</li> </ul>	<ul style="list-style-type: none"> <li>For DPPC, 17-β-estradiol increased the order of the bilayers in the gel and liquid crystalline phases at low molar ratio.</li> <li>Whereas, a contrary effect was observed at higher molar ratio for</li> </ul>

				DPPC and DMPC membranes. • In liver tissue, 17- $\beta$ -estradiol increased the order of the membrane of the treated cells.
Finasteride <sup>5</sup>	DPPC/Finasteride: 100/0.5	• At 32°C: slight shift to lower frequency. • At 50°C: significant shift to lower frequency.	• At 32°C: decrease of the bandwidth. • At 50°C: increase of the bandwidth.	Finasteride decreased the order of the system at the liquid crystalline phase.
17-OHPG <sup>1</sup>	DPPC/17-OHPG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	• At 28°C: significant shift to higher frequency at 100/5. • At 41°C: negligible variation. • At 50°C: negligible variation.	• At 28°C: significant decrease of the bandwidth at 100/2.5, 100/5 and 100/10. • At 41°C: negligible variation. • At 50°C: negligible variation.	17-OHPG increased the order of the system at the gel phase.
21-OHPG <sup>1</sup>	DPPC/21-OHPG: 100/1, 100/2.5, 100/5, 100/10 and 100/25	At 28°C, 41°C and 50°C negligible variations.	At 28°C, 41°C and 50°C negligible variations.	21-OHPG increased the order of the system at the gel, transition and liquid phases.
MP <sup>1</sup>	DPPC/MP: 100/1, 100/2.5, 100/5, 100/10 and 100/25	• At 28°C: significant shift to higher frequency at 100/5. • At 41°C and 50°C negligible variations.	• At 28°C: significant decrease of the bandwidth at 100/1, 100/10 and 100/25. • At 41°C: negligible variation.	MP increased the order of the system at the gel and liquid phases.

			<ul style="list-style-type: none"> <li>At 50°C: significant decrease of the bandwidth at 100/2.5.</li> </ul>	
MPA <sup>1</sup>	DPPC/MPA: 100/1, 100/2.5, 100/5, 100/10 and 100/25	At 28°C, 41°C and 50°C negligible variations.	<ul style="list-style-type: none"> <li>At 28°C: significant increase of the bandwidth at 100/1.</li> <li>At 41°C: negligible variation.</li> <li>At 50°C: significant increase of the bandwidth at 100/2.5.</li> </ul>	MPA decreased the order of the system at the gel and liquid phases.
PG <sup>1, 19, 20</sup>	<ul style="list-style-type: none"> <li>DPPC/PG: 100/1, 100/2.5, 100/5, 100/10 and 100/25<sup>1</sup></li> <li>DPPC/PG: 100/1, 100/3, 100/6, 100/9, 100/12, 100/18, 100/24<sup>19, 20</sup></li> </ul>	<ul style="list-style-type: none"> <li>At 28°C: significant shift to higher frequency at 100/2.5, 100/10 and 100/25<sup>1</sup>.</li> <li>At 41°C and 50°C, negligible variations<sup>1</sup>.</li> <li>At 28°C: negligible variation at 100/1<sup>19, 20</sup>.</li> <li>At 41°C: significant shift to higher frequency at 100/1<sup>19, 20</sup>.</li> <li>At 50°C: negligible variation at 100/1<sup>19, 20</sup>.</li> <li>At 28, 41 and 50 °C shift to higher frequency for 100/3, 100/6, 100/9, 100/12, 100/18, 100/24<sup>19, 20</sup>.</li> </ul>	<ul style="list-style-type: none"> <li>At 28°C: significant increase of the bandwidth at 100/1<sup>1</sup>.</li> <li>At 41°C: significant increase of the bandwidth at 100/10.</li> <li>At 50°C: negligible variations<sup>1</sup>.</li> <li>At 28°C, 41°C and 50°C decrease of the bandwidth at 100/1, 100/12, 100/18 and 100/24. Whereas, significant increase of the bandwidth at 100/3, 100/6 and 100/9<sup>19, 20</sup>.</li> </ul>	At the gel, transition and liquid phases, PG increased the order of the system at very low and high concentrations. Whereas, it decreased it at low concentrations.

[1] Abboud et al., 2015; [5] Biruss et al., 2007; [6] Boyar and Severcan 1997; [8] Cakmak et al., 2006; [10] Dicko et al., 1999; [19] Korkmaz et al., 2005; [20] Korkmaz and Severcan 2005.

## **Effect of human steroid hormones on liposome membranes fluidity measured by EPR**

Electron paramagnetic resonance (EPR) spectroscopy is a well-known method for the examination of the local structure and dynamics of paramagnetic centers in biological samples (Prisner et al., 2001). The fatty acid spin-label agents 5-doxyl stearate and 16-doxyl stearate are used as paramagnetic probes. 5-doxyl stearate is believed to be anchored at the lipid– aqueous interface of the cell membranes by its carboxyl end, while the doxyl group of 16-doxyl stearate is incorporated deeper in the hydrophobic core of the membrane (Zamoon et al., 2003; Sommer et al., 2014). The nitroxide group moves rapidly through a restrict angle around the point of attachment. Therefore, the EPR spectra of the fatty acid spin-label agents are used to detect an alteration in the freedom of motion in biological membranes and to provide an indication of membrane fluidity (Tsuda et al., 2002). For indexes of membrane fluidity, the order parameter ( $S$ ) is calculated for the probes and the peak height ratio ( $h_0/h_{-1}$ ) value is used as an index of the membrane fluidity. The greater the values of the peak height ratio ( $h_0/h_{-1}$ ), the lesser the freedom of motion of the spin labels in the biomembrane bilayers indicating the lower membrane fluidity (Tsuda et al., 2002). Whereas, the order parameter ( $S$ ) is a structural parameter giving information about lipid acyl chain flexibility.

Table 4 presents the effect of some human steroid hormones on the order parameter and the peak height ratio, obtained by EPR. It has been shown that progesterone (Tsuda et al., 2002),  $17\beta$ -estradiol (Tsuda et al., 2001a) and estriol (Tsuda et al., 2001b) decreased the order parameter and the peak height ratio in a dose-dependent manner, which means an increment of the lipid acyl chain flexibility of erythrocyte membranes (Tsuda et al., 2001a; 2001b and 2002). The additional hydroxyl group at C16 of estriol did not produce additional effect on the lipid acyl chain flexibility of erythrocyte compared to  $17\beta$ -estradiol. Furthermore,  $17\alpha$ -estradiol showed no effect on lipid acyl chain flexibility of erythrocyte (Tsuda et al., 2001a). Once again, the  $\alpha$ /trans or  $\beta$ /cis configuration at C-17 seems to control the effect of estradiol on membrane ordering. On the other hand, with respect to  $17\beta$ -estradiol and estriol, the presence of a conjugated 3-Keto group in the progesterone molecule did not induce a remarkable effect on the lipid acyl chain flexibility of erythrocyte.

Besides, methylprednisolone induced the granulocyte membrane ordering in a dose dependent manner (Lamche et al., 1990). We again propose that the formation of an intramolecular hydrogen bond between the oxygen atom at C20 and the hydroxyl group at C21 or C17 may

deplete the effect of methylprednisolone on the flexibility of the lipid acyl chains in membrane.

**Table 4** Effect of human steroid hormones on the order parameter and the peak height ratio obtained by EPR.

Steroids	Phospholipid/Steroid molar ratio	Order Parameter (S)	Peak Height Ratio ( $h_0/h_{-1}$ )	Conclusions
Estradiol <sup>27</sup> and E <sub>3</sub> <sup>28</sup>	Human erythrocyte membranes ( $10^{-6}$ ; $10^{-7}$ M of E <sub>2</sub> and E <sub>3</sub> )	Decreased significantly at $10^{-6}$ and $10^{-7}$ M	Decreased significantly at $10^{-6}$ and $10^{-7}$ M	E <sub>2</sub> and E <sub>3</sub> increased the lipid acyl chain flexibility and the membrane fluidity of erythrocyte.
MPd <sup>21</sup>	Human granulocyte membranes (0.5, 1 and 2 mg/ml)	Increased significantly at 0.5, 1 and 2 mg/ml		MPd decreased the lipid acyl chain flexibility of granulocyte.
PG <sup>29</sup>	Human erythrocyte membranes ( $10^{-9}$ , $10^{-8}$ , $10^{-7}$ , $10^{-6}$ M of PG)	Decreased significantly at $10^{-9}$ , $10^{-8}$ , $10^{-7}$ , $10^{-6}$ M	Decreased significantly at $10^{-9}$ , $10^{-8}$ , $10^{-7}$ and $10^{-6}$ M	PG increased the lipid acyl chain flexibility and the membrane fluidity of erythrocyte.

[21] Lamche et al., 1990; [27] Tsuda et al., 2001a; [28] Tsuda et al., 2001b; [29] Tsuda et al., 2002.

### **Effect of human steroids hormones on liposome membranes fluidity determined by fluorescence anisotropy**

Anisotropy measurements are commonly used in the biochemical applications of fluorescence (Lacowicz 2006). They are based on the principle of photoselective excitation of fluorophores by polarized light. Fluorophores preferentially absorb photons whose electric vectors are aligned parallel to the transition moment of the fluorophore. The transition moment has a defined orientation with respect to the molecular axis. In an isotropic solution, the fluorophores are oriented randomly. Upon excitation with polarized light, one selectively excites the fluorophore molecules whose absorption transition dipole is parallel to the electric vector of the excitation. This selective excitation results in a partially oriented population of fluorophores (photoselection), and in partially polarized fluorescence emission. Emission also occurs with the

light polarized along a fixed axis in the fluorophore. The relative angle between these moments determines the maximum measured anisotropy. The fluorescence anisotropy ( $r$ ) and polarized ( $P$ ) are defined by

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where  $I_{\parallel}$  and  $I_{\perp}$  are the fluorescence intensities of the vertically ( $\parallel$ ) and horizontally ( $\perp$ ) polarized emission, when the sample is excited with vertically polarized light. Anisotropy and polarization are both expressions for the same phenomenon (Lakowicz 2006).

Membranes typically do not display intrinsic fluorescence. For this reason, they are marked with probes which are inserted in the membrane (Lakowicz 2006). The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and its derivatives represent popular membrane probes for monitoring organization and dynamics in membranes (Rodriguez et al., 1997; Pucadyil and Chattopadhyay 2006; Lakowicz 2006). DPH is a rod-like molecule and partitions into the interior of the bilayer (Pucadyil and Chattopadhyay 2006). The derivative of DPH, 4-trimethyl-ammonio-1,6-diphenyl-1,3,5-hexatriene (TMA-DPH) has a cationic moiety attached to the para -position of one of the phenyl rings. The amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid–water interface (Rodriguez et al., 1997; Pucadyil and Chattopadhyay 2006). Moreover other fluorescent membrane probes are used such as Laurdan, which is located in the glycerol backbone of the lipid layer (Socaciu et al., 2002). 12-(9-antroiloxy)-Stearic acid (12-AS) is incorporated into the hydrophobic regions of the lipid bilayer (Marczak et al., 2009), 1-anilino-8-naphthalene sulfonate (ANS) binds to phospholipids in such a way that the anionic sulfonate group is oriented toward the hydrophilic head group of the lipid (Marczak et al., 2009) and 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, curcumin, which is incorporated into the nonpolar region of the membrane (Patra et al., 2012). The anisotropy parameter is inversely related to the membrane fluidity, that is, lower anisotropy values indicate an increase in fluidity (Park et al., 2006).

Table 5 resumes the results of anisotropy obtained by fluorescence spectroscopy regarding the effect of human steroid hormones on membranes fluidity.

An increase in the membrane fluidity of DPPC was observed at temperatures ranging from 28 to 50°C with betamethasone (0.1%) (Ghosh et al., 1996), cortisol (0.1; 1; 2.5 and 10%) (Ghosh et al., 1996; Abboud et al., 2016), dexamethasone (0.1%) (Ghosh et al., 1996), 9-fluorocortisol

acetate (1; 2.5 and 10%) (Abboud et al., 2016) and prednisolone (0.1; 1; 2.5 and 10%) (Ghosh et al., 1996; Abboud et al., 2016). Similarly, androstenol at 33% produced an increase in DPPC membrane fluidity at 36°C while a contrary effect was obtained at 52°C (Xu and London 2000). Besides, dexamethasone increased the fluidity of intestinal (Brasitus et al., 1987), liver (Kapitulnik et al., 1986) and leukemia cells (Kiss et al., 1990) membranes. The fluidizing effect of the mentioned corticosteroids lies with the presence of a conjugated 3-Keto group in betamethasone, cortisol, dexamethasone, 9-fluorocortisol acetate and prednisolone promoting a destabilization of the bilayers (Gallay and De Kruijff 1984; Wenz 2012) and consequently a fluidizing effect. Cortisol didn't affect the membrane fluidity of erythrocytes (Golden et al., 1999), and this can be due to the difference in membrane composition.

Progesterone induced a decrease in the membrane fluidity of DPPC (Whitting et al., 2000 and Liang et al., 2001), hamster sperm plasma membrane (SPM), sperm acrosomal membrane (SAM) (Shivaji and Jagannadham 1992), synaptosomal plasma membrane (SyPM) from brain tissue and sarcoplasmic reticulum (SR) membrane from skeletal muscle (Whitting et al., 2000). Whatever was the membrane type, the testosterone effect on the membrane fluidity depends on its concentration (Table 5).

The effect of 17 $\beta$ -estradiol on the fluidity of biological membranes depends on its concentration (Reddy et al., 1989; Shivaji and Jagannadham 1992; Schwartz et al., 1996; Whiting et al., 2000; Liang et al., 2001) and on the biological membrane composition. Indeed, 17 $\beta$ -estradiol increased the membrane fluidity of DPPC, SyPM, SR and rat vaginal epithelial cells (Reddy et al., 1989; Whiting et al., 2000), decreased that of rat chondrocytes cells (Schwartz et al., 1996) and didn't affect that of erythrocytes (Golden et al., 1999). Moreover, Scheidt et al., 2010 proved that 17 $\beta$ -estradiol had no influence on the molecular order of POPC lipid chains proved by NMR (Scheidt et al., 2010). Besides, 17 $\alpha$ -estradiol had no effect on the rat chondrocytes cells (Schwartz et al., 1996).

17 $\alpha$ -estradiol, E3ol and 17-estradiol octyl ether that differ by the substitution at C16 and C17 presented the same effect on the membrane fluidity of HT-22 cells (a mouse transformed hippocampal cell line) (Liang et al., 2001), indicating that the substitution at these carbons did not modulate the membrane properties.

**Table 5** Anisotropy measured by Fluorescence spectroscopy of lipid membranes incorporating human steroid hormones.

Steroids	Phospholipid/Steroid molar ratio	Anisotropy ( <i>r</i> )	Conclusions
BM <sup>16</sup>	DPPC/BM: 100/0.1	Decreased throughout the experimental temperature ranging from 30 to 50°C	BM increased the membrane fluidity throughout the temperature range.
Co <sup>2, 16</sup>	DPPC/Co: • 100/0.1 <sup>16</sup> • 100/1, 100/2.5 and 100/10 <sup>2</sup>	Decreased at 28, 41 and 50 °C	Co increased the membrane fluidity throughout the temperature range.
DEX <sup>7, 16, 17, 18</sup>	• Proximal-small-intestinal brush-border membranes of rats (100 µg/day per 100 g body weight for 4 days) <sup>7</sup> • DPPC/Drug: 100/0.1 <sup>16</sup> • Fetal liver microsomal membranes of rats (injections of 0.4 mg on days 14-18 or 15-19 of pregnancy) <sup>17</sup> • Membrane of leukemia cells 20 nM <sup>18</sup>	Decreased	DEX increased the membrane fluidity for synthetic and biological membranes.

Estradiol <sup>22, 24, 25, 26, 30</sup>	<ul style="list-style-type: none"> <li>• PC and HT-22 cells/30µM of 17α and 17β-estradiol <sup>22</sup></li> <li>• Membrane of the rat vaginal epithelial cells (0.1µg/g of 17β-estradiol body weight) <sup>24</sup></li> <li>• Hamster sperm plasma membrane (SPM) and sperm acrosomal membrane (SAM) (0; 5; 10; 15; 20; 24 and 30 µg/ml) <sup>25</sup></li> <li>• Plasma membrane of chondrocyte's cells from rat (<math>10^{-8}</math>; <math>10^{-9}</math>; <math>10^{-10}</math> M of 17α and 17β-estradiol) <sup>26</sup></li> <li>• DPPC, synaptosomal plasma membranes (SyPM) and sarcoplasmic reticulum (SR) membranes /17-β-estradiol: 100/10, 100/20, 100/40, 100/60 <sup>30</sup></li> </ul>	<ul style="list-style-type: none"> <li>• Increased <sup>22, 26</sup></li> <li>• Decreased <sup>24, 30</sup></li> <li>• No effect <sup>25, 26</sup></li> </ul>	<ul style="list-style-type: none"> <li>• 17α-estradiol enhanced the ordering of the PC and HT-22 cell membranes. While 17β-estradiol had no effect <sup>22</sup>.</li> <li>• 17β-estradiol enhanced the ordering of the plasma membrane of chondrocyte's cells. Whereas, 17α-estradiol had no effect <sup>26</sup>.</li> <li>• 17β-estradiol had no effect on the fluidity of SPM and SAM <sup>25</sup>.</li> <li>• 17β-estradiol increased the fluidity of the membranes of the rat vaginal epithelial cells, DPPC, SyPM and SR <sup>24, 30</sup>.</li> </ul>
17 Estradiol octyl ether <sup>22</sup>	PC and HT-22 cells/30µM	Increased	17 Estradiol octyl ether enhanced the ordering of the PC and HT-22 cells membranes.
E3ol <sup>22</sup>	PC and HT-22 cells/30µM	Increased	E3ol enhanced the ordering of the PC and HT-22 cells membranes.
9-FA <sup>2</sup>	DPPC/9-FA: 100/1, 100/2.5 and 100/10	Decreased at 28, 41 and 50 °C	9-FA increased the membrane fluidity throughout the temperature range.

Pd <sup>2, 16</sup>	DPPC/Pd: • 100/1, 100/2.5 and 100/10 <sup>2</sup> • 100/0.1 <sup>16</sup>	Decreased at 28, 41 and 50 °C	PD increased the membrane fluidity throughout the temperature range.
PG <sup>22, 25, 30</sup>	• PC and HT-22 cells/30µM <sup>22</sup>  • Hamster sperm plasma membrane and sperm acrosomal membrane (0; 5; 10; 15; 20; 24 and 30 µg/ml) <sup>25</sup>  • DPPC, synaptosomal plasma membranes and sarcoplasmic reticulum membranes /Progesterone: 100/10, 100/20, 100/40, 100/60 <sup>30</sup>	Increased <sup>22, 25, 30</sup>	PG enhanced the ordering of the systems.
T <sup>22, 25, 30</sup>	• PC and HT-22 cells/30µM <sup>22</sup>  • Hamster sperm plasma membrane and sperm acrosomal membrane (0; 5; 10; 15; 20; 24 and 30 µg/ml) <sup>25</sup>  • DPPC, synaptosomal plasma membranes and sarcoplasmic reticulum membranes /T: 100/10, 100/20, 100/40, 100/60 <sup>30</sup>	• Increased <sup>22</sup> • No effect <sup>25, 30</sup>	T enhanced the ordering of PC and HT-22 membranes.

[2] Abboud et al., 2016; [7] Brasitus et al., 1987; [16] Ghosh et al., 1996; [17] Kapitulnik et al., 1986; [18] Kiss et al., 1990; [22] Liang et al., 2001; [24] Reddy et al., 1989; [25] Shivaji and Jagannadham 1992; [26] Schwartz et al., 1996; [30] Whiting et al., 2000.

## Effect of human steroid hormones on domain formation by fluorescence quenching measurements

To detect the effect of an active molecule on lipid rafts formation, vesicles composed of either saturated or unsaturated acyl chains, DPH and 12SLPC are prepared in many studies in literature (Xu and London 2000; Xu et al., 2001; Wenz and Barrantes 2003). 1-palmitoyl-2- (12 doxyl) stearoylphosphatidylcholine (12SLPC) is a fluorescence-quenching phospholipid that exhibits phase behavior similar to that of an unsaturated lipid. When membranes are made up of an unsaturated lipid (the  $T_m$  of both lipids is below 0 °C), all components mix and distribute randomly in the bilayer at temperatures above their  $T_m$ . When the unsaturated lipid is replaced by a saturated one, saturated phospholipids molecules segregate from the quencher molecules at temperatures below their  $T_m$  (Wenz and Barrantes 2003).

The degree of domain formation is related to  $\Delta F/F_0$ , the difference between the fraction of DPH fluorescence that is unquenched in a sample containing saturated acyl chain lipid-rich domains and the fraction of unquenched fluorescence in a sample having the same amount of quencher lipid but lacking such domains ( $\Delta F/F_0 = F/F_0$  <sub>saturated lipid/12SLPC mixture</sub> -  $F/F_0$  <sub>unsaturated lipid /12SLPC mixture</sub>). F corresponds to samples with quencher, containing a saturated lipid and 12SLPC or unsaturated lipid and 12SLPC, with or without mol % of drug. Whereas, samples without quencher are the  $F_0$  samples containing saturated/unsaturated lipids or unsaturated lipid, with or without mol % of drug. Fluorescence in F and  $F_0$  samples is measured at different temperatures and  $\Delta F/F_0$  values are calculated. Therefore, a high value of  $\Delta F/F_0$ , is an indicative of a high “extent” of domain formation (Xu and London 2000; Xu et al., 2001). Quenching measurements demonstrated that some steroids promote formation of lipid-enriched domains, while others inhibit it.

Androstenol (Xu and London 2000), 11-hydroxyprogesterone, 17-hydroxyprogesterone, 17 $\beta$ -estradiol, cortisol, cortisone, deoxycortisol, progesterone, pregnenolone and promegestone (Wenz and Barrantes 2003) are studied for their effects on domain formation. These steroids display lipid domain-disrupting activity. The studied steroid hormones lack the aliphatic side chain, which is present in domain promoting steroids such as cholesterol and 25-hydroxycholesterol (Wenz and Barrantes 2003).

### **Effect of human steroid hormones hydrophobicity on membrane ordering**

Wenz 2015 studied the activity of 82 steroids on membranes in relation to their ordering, rigidifying, condensing and/or raft promoting ability. The series included the following human steroid hormones: cortisol, cortisone, 17 $\beta$ -estradiol, 17-hydroxyprogesterone, 21-hydroxyprogesterone, progesterone androsterol and androstenol. Using a semi-empirical procedure and 245 molecular descriptors, the author developed a sample model to estimate and predict the steroid activity as a function of steroid molecular properties. Wenz concluded that the most relevant properties in governing and predicting the activity of steroids on membranes are area and Log P. Thus, a logistic regression model (p) as a function of the area and log P of the steroids was proposed. When applied to human steroid hormones to which area and Log P are published (Wenz 2015), all of them demonstrated p values below 0.65 (which is the cut value) suggesting that they are membrane disrupters. According to Wenz's logistic regression model, the p value is 0.0002, 0.0005, 0.004, 0.017, 0.021 and 0.23 respectively for 17 $\beta$ -estradiol, androsterol, progesterone, 17-hydroxyprogesterone, 21-hydroxyprogesterone and cortisol. The findings are consistent with experimental literature data (Table 1) described in detail in this revue. Considering the only Log P parameter, among the series that produced a higher fluidifying effect (21-hydroxyprogesterone, progesterone, pregnanedione, testosterone, testosterone acetate, cyproterone acetate, finasteride, 17 $\beta$ -estradiol and cortisone hexadecanoate), the range of log P values ranged between 2.88 and 5.6 (Table 6). Also among the series that produced a lower fluidifying effect on the membrane (beclomethasone dipropionate, cortisol, 9-fluorocortisol acetate and prednisolone), the range of log P values ranged between 1.32 and 4.59 (Table 6). The findings altogether demonstrate that the Log P value is probably not the main factor that controls the effect of human steroid hormone molecule on membrane ordering.

**Table 6** logP values of human steroid hormones that appear throughout the manuscript.

Steroids	logP	References
BM	1.83	Alvarez Nunez and Yalkowsky 1997
BDP	4.59	Chemspider
Co	1.61	Alvarez Nunez and Yalkowsky 1997
	1.42	Kilford et al., 2008
Cn	1.47	Alvarez Nunez and Yalkowsky 1997
Cpa	3.40	Rezaei et al., 2010
DEX	1.83	Alvarez Nunez and Yalkowsky 1997
DYG	3.58	Torres-Cartas et al., 2000
E <sub>2</sub>	4.01	Alvarez Nunez and Yalkowsky 1997
E <sub>3</sub>	2.45	Alvarez Nunez and Yalkowsky 1997
Finasteride	3	Pubchem
9-FA	1.70	Pubchem
17-OHPG	3.17	Alvarez Nunez and Yalkowsky 1997
21-OHPG	2.88	Alvarez Nunez and Yalkowsky 1997
MPd	1.73	Marczak et al., 2015
MP	3.38	Alvarez Nunez and Yalkowsky 1997
MPA	4.11	Torres-Cartas et al., 2000
Pd	1.62	Alvarez Nunez and Yalkowsky 1997
	1.32	Marczak et al., 2015
Pdn	5.6	Pubchem
PG	4.04	Torres-Cartas et al., 2000
T	3.32	Alvarez Nunez and Yalkowsky 1997
	3.47	Kilford et al., 2008
TA	3.9	Pubchem

## Conclusion

In this work, we reviewed the literature using biophysical methods on the interaction of human steroid hormones with membranes. The analysis of DSC results allowed the classification of molecules into different groups according to their effect on the calorimetric parameters of the main transition phase. Steroids containing some features (a conjugated 3-keto group, free polar groups at ring D, bulkier structure at rings A or D) seem to increase the acyl chain flexibility of

the membrane. FTIR results analysis did not give additional deeper information regarding the structural characteristics of a steroid that can affect the membrane ordering. The hydrophobicity of the human steroid hormone seems not to be the main factor that controls the membrane disruption effect. Also, it is worthy noting that the effect of human steroid hormones on the membrane is controlled by membrane composition and steroids concentration.

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## **1.2 Effect of progesterone, its hydroxylated and methylated derivatives, and dydrogesterone on lipid bilayer membranes**

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**Journal of Membrane Biology (2015) 248: 811-824**

## Effect of Progesterone, Its Hydroxylated and Methylated Derivatives, and Dydrogesterone on Lipid Bilayer Membranes

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Received: 7 November 2014 / Accepted: 12 April 2015  
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**Abstract** The interaction of progesterone (PG), 17-hydroxyprogesterone (17-OHPG), 21-hydroxyprogesterone (21-OHPG), medroxyprogesterone (MP), medroxyprogesterone acetate (MPA), and dydrogesterone (DYG), with zwitterionic dipalmitoyl phosphatidylcholine (DPPC) multilamellar liposome, was investigated as a function of drug concentration using Fourier transform infrared spectroscopy and differential scanning calorimetry. The results reveal that progesterone and its derivatives changed the physical properties of the DPPC bilayers by decreasing the main phase-transition temperature ( $T_m$ ) and enthalpy ( $\Delta H_m$ ), abolishing the pre-transition and disordering the membrane. From the thermodynamic parameters analysis, we concluded that PG, 21-OHPG, and MPA are localized inside the membrane. Whereas, the insertion of 17-OHPG in the lipid bilayers cannot be excluded in view of the significant decrease in the transition enthalpy at two molar ratios. MP and DYG are rather localized near the polar heads of phospholipids at the interface water-lipid bilayer. PG derivatives increase the membrane fluidity in the order: PG  $\approx$  21-OHPG  $\approx$  MPA  $>$  17-OHPG  $>$  MP  $\approx$  DYG. The distinct effects produced by steroids are discussed in terms of hydrophobicity and chemical structure.

**Keywords** Dydrogesterone · Hydroxyprogesterone · Liposome · Medroxyprogesterone · Medroxyprogesterone acetate · Progesterone

### Introduction

Steroids hormones are very efficient drugs. They generally possess a non-genomic and a genomic mechanism of action. In the classic genomic pathway of steroid action, a steroid enters a cell through the plasma membrane and binds to specific steroid receptor, and the complex transported into the nucleus where it regulates gene transcription (Korkmaz and Severcan 2005; Vijayan and Biggin 2008). In the non-genomic case, the cell membrane acts as mediator and interactions take place with membrane bound receptors (McEwen 1994) or steroids bind to neurotransmitter receptors (Iswari et al. 1986) or intercalate into the membrane of target tissues resulting in perturbation of lipid-lipid interactions (Korkmaz and Severcan 2005). Endogenous progesterone derivatives and clinical progesterone were used to define if the structure may play a role in the membrane modulating properties and so explain the potency between various derivatives. In this point of view, the determination of the role of a functional group would be crucial. Previous studies showed that steroids are able to modulate the membranes properties such as lipid fluidity (Mendoza et al. 1996), protein mobility (Ehring et al. 1998) and  $\text{Ca}^{2+}$ -ATPase activity (Korkmaz and Severcan 2005).

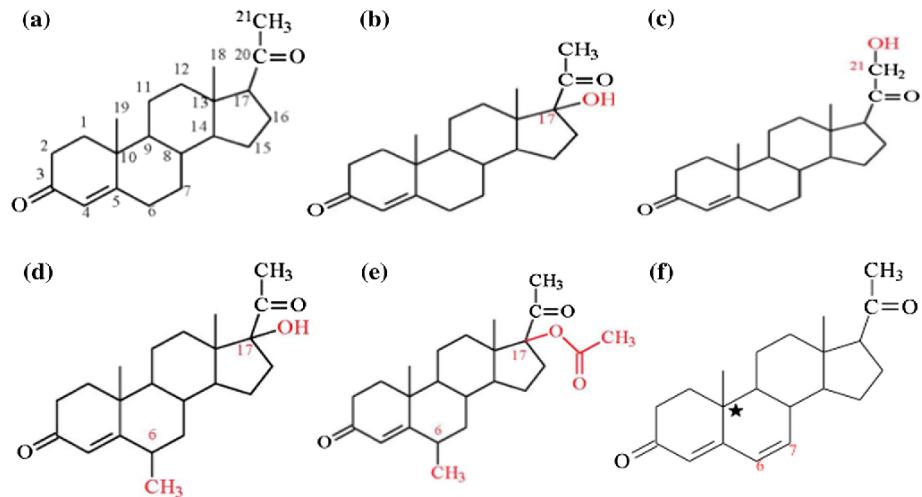
Progesterone (PG) (Fig. 1a) is a C21 steroid hormone, also known as P4 (Pregn-4-ene-3, 20-dione). PG belongs to a class of hormones called progestogens and is the major naturally occurring human one (Al-Asmakh 2007). It is involved in control of ovulation, preparation and stabilization of the endometrium before implantation, regulation

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**Fig. 1** Chemical structure of (a) Progesterone, (b) 17-hydroxyprogesterone, (c) 21-hydroxyprogesterone, (d) Medroxyprogesterone, (e) Medroxyprogesterone acetate, (f) Dydrogesterone



of the implantation process, and maintenance of pregnancy (Olive 2002; Zhao et al. 2012). In addition to these reproductive effects, PG acts systemically to affect organs such as the breast, cardiovascular system, and central nervous system (Olive 2002; Sun et al. 2012). PG is used in menopausal hormone replacement therapy and reproductive control function (Skiba et al. 2006).

PG enhances  $\text{Ca}^{2+}$  and  $\text{K}^+$  uptake of membranes, prevents apoptosis of numerous cells through a direct non-genomic mechanism, and has sedative and anesthetic effects (Ehring et al. 1998; Soderpalm et al. 2004). Despite their importance, the studies on PG-membrane interaction are very limited (Carlson et al. 1983; Whiting et al. 2000; Liang et al. 2001; Korkmaz and Sevencan 2005; Korkmaz et al. 2005). They dealt mainly with membrane fluidity and aimed to determine the location of PG in membranes, which are not in agreement with each other.

PG derivatives, naturally occurring or synthesized, show a broad range of biological properties and their backbone substitutions can have an enormous impact on their specific activities (Zhang et al. 2008).

17-Hydroxyprogesterone (17-OHPG) (Fig. 1b) is a natural progestogen, which increases in the third trimester of pregnancy is primarily due to fetal adrenal production. It is derived from PG via 17-hydroxylase, a P450c17 enzyme, or from 17-hydroxypregnенolone via  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase (Dunn et al. 1981). In healthy men, it is synthesized as a precursor of testosterone in the testes. In healthy women, 17-OHPG is produced in small quantities as a precursor of estradiol in the ovary (Dunn et al. 1981). For the progestogenic activity the C17 position is of key importance. Progesterone loses its progestogenic activity with the introduction of the hydroxyl group at C17. While 17-OHPG is hormonally

inactive, its esterification on the one hand leads to weak progestogenic activity, while on the other hand esterification with caproate leads to a highly active progestin (Schindler et al. 2003).

21-Hydroxyprogesterone (21-OHPG) (Fig. 1c), also known as deoxycorticosterone, is produced by the adrenal gland. It possesses mineralocorticoid activity and is a precursor of aldosterone. It has no significant glucocorticoid activity. It is produced from PG by  $21\beta$ -hydroxylase and is converted to corticosterone by  $11\beta$ -hydroxylase (Wood 2006).

Orally administered, PG shows low plasma levels (De Ziegler 2000), as it is readily inactivated by the liver undergoing an extensive first-pass hepatic metabolism (De Ziegler 2000). Especially for this reason, synthetic progestins were developed. After oral administration, synthetic progestins are in general rapidly absorbed and reach a maximum serum concentration within 2–5 h, they have a longer half-life than PG and display stable plasma levels on long-term use (Schindler et al. 2003).

Medroxyprogesterone (MP) (Fig. 1d) is a progestin, which is commonly a component of hormonal contraceptives. MP, along with other progestins, was developed to allow the hormones to be taken orally.

After oral intake, medroxyprogesterone acetate (MPA) (Fig. 1e) does not undergo any first-pass effect. The bioavailability is nearly 100 %. MPA is bound to human serum albumin at 88 % (Schindler et al. 2003) and is essentially metabolized through hydroxylation (Schindler et al. 2003) at C6, C17 and C21.

Dydrogesterone (DYG) (Fig. 1f) is a retroprogesterone, a stereoisomer of PG, with an additional double bond between C6 and C7. The PG molecule is almost “flat”; the retroprogesterone molecule is bent by a change of the

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methyl group at C10 from the  $\beta$  to the  $\alpha$ -position and the H at C9 from the  $\alpha$  to the  $\beta$ -position. These changes in configuration make DYG metabolically stable and orally effective (Schindler et al. 2003). In human, DYG is metabolized by reduction at C20 to the  $20\alpha$ -hydroxy-derivative and by hydroxylation at the C21-methyl group and at the C16 $\alpha$ -position (Schindler et al. 2003). As DYG, DYG metabolites maintain the retrosteroid structure.

In the current study, we studied the effect of different concentrations of PG, 17-OHPG, 21-OHPG, MP, MPA, and DYG on zwitterionic dipalmitoyl phosphatidylcholine (DPPC) multilamellar liposomes (MLVs) using Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC), in order to understand the pharmacodynamics effect of steroids and to determine their functional groups, which can disturb the lipid membrane properties. We decided to choose different progesterone derivatives. The effect of the chemical structure on the steroid-bilayer interaction will be also described so that a structure activity relationship can be defined. To our knowledge, our study is the first one to investigate the interaction of a series of PG derivatives with lipidic bilayer membranes. The present work deals with the effect of the hydrophobicity and the chemical structure of the PG derivatives (Fig. 1) on their interaction with DPPC membranes.

## Materials and Methods

### Chemicals

DPPC was from Lipoid GmbH (Germany). PG, 21-OHPG, 17-OHPG, MP, MPA, DYG, and the organic solvents: chloroform and methanol were purchased from Sigma-Aldrich, France. The buffer reagent Trizma base was from Sigma-Aldrich, France, and HCl from VWR, France.

## Methods

### Preparation of Multilamellar Vesicles

MLVs were prepared by the thin film hydration technique described by Bangham et al. (1965). DPPC and PG were mixed in molar ratios of 100:0; 100:1; 100:2.5; 100:5; 100:10, and 100:25. DPPC (25 mg/ml) and PG were dissolved in a solvent mixture of chloroform/methanol 2:1 (v/v). The preparation was then evaporated in a rotary evaporator (BÜCHI Rotavapor R-124) at a temperature above the phase-transition temperature of the lipid (55 °C) to form a thin lipid film. The latter was then hydrated with

2 ml of Tris-HCl (pH 7.4, 0.1 M). The preparations were alternatively vortexed for 5 min and warmed in a water bath at 55 °C for 5 min. The cycle was repeated three times. The preparations in which DPPC and PG were at a molar ratio of 100:0 were considered as controls. For each molar ratio, three MLV batches were prepared.

### Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was used to study the effect of progesterone and progesterone derivatives on the frequency of CO and  $\text{PO}_2^-$  of the lipid head group and  $\text{CH}_2$  symmetric and antisymmetric of the lipid acyl chain. The experiments were performed at 28, 41 and 50 °C. FTIR spectra were obtained in the frequency range between 4000 and 400  $\text{cm}^{-1}$ , and were recorded on a JASCO 6300 spectrophotometer, Japan. FTIR samples were prepared in the form of potassium bromide pellets. Interferograms were averaged for 100 scans at 4  $\text{cm}^{-1}$  resolution. The water and the  $\text{CO}_2$  bands were subtracted.

### Differential Scanning Calorimetry

Thermal scans were obtained using a DSC Q200 scanning calorimeter (TA Instruments, France). The samples (10  $\mu\text{l}$ ) were encapsulated in hermetically sealed standard aluminum DSC pans. An empty pan was used for the reference during the analysis. Samples were run at a scan rate of 5 °C/min in a temperature range of 20–50 °C. DSC data were analyzed with the instrument software package. The measured parameters were as follows: the transition temperature ( $T_m$ ) determined from the maximum of the recorded heat capacity, transition enthalpy ( $\Delta H_m$ ) obtained from the area under the peak, the respective parameters for the pre-transition ( $T_p$  and  $\Delta H_p$ ), and the temperature width at half peak height,  $\Delta T_{1/2}$ , which measures the sharpness of the phase transition. This latter is very sensitive to the presence of any additives. It will be taken as a measure for the cooperativity of the transition.  $\Delta T_{1/2}$  is inversely proportional to the cooperativity (El Maghraby et al. 2005).

### Statistics

Values are expressed as mean  $\pm$  SD. The differences between the means of thermotropic parameters determined for liposomes loaded with PG or its derivatives and their corresponding controls were tested with *t* test. A *P* value  $< 0.05$  was accepted as the level of significance.

## Results

FTIR results obtained are presented below in Tables 1 and 2. Table 1 showed the frequency of the characteristic lipid functional groups C=O and PO<sub>2</sub><sup>-</sup> of the head phospholipid groups, while Table 2 presented the CH<sub>2</sub> symmetric and the bandwidth of the CH<sub>2</sub> antisymmetric stretching bands of normalized spectra in the absence or presence of the steroid hormones at 28, 41, and 50 °C.

The C=O stretching band was analyzed to examine the interaction of the investigated steroid hormones with

glycerol backbone near the head group of phospholipids in the interfacial region. According to the empirical rules, a decrease in the frequency implies either the strengthening of existing hydrogen bonding or formation of new hydrogen bonding between the components (Korkmaz et al. 2005).

In the gel and the liquid crystalline phases, the characteristic lipid peak C=O of DPPC glycerol backbone slightly shifted to higher frequency values in the presence of the steroid hormones (Table 1) suggesting a decrease in the hydrogen bond between the components of the system. Results are in agreement with published data for PG-DPPC

**Table 1** Frequency of the characteristic lipid functional groups in the absence or presence of the steroid hormones at 28, 41, and 50 °C

MLVs	28 °C		41 °C		50 °C	
	C=O	PO <sub>2</sub>	C=O	PO <sub>2</sub>	C=O	PO <sub>2</sub>
DPPC (100/0)	1636.19 ± 0.80	1231.11 ± 1.03	1638.44 ± 1.03	1238.71 ± 0.55	1637.26 ± 0.96	1234.54 ± 2.94
DPPC/PG						
DPPC/PG (100/1)	1637.59 ± 2.00	1222.00 ± 1.11*	1638.23 ± 1.67	1245.14 ± 4.01*	1638.23 ± 1.67	1235.18 ± 1.93
DPPC/PG (100/2.5)	1636.30 ± 0.96	1224.57 ± 0.96*	1638.23 ± 1.36	1232.61 ± 2.22*	1640.48 ± 2.42	1236.47 ± 0.55
DPPC/PG (100/5)	1641.44 ± 5.89	1228.43 ± 1.66*	1636.62 ± 1.47	1231.65 ± 0.55*	1637.59 ± 0.55	1235.50 ± 1.47
DPPC/PG (100/10)	1639.52 ± 2.00*	1238.4 ± 0.55*	1639.19 ± 1.36	1237.43 ± 3.65	1638.23 ± 1.67	1230.36 ± 0.96
DPPC/PG (100/25)	1637.91 ± 2.94	1232.61 ± 1.10	1637.91 ± 1.47	1232.29 ± 1.66*	1637.91 ± 1.47	1236.14 ± 0.96
DPPC/21-OHPG						
DPPC/21-OHPG (100/1)	1637.48 ± 0.66	1225.55 ± 2.51*	1638.23 ± 0.96	1225.54 ± 1.66*	1637.69 ± 1.21	1240 ± 1.36*
DPPC/21-OHPG (100/2.5)	1638.98 ± 1.88	1224.57 ± 1.66*	1637.80 ± 1.45	1221.68 ± 3.85*	1638.12 ± 0.98	1241.61 ± 1.47*
DPPC/21-OHPG (100/5)	1636.73 ± 1.64	1236.47 ± 2.42*	1638.55 ± 2.22	1222.88 ± 1.02	1637.58 ± 0.55	1228.80 ± 3.25
DPPC/21-OHPG (100/10)	1637.91 ± 1.11	1224.36 ± 2.91*	1638.33 ± 1.77	1233.57 ± 1.47*	1640.59 ± 1.82*	1222.80 ± 1.13*
DPPC/21-OHPG (100/25)	1637.58 ± 0.55*	1229.17 ± 3.70	1638.76 ± 1.21	1229.4 ± 2.72	1638.76 ± 3.72	1219.75 ± 3.85*
DPPC/17-OHPG						
DPPC/17-OHPG (100/1)	1638.71 ± 3.41	1237.94 ± 1.92*	1637.05 ± 1.61	1226.34 ± 1.13*	1638.12 ± 1.58	1227.71 ± 0.33*
DPPC/17-OHPG (100/2.5)	1636.94 ± 1.47	1231.61 ± 0.67	1637.37 ± 1.58	1230.36 ± 2.54 *	1637.05 ± 2.45	1236.14 ± 7.52
DPPC/17-OHPG (100/5)	1637.59 ± 1.47	1232.50 ± 2.00	1637.59 ± 1.47	1232.50 ± 3.21*	1638.33 ± 0.81	1238.80 ± 1.89
DPPC/17-OHPG (100/10)	1639.52 ± 1.47*	1233.86 ± 2.90	1637.05 ± 1.30	1229.72 ± 1.47*	1638.66 ± 3.03	1221.84 ± 1.82*
DPPC/17-OHPG (100/25)	1636.84 ± 1.33	1234.64 ± 2.94	1636.08 ± 0.66*	1222.65 ± 0*	1637.37 ± 0.18	1241.29 ± 0.55*
DPPC/MP						
DPPC/MP (100/1)	1639.52 ± 0.55*	1232.61 ± 0.55	1637.91 ± 1.47	1236.47 ± 2.00	1638.23 ± 1.67	1234.53 ± 1.11
DPPC/MP (100/2.5)	1640.48 ± 1.11*	1236.14 ± 1.66*	1635.98 ± 2.00	1239.04 ± 1.66	1642.41 ± 2.22*	1235.18 ± 0
DPPC/MP (100/5)	1636.30 ± 0.96	1230.68 ± 1.11	1637.59 ± 0.55	1232.29 ± 0.56*	1638.23 ± 0.96	1233.25 ± 0.96
DPPC/MP (100/10)	1637.91 ± 2.94	1229.39 ± 0.96	1637.59 ± 0.55	1234.86 ± 1.47*	1636.94 ± 2.00	1227.47 ± 0*
DPPC/MP (100/25)	1637.26 ± 1.67	1230.36 ± 0.96	1635.66 ± 1.47*	1235.82 ± 1.47*	1636.30 ± 0.96	1232.61 ± 1.47
DPPC/MPA						
DPPC/MPA (100/1)	1636.30 ± 0.96	1231.33 ± 0	1636.94 ± 1.11	1227.97 ± 0.55*	1636.30 ± 0.96	1241.61 ± 2.94*
DPPC/MPA (100/2.5)	1636.94 ± 1.11	1229.4 ± 1.93	1638.87 ± 0.56	1238.71 ± 1.47	1641.44 ± 4.75*	1238.39 ± 3.38
DPPC/MPA (100/5)	1637.91 ± 1.11	1227.14 ± 1.47*	1638.87 ± 1.11	1240.64 ± 1.11*	1637.91 ± 0.55	1242.89 ± 1.66*
DPPC/MPA (100/10)	1637.91 ± 2.42	1228.75 ± 1.11*	1636.63 ± 1.11	1235.50 ± 1.47*	1638.23 ± 0.96	1236.79 ± 1.11
DPPC/MPA (100/25)	1636.30 ± 0.96	1230.04 ± 1.11	1637.26 ± 0.96	1250.93 ± 0.56*	1641.45 ± 2.42*	1245.78 ± 1.66*
DPPC/DYG						
DPPC/DYG (100/1)	1638.44 ± 3.40	1234.06 ± 2.95	1636.73 ± 1.65	1233.74 ± 3.40	1640.05 ± 3.61	1226.18 ± 2.22*
DPPC/DYG (100/2.5)	1638.66 ± 2.14	1232.18 ± 0.97	1638.76 ± 1.64	1223.61 ± 4.82*	1639.09 ± 2.09	1219.27 ± 2.04*
DPPC/DYG (100/5)	1635.87 ± 0.92	1235.18 ± 1.66*	1637.48 ± 1.52	1239.68 ± 3.38	1636.84 ± 0.74	1223.13 ± 0.67*
DPPC/DYG (100/10)	1636.94 ± 0.45	1233.73 ± 2.04	1636.51 ± 1.03*	1224.25 ± 3.09*	1640.91 ± 3.83	1227.46 ± 1.36*
DPPC/DYG (100/25)	1638.12 ± 0.18*	1232.61 ± 2.00	1637.91 ± 1.11	1223.93 ± 2.00*	1640.27 ± 3.34	1228.75 ± 2.00*

Values are expressed as the means of three repetitions ± SD

\* P < 0.05 compared with the control value

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**Table 2** Frequency of the characteristic lipid functional groups and bandwidth of the CH<sub>2</sub> antisymmetric stretching bands in the absence or presence of the steroid hormones at 28, 41, and 50 °C

MLVs	28 °C		41 °C		50 °C	
	Bandwidth (cm <sup>-1</sup> )	Wavenumber CH <sub>2</sub> Symm (cm <sup>-1</sup> )	Bandwidth (cm <sup>-1</sup> )	Wavenumber CH <sub>2</sub> Symm (cm <sup>-1</sup> )	Bandwidth (cm <sup>-1</sup> )	Wavenumber CH <sub>2</sub> Symm (cm <sup>-1</sup> )
DPPC (100/0)	21.58 ± 0.91	2846.41 ± 0.96	20.50 ± 0.77	2847.81 ± 0.49	19.57 ± 0.31	2848.34 ± 0.96
DPPC/PG						
DPPC/PG (100/1)	25.23 ± 1.66*	2847.06 ± 0.55	20.47 ± 3.50	2847.57 ± 0.69	20.48 ± 0.98	2848.34 ± 0.96
DPPC/PG (100/2.5)	22.41 ± 2.89	2847.70 ± 1.47	20.92 ± 1.83	2847.70 ± 1.47	18.42 ± 1.47	2851.24 ± 1.93
DPPC/PG (100/5)	16.78 ± 1.50*	2848.67 ± 0.55*	21.80 ± 1.00	2847.70 ± 0.56	19.69 ± 1.11	2848.34 ± 0.96
DPPC/PG (100/10)	20.45 ± 0.99	2848.34 ± 0.96*	22.22 ± 0.40*	2848.98 ± 1.47	19.61 ± 1.23	2848.66 ± 1.47
DPPC/PG (100/25)	21.33 ± 0.67	2848.99 ± 1.10*	19.31 ± 1.30	2847.70 ± 1.11	19.84 ± 0.12	2848.67 ± 0.55
DPPC/21-OHPG						
DPPC/21-OHPG (100/1)	19.89 ± 1.54	2846.41 ± 1.67	21.45 ± 1.33	2846.41 ± 0.96	17.63 ± 1.40	2849.52 ± 2.18
DPPC/21-OHPG (100/2.5)	21.53 ± 1.42	2847.38 ± 0.96	19.94 ± 0.43	2848.13 ± 1.03	20.80 ± 1.77	2848.98 ± 1.47
DPPC/21-OHPG (100/5)	20.93 ± 1.62	2846.52 ± 1.13	21.20 ± 0.76	2848.02 ± 0.56	18.30 ± 0.88	2848.02 ± 2.00
DPPC/21-OHPG (100/10)	20.48 ± 3.11	2847.05 ± 1.47	20.77 ± 0.4	2847.70 ± 0.55	16.75 ± 2.71	2848.66 ± 1.47
DPPC/21-OHPG (100/25)	18.87 ± 2.76	2848.13 ± 1.03	21.16 ± 0.80	2848.45 ± 0.80	19.55 ± 0.13	2849.41 ± 1.58
DPPC/17-OHPG						
DPPC/17-OHPG (100/1)	20.94 ± 1.75	2847.13 ± 1.29	19.68 ± 1.99	2847.06 ± 0.55	19.13 ± 0.54	2849.52 ± 0.66
DPPC/17-OHPG (100/2.5)	18.94 ± 0.88*	2847.27 ± 0.98	18.70 ± 1.90	2847.81 ± 0.92	19.89 ± 0.41	2848.24 ± 0.81
DPPC/17-OHPG (100/5)	19.80 ± 0.43*	2847.91 ± 0.49*	20.60 ± 1.72	2847.49 ± 0.81	19.68 ± 0.64	2848.98 ± 0.55
DPPC/17-OHPG (100/10)	18.01 ± 0.22*	2847.81 ± 1.21	19.86 ± 2.40	2848.24 ± 0.18	20.03 ± 1.58	2849.73 ± 0.92
DPPC/17-OHPG (100/25)	19.92 ± 1.05	2848.13 ± 1.29	20.86 ± 0.74	2848.77 ± 0.92	19.05 ± 0.34	2849.52 ± 1.29
DPPC/MP						
DPPC/MP (100/1)	17.60 ± 1.65*	2847.70 ± 0.56	20.56 ± 1.55	2848.35 ± 0	19.57 ± 0.77	2848.67 ± 0.55
DPPC/MP (100/2.5)	20.53 ± 0.53	2847.70 ± 0.56	20.46 ± 1.70	2848.35 ± 0	18.84 ± 0.40*	2849.31 ± 0
DPPC/MP (100/5)	21.40 ± 1.79	2848.02 ± 0.56*	22.32 ± 1.97	2848.34 ± 0.96	18.90 ± 1.55	2848.02 ± 0.56
DPPC/MP (100/10)	18.27 ± 1.07*	2848.34 ± 1.92	20.58 ± 0.84	2848.02 ± 0.56	19.78 ± 1.31	2848.34 ± 0.96
DPPC/MP (100/25)	19.11 ± 1.36*	2848.35 ± 0*	22.44 ± 1.88	2847.70 ± 0.56	19.74 ± 0.33	2848.67 ± 0.55
DPPC/MPA						
DPPC/MPA (100/1)	23.74 ± 0.08*	2846.41 ± 0.96	19.51 ± 0.93	2847.70 ± 0.56	19.58 ± 0.44	2848.67 ± 0.55
DPPC/MPA (100/2.5)	22.65 ± 0.58	2846.74 ± 0.55	20.04 ± 0.57	2847.70 ± 0.56	21.40 ± 0.42*	2848.67 ± 0.55
DPPC/MPA (100/5)	22.65 ± 1.05	2846.74 ± 0.55	20.78 ± 0.07	2848.02 ± 1.47	20.40 ± 1.22	2849.31 ± 0
DPPC/MPA (100/10)	23.84 ± 1.56	2846.74 ± 0.55	21.19 ± 2.29	2847.70 ± 1.47	20.56 ± 0.73	2848.67 ± 0.55
DPPC/MPA (100/25)	22.61 ± 1.06	2846.74 ± 0.55	19.73 ± 0.94	2848.02 ± 0.56	19.58 ± 1.05	2848.67 ± 0.55
DPPC/DYG						
DPPC/DYG (100/1)	20.07 ± 0.64*	2847.91 ± 0.92	18.51 ± 0.47*	2849.41 ± 1.77	21.15 ± 1.62	2848.23 ± 0.98
DPPC/DYG (100/2.5)	20.93 ± 1.29	2848.13 ± 1.03	16.96 ± 1.24*	2847.70 ± 1.47	19.11 ± 0.93	2848.13 ± 1.03
DPPC/DYG (100/5)	20.57 ± 0.51	2847.17 ± 1.29	18.41 ± 0.84*	2848.88 ± 0.70	20.01 ± 0.65	2847.81 ± 0.49
DPPC/DYG (100/10)	20.82 ± 1.17	2848.34 ± 0.96*	19.13 ± 1.68	2848.66 ± 1.11	19.59 ± 1.53	2848.02 ± 0.55
DPPC/DYG (100/25)	20.93 ± 0.54	2848.56 ± 1.03*	17.49 ± 0.37*	2849.41 ± 0.80*	19.10 ± 0.80	2848.67 ± 2.42

Values are expressed as the means of three repetitions ± SD

\* P &lt; 0.05 compared with the control value

interaction using FTIR as a technique (Korkmaz et al. 2005; Korkmaz and Severcan 2005). Results suppose that the steroid hormones displaced some H<sub>2</sub>O molecules from the interfacial region resulting in an increase in the number of free carbonyl groups. However, at the transition phase, all the steroids shifted the peak position to lower frequency values (Table 1) suggesting a strengthening of the

hydrogen bonds or even a formation of new hydrogen bonds between the components. We suggest that in both cases (increase or decrease of the peak position), the steroid hormones disturbed the glycerol backbone.

Besides, the interaction between the selected steroid hormones and the head group of the lipid was investigated by the observation of the PO<sub>2</sub><sup>-</sup> antisymmetric double

stretching band at  $1230\text{ cm}^{-1}$  (Table 1). The frequencies of the  $\text{PO}_2^-$  stretching modes are sensitive to the state of hydration of phospholipid bilayers and hydrogen bonding interactions at the surfaces of hydrated phospholipid assemblies. It can be seen that the frequency shifted to lower and higher values by the presence of the selected steroid hormones in the gel, transition, and liquid phases suggesting interactions of these molecules with the head group of the lipid bilayer. Dehydration results in band shifts of  $\text{PO}_2^-$  antisymmetric towards higher wavenumbers (Kazanci et al. 2001), while a strengthening of the hydrogen bonding interactions with water results in band shifts towards lower wavenumbers (Lewis and McElhaney 1998).

Table 2 showed the bandwidth of the  $\text{CH}_2$  antisymmetric and the frequency of  $\text{CH}_2$  symmetric stretching bands at 28, 41, and  $50\text{ }^\circ\text{C}$ .

On the overall, the steroid hormones did not affect significantly the C–H stretching modes at  $2800\text{--}3000\text{ cm}^{-1}$  for 28, 41, and  $50\text{ }^\circ\text{C}$ . Increases in hydrocarbon chain conformational disorder are accompanied by increases in the frequencies of infrared absorption bands arising from the symmetric stretching vibrations of the methylene groups on lipid hydrocarbon chains (Lewis and McElhaney 1998) leading to an increase in the number of gauche conformers and in the disorder of the bilayer (Boyar and Sevencan 1997).

At the gel phase, PG and DYG increased significantly the frequency of the C–H stretching mode at three and two highest molar ratios, respectively. 17-OHPG at molar ratio of 5 and MP at molar ratios of 5 and 25 produced similar effect.

At the transition and the liquid crystalline phases, none of the studied steroids affected significantly the C–H stretching mode. Exception can be observed in the liquid crystalline phase for DYG at the highest molar ratio. At the transition phase and compared to control, the bandwidth of the  $\text{CH}_2$  antisymmetric of DPPC-PG, DPPC-MP, and DPPC-DYG varied remarkably. PG at molar ratio 100:10 increased in the significant way the membrane dynamics, while DYG had a remarkable stabilizing effect at all molar ratios. At the liquid crystalline phase, MPA at a molar ratio 100:2.5 increased the membrane dynamics, while MP produced the contrary effect at the same molar ratio.

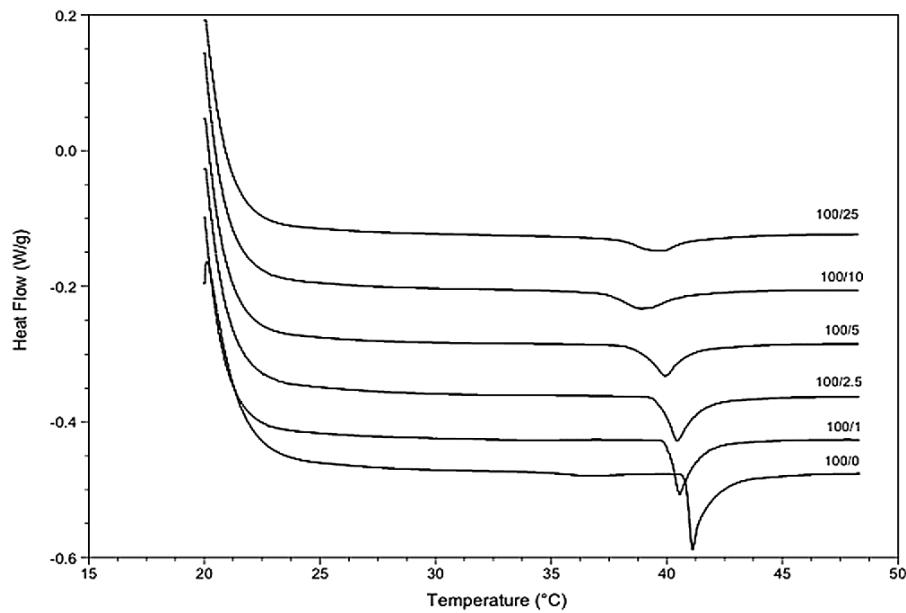
A slight increase in the frequency of C–H stretching mode of the lipid acyl chain was reported for estradiol ( $\text{E}_2$ ) loaded DPPC and DMPC membrane at 28 and 50 (Boyar and Sevencan 1997; Dicko et al. 1999).

Besides, the increase of the bandwidth of the  $\text{CH}_2$  stretching bands measured at 50 % of the peaks height is the indication of an increase in membrane dynamics (Korkmaz and Sevencan 2005; Korkmaz et al. 2005).

At the gel phase, 21-OHPG, 17-OHPG, MP, and DYG tend to stabilize the bilayer while a contrary effect was observed for MPA and PG. Our results are in accordance with (Korkmaz and Sevencan 2005; Korkmaz et al. 2005) regarding the effect of PG on the membrane dynamics.

Based on FTIR results, it is obvious that the steroid hormones interact with glycerol backbone and the head groups of the DPPC at the investigated temperatures (Table 1) and are able to modulate the membrane dynamics.

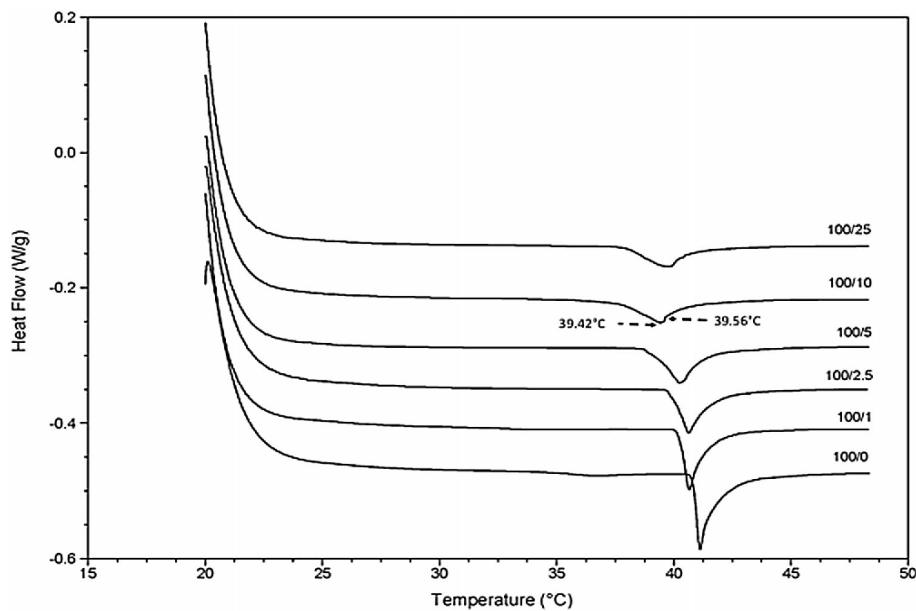
**Fig. 2** DSC scans of DPPC:PG liposomes at molar ratios of 100:0; 100:1; 100: 2.5; 100:5; 100:10; and 100:25



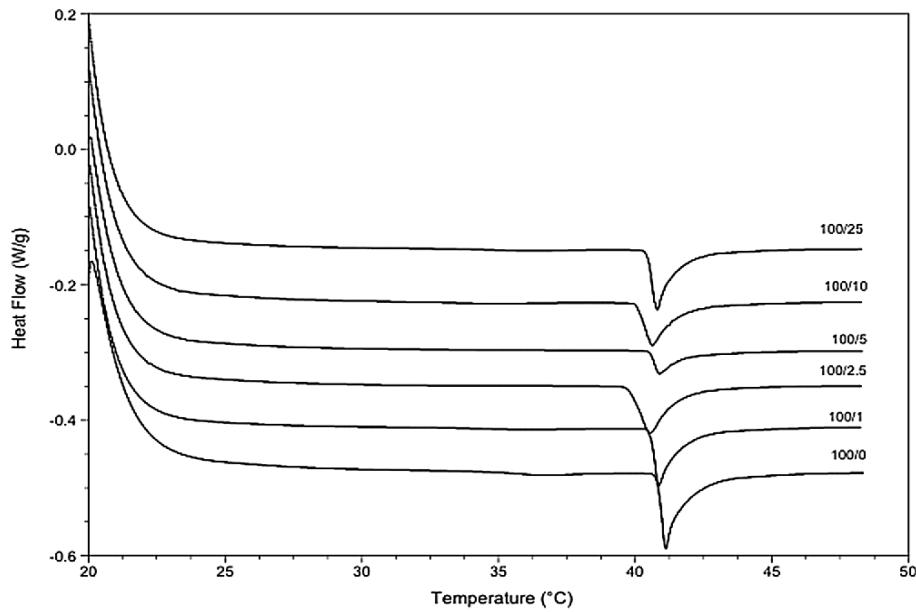
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**Fig. 3** DSC scans of DPPC:21-OHPG liposomes at molar ratios of 100:0; 100:1; 100:2.5; 100:5; 100:10; and 100:25



**Fig. 4** DSC scans of DPPC and DPPC:17-OHPG liposomes at molar ratios of 100:0; 100:1; 100:2.5; 100:5; 100:10; and 100:25



Figures 2, 3, 4, 5, 6, and 7 show the DSC curves of DPPC:PG liposomes containing, respectively, PG, 21-OHPG, 17-OHPG, MP, MPA, and DYG in molar ratios of 100:0; 100:1; 100:2.5; 100:5; 100:10; and 100:25.

Table 3 summarizes the calorimetric parameters, which displays the average of three independent experiments. Blank liposomes displayed a low enthalpic pre-transition

( $L_{\beta'}$  to  $P_{\beta'}$ ) at  $36.77 \pm 0.12$  °C and a sharp main transition ( $P_{\beta'}$  to  $L_{\alpha}$ ) at  $41.06 \pm 0.06$  °C. The enthalpy of the main transition was much higher ( $\approx 45$  J/g) than that of the pre-transition ( $\approx 0.41$  J/g). The sharpness of the main transition peak obtained for DPPC liposomes with  $\Delta T_{1/2}$  value of 0.59 °C is an indication of the purity of the system and a highly cooperative transition.

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**Table 3** DSC data of DPPC vesicles incorporating steroids

MLV	$T_m$ (°C)	$\Delta H_m$ (J/g)	$T_p$ (°C)	$\Delta H_p$ (J/g)	$\Delta T_{1/2}$ (°C)
DPPC (100/0)	41.06 ± 0.06	45.47 ± 0.41	36.77 ± 0.12	0.41 ± 0.41	0.59 ± 0.03
DPPC/PG					
DPPC/PG (100/1)	40.70 ± 0.01*	36.98 ± 0.41			0.84 ± 0.00*
DPPC/PG (100/2.5)	40.36 ± 0.24*	38.85 ± 0.41			1.01 ± 0.00*
DPPC/PG (100/5)	40.03 ± 0.09*	30.06 ± 0.41*			1.29 ± 0.00*
DPPC/PG (100/10)	38.94 ± 0.12*	25.02 ± 0.82*			1.98 ± 0.06*
DPPC/PG (100/25)	30.90 ± 0.12*	23.61 ± 2.46*			1.76 ± 0.05*
DPPC/21-OHPG					
DPPC/21-OHPG (100/1)	40.70 ± 0.02*	41.74 ± 4.92			0.81 ± 0.01*
DPPC/21-OHPG (100/2.5)	40.55 ± 0.04*	36.52 ± 1.23			1.05 ± 0.01*
DPPC/21-OHPG (100/5)	40.39 ± 0.41*	45.1 ± 9.02			1.22 ± 0.01*
DPPC/21-OHPG (100/10)	39.58 ± 0.06*	28.94 ± 0.82*			1.59 ± 0.02*
DPPC/21-OHPG (100/25)	39.67 ± 0.12*	32.6 ± 0.41			1.75 ± 0.09*
DPPC/17-OHPG					
DPPC/17-OHPG (100/1)	40.55 ± 0.47*	38.16 ± 2.05			0.9 ± 0.34*
DPPC/17-OHPG (100/2.5)	40.56 ± 0.13*	42.23 ± 2.46			0.85 ± 0.14*
DPPC/17-OHPG (100/5)	40.83 ± 0.09*	21.97 ± 6.56*			0.74 ± 0.05*
DPPC/17-OHPG (100/10)	40.75 ± 0.12*	33.62 ± 3.64			0.77 ± 0.12*
DPPC/17-OHPG (100/25)	41.41 ± 1.07	31.98 ± 3.69*			0.7 ± 0.07*
DPPC/MP					
DPPC/MP (100/1)	40.89 ± 0.07	39.6 ± 5.33			0.84 ± 0.04*
DPPC/MP (100/2.5)	40.77 ± 0.11*	41.69 ± 0.32			0.89 ± 0.07*
DPPC/MP (100/5)	40.92 ± 0.16	36.49 ± 1.35			0.94 ± 0.09*
DPPC/MP (100/10)	40.93 ± 0.17	38.54 ± 2.46			0.94 ± 0.12*
DPPC/MP (100/25)	40.81 ± 0.11*	39.77 ± 0.41			0.85 ± 0.02*
DPPC/MPA					
DPPC/MPA (100/1)	40.83 ± 0.03*	38.95 ± 3.28			0.73 ± 0.06*
DPPC/MPA (100/2.5)	40.62 ± 0.13*	34.44 ± 3.15			0.95 ± 0.06*
DPPC/MPA (100/5)	40.67 ± 0.09*	30.75 ± 3.15*			0.98 ± 0.1*
DPPC/MPA (100/10)	40.88 ± 0.24	31.98 ± 2.05*			0.95 ± 0.26*
DPPC/MPA (100/25)	40.79 ± 0.1*	30.75 ± 5.74*			0.77 ± 0.1*
DPPC/DYG					
DPPC/DYG (100/1)	40.81 ± 0.11*	37.35 ± 0.41			0.82 ± 0.03*
DPPC/DYG (100/2.5)	40.85 ± 0.14	40.55 ± 2.46			0.81 ± 0.1*
DPPC/DYG (100/5)	40.67 ± 0.07*	33.34 ± 2.46			0.84 ± 0.04*
DPPC/DYG (100/10)	40.57 ± 0.13*	36.08 ± 1.23			0.93 ± 0.08*
DPPC/DYG (100/25)	40.53 ± 0.01*	42.92 ± 0.73			0.93 ± 0.00*

Values are expressed as the means of three repetitions ± SD

\*  $P < 0.05$  compared with the control value**Progesterone**

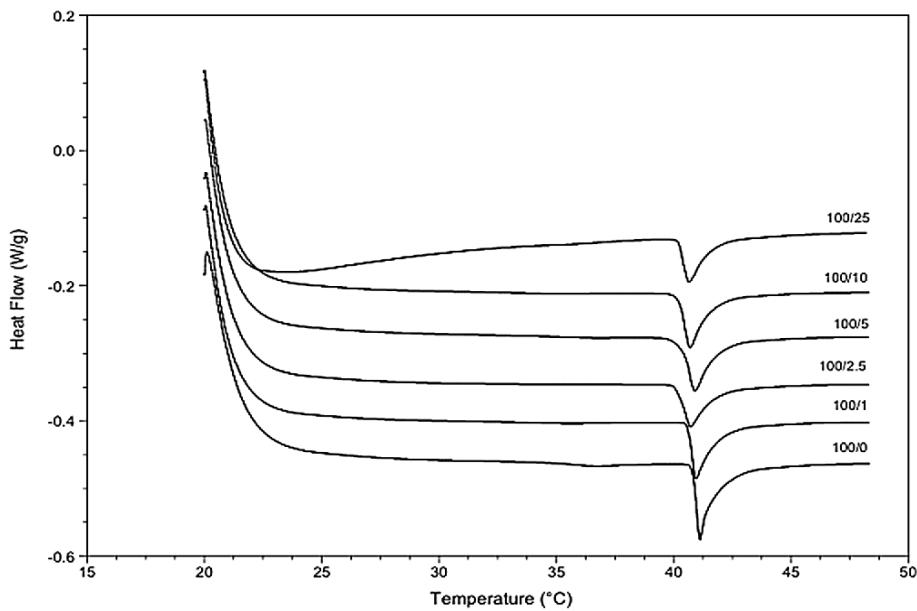
With the addition of PG, the main phase-transition temperature shifted to the lower values, and the pre-transition completely diminished at all PG concentrations (Fig. 2). Furthermore, the addition of PG broadened the transition peaks at all molar ratios. The decrease in  $T_m$  and  $\Delta H_m$  with the addition of PG was in a concentration-dependent

manner. Those findings were validated by the measurement of  $\Delta T_{1/2}$  showing a clear increase versus PG concentration (Table 3).

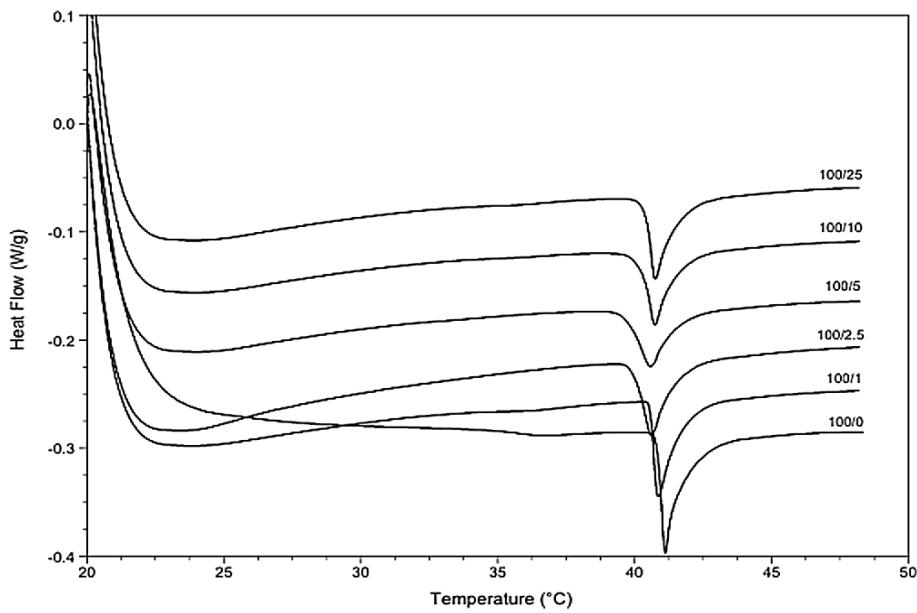
**21-Hydroxyprogesterone**

The incorporation of 21-OHPG in the lipid membrane abolished the pre-transition peak (Fig. 3), reduced the

**Fig. 5** DSC scans of DPPC and DPPC:MP liposomes at molar ratios of 100:0; 100:1; 100: 2.5; 100:5; 100:10; and 100:25



**Fig. 6** DSC scans of DPPC and DPPC:MPA liposomes at molar ratios of 100:0; 100:1; 100: 2.5; 100:5; 100:10; and 100:25

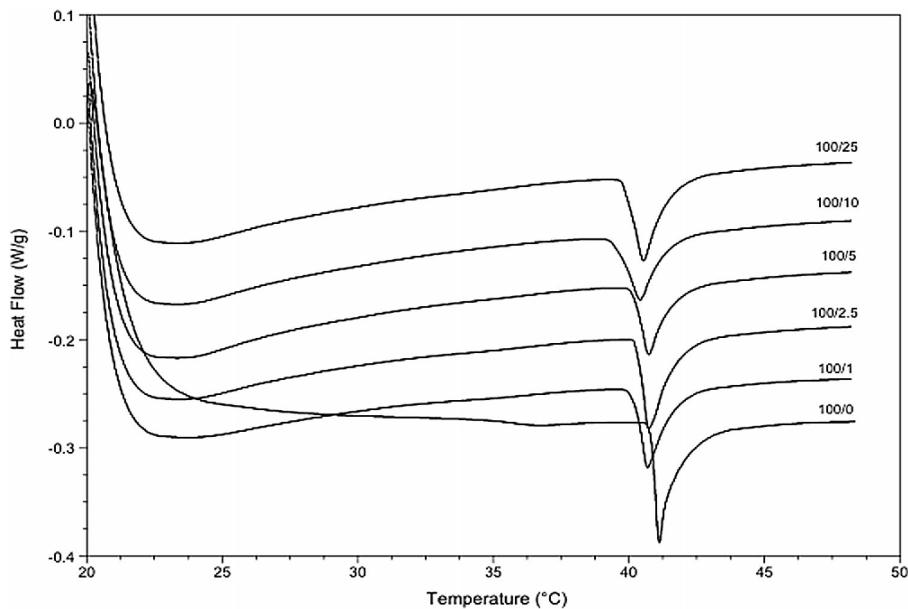


temperature and the enthalpy of the main phase transition, broadened the main transition peak, and induced the splitting of the main calorimetric peak into two peaks at the molar ratio 100:10 ( $T_{m1}$  39.42 and  $T_{m2}$  39.56 °C). Besides, an increase of  $\Delta T_{1/2}$  in a concentration-dependent manner is obtained (Table 3).

### 17-Hydroxyprogesterone

Similarly to PG and 21-OHPG, the addition of 17-OHPG abolished the pre-transition peak and shifted the peak of the main transition to lower values at molar ratios of 100:1; 100:2.5; 100:5; and 100:10 (Fig. 4). No shoulders were

**Fig. 7** DSC scans of DPPC and DPPC:DYG liposomes at molar ratios of 100:0; 100:1; 100:2.5; 100:5; 100:10; and 100:25



observed in the main transition peak. Besides, the decrease in  $T_m$  and  $\Delta H_m$  with the addition of 17-OHPG was not concentration dependent (Table 3).

#### Medroxyprogesterone

Only at molar ratios of 100:2.5 and 100:25, the addition of MP significantly changed the main transition temperature ( $T_m$ ), (Fig. 5). The enthalpy of the main transition of DPPC bilayers was not affected at all molar ratios. Compared to pure liposomes, the pre-transition was abolished and  $\Delta T_{1/2}$  increased (Table 3).

#### Medroxyprogesterone Acetate

Compared to the pure liposomes, the main phase-transition temperature ( $T_m$ ) shifted to lower values in the presence of MPA (Fig. 6; Table 3). Besides, the enthalpy of the main phase transition showed a significant decrease at molar ratios of 100:5; 100:10; and 100:25. A disappearance of the pre-transition peak and an increased of the  $\Delta T_{1/2}$  were also observed (Fig. 6; Table 3).

#### Dydrogesterone

Compared to pure liposomes, the presence of DYG in the liposome formulation produced a significant decrease of the main phase  $T_m$  at molar ratios of 100:1; 100:5; 100:10; and 100:25 (Fig. 7). The enthalpy of the main transition  $\Delta H_m$  was not affected by the DYG addition at the different

molar ratios, whereas the pre-transition temperature  $T_p$  was abolished and  $\Delta T_{1/2}$  increased (Fig. 7; Table 3).

## Discussion

### Localization of the Interaction

The DSC thermogram of DPPC liposomes demonstrates two transitions: the pre-transition, which corresponds to the conversion of a lamellar gel phase to a rippled gel phase, is mainly related to the polar region of phospholipids and the main phase transition which reflects the change from a rippled gel phase to a liquid-crystal phase and is closely related to the acyl chains of phosphatidylcholine bilayers. The main transition can be used to probe the interaction between the acyl chains of phosphatidylcholine and exogenous substances. Moreover, the width of its DSC peak is an index of the cooperativity of this conversion: the narrower the peak, the higher the cooperativity (Cong et al. 2009). Accordingly with literature (Biruss et al. 2007), for DPPC liposomes, the pre-transition is a flat endothermic peak and the main transition represents a sharp, intense endotherm (Fig. 2). The location of the molecules within the liposomes can be classified into three categories: the strongly hydrophilic ones, which are localized in the aqueous domains of liposomes and can exert some interaction with the head groups, the less hydrophilic molecules that adsorb at the water-lipid bilayer interface with some degree of penetration into the bilayer, and the strongly lipophilic drugs, which locate in the bilayer

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itself (El Maghraby et al. 2005). Any compound that interacts with the head groups will affect the pre-transition (Heimburg 2000) and the main transition is a consequence of the chain melting which can be affected by the incorporation of a drug inside the lipid bilayers (El Maghraby et al. 2005).

Our results showed the disappearance of the pre-transition peak upon addition of PG and PG derivatives even at low concentration (100:1). This phenomenon is associated with an interaction of these molecules with the polar head groups of phospholipids, in agreement with published data for some steroid molecules such as dexamethasone (Bhardwaj and Burgess 2010), beclomethasone dipropionate (Elhissi et al. 2006), and PG (Korkmaz and Severcan 2005). Similar results were reported for other triterpene molecules such as cucurbitacin E (Habib et al. 2013), oleanolic acid, and its derivatives maslinic and ursolic acids (Prades et al. 2011).

Incorporation of PG into DPPC liposomes causes simultaneous decrease in  $T_m$  and  $\Delta H_m$  values in a concentration-dependent manner. PG decreased the main-phase transition temperature (around 41 °C) and broadened the peak. The broadening of peak profile and lowering transition temperature demonstrate that both the size and packing of bilayers are modified, and the system is disordered (Jain and Wu 1977). Due to its lipophilic nature, PG molecules were incorporated into lipid bilayers, disturbing the strong hydrophobic interactions between the lipid molecules. In literature, there is confliction about the location of PG in the membrane. Using DSC, Carlson et al. (1983) suggested that PG associates mainly with the hydrophilic part of the lipidic membrane. While Sanchez-Bueno (1991) proved by NMR study that the PG molecules are rather localized near a double bound or ester moiety in lipid molecules.

21-OHPG is also found to decrease the  $T_m$  in a significant way at all molar ratios and the enthalpy of the main phase transition. But this diminution was not concentration dependent (Table 3). A splitting of the peak, observed clearly at molar ratio of 10, suggested a phase separation and incorporation into lipid bilayers. Two transitions occur: a first transition near  $T_m$  of MLV control and a second transition at a temperature below  $T_m$ . We suggest that the peak observed at lower temperature most likely represent the transition of 21-OHPG rich domains, while the peak located at higher temperature may arise from 21-OHPG poor domains.

Compared to PG and 21-OHPG, the effect of MPA on the main transition enthalpy and the main transition temperature value were weaker suggesting a moderate insertion in the bilayers.

Whereas, 17-OHPG did not show a significant decrease in the main transition enthalpy; suggesting that it is not incorporated in the bilayer. This fact could be due to the hydroxyl group on C17, which makes this molecule more polar than PG. But its interaction with the hydrophobic region of lipid

bilayers cannot be excluded in view of the significant decrease in the transition enthalpy at 5 and 25 mol%.

Moreover, MP and DYG did not produce significant decreases in the main transition enthalpy or formed separate domains in the bilayer. They are rather localized near the polar heads of the phospholipids at the interface of the lipid bilayer. Additionally, other oxygenated triterpenes oleanolic, maslinic, and ursolic were also proved to be located close to the lipid head groups at the bilayer interface (Prades et al. 2011).

In the present study,  $\Delta T_{1/2}$  increased with PG and its derivatives compared to pure DPPC liposomes at all molar ratios, indicating a disorder of the bilayers. These findings suggest that PG and its derivatives formed separate domains within the bilayers, which caused non-ideal mixing, and phase separation between phospholipid and steroids in agreement with published data for some steroid molecules such as beclomethasone dipropionate (Elhissi et al. 2006). This phase separation of lipids probably produced phases with different ratios of steroid to phospholipid suggesting the co-existence of more than one domain (steroid rich domain and steroid poor domain) (Korkmaz and Severcan 2005). In addition, we can assume that our tested molecules destabilize or disorder the bilayers due to the conjugated 3-Keto group, as been demonstrated by Gallay and De Kruijff (1984). Reinforcing this finding, all steroids possessing a Keto at C3, a double bond at C4, and substituents at C17 are known to display membrane-disrupting activity (Wenz 2012).

## Fluidity

The membrane fluidity is a physicochemical feature of biomembranes and is an important factor in modulating the cell rheological behavior (Tsuda et al. 2002). The effect of PG on membrane fluidity of lipid bilayers or erythrocytes was assessed in many studies using various techniques including electron paramagnetic resonance, FTIR, turbidimetry, and DSC.

Korkmaz et al. (2005) and Korkmaz and Severcan (2005) have determined the effect of PG on fluidity of DPPC liposomes using FTIR and turbidimetry. An increase in bandwidth of  $\text{CH}_2$  antisymmetric stretching modes of DPPC liposomes is the indication of an increase in membrane dynamics (Korkmaz et al. 2005) and a decrease in turbidimetry reflects an easier transition of membranes from the gel phase to the liquid-crystalline phase (increase in fluidity). Using various PG concentrations, the authors concluded that PG can modulate the membrane dynamics. In another study using electron paramagnetic resonance, Tsuda et al. (2002) showed that PG increased the fluidity of human erythrocytes membranes. Compared to blank DPPC liposomes, the variation of the bandwidth of the  $\text{CH}_2$

antisymmetric of DPPC-PG at 28 and 50 °C which monitor the gel and liquid-crystalline phases (Table 2), and the decrease in the  $T_m$  and  $\Delta H_m$  values suggest that this hormone increases the membrane fluidity.

Also, 21-OHPG showed a decrease of the  $T_m$  and of the enthalpy of the main transition, in a significant way at all molar ratio, indicating an increase in fluidity. Whereas the decrease in the main transition parameters ( $T_m$  and  $\Delta H_m$ ) was not concentration dependent for 17-OHPG, indicating a very slight effect on fluidity, and suggests that the added amount does not imply an additional effect on the acyl chains. 17-β-estradiol with a hydroxyl function at C17 induces an important disorder and perturbations in liposomes membrane and thus an increase in membrane fluidity. According to the authors, this effect could not be due to an interaction of the hydroxyl group at C17 with an electronegative group of phospholipids (the oxygen atoms of C=O and PO<sub>2</sub><sup>-</sup> groups) but rather to the presence of the highly hydrophilic hydroxyl at C17 of estradiol in contact with the hydrophobic chains of phospholipids. This is in agreement with our results showing an increase in membrane fluidity upon addition of PG having a hydrophilic carbonyl in contact with the lipid acyl chains. The presence in 17-OHPG, MP, and MPA structures of an oxygen atom at position C20 very close to the 17-hydroxy group may favor an intramolecular hydrogen bond and thus deplete the effect of these compounds on fluidity. Our results showed a more important effect of 21-OHPG than 17-OHPG on fluidity, suggesting that the formation of the intramolecular hydrogen bond in 21-OHPG is less favored due to the increased distance between the hydroxyl group and the oxygen atom at position C21. So the intramolecular interactions between the carbonyl and hydroxyl group of 17-OHPG and MPA, and to a smaller extent in 21-OHPG may mask the presence of the highly hydrophilic carbonyl moiety in PG, even with the presence of two hydrophilic moiety instead of one, close to cyclopentane in 17-OHPG and 21-OHPG in comparison with PG; the interaction of the carbonyl group with the hydroxyl may change the electron distribution of these groups and mask the partial charges present at the hydrogen atom (+δ) of the hydroxyl and the oxygen atom (-δ) of the carbonyl leading to a less perturbation than that exerted by the carbonyl alone in PG or the hydroxyl alone in 17-β-estradiol. Gallay and De Kruijff (1984) reported that 3-Keto group (PG) promotes a destabilization of the bilayers compared to the 3β-OH bearing-compounds (pregnenolone) and that the hydroxylation of PG at C21 leads to a destabilization of the bilayers, while the hydroxylation at C17 cancels out the destabilization effect (Gallay and De Kruijff 1984).

As far as for MPA, the decrease in the main transition parameters ( $T_m$  and  $\Delta H_m$ ) is not concentration dependent

which indicates a slight effect on fluidity, compared to PG and 21-OHPG. The presence of an acetyl function may produce a steric effect that prohibits the incorporation of MPA inside the bilayers.

However, the main transition parameters ( $T_m$  and  $\Delta H_m$ ) were not affected by MP and DYG, suggesting their lower impact on fluidity. Similarly to 17-OHPG, and as described above, the intramolecular hydrogen bond might deplete the effect of MP on fluidity. Whereas, the inverted configuration at C9 and C10 and the additional C6-C7 double bond are the two structural features, which differentiate DYG from PG (Colombo et al. 2006). A bent shape has been suggested for DYG while PG has an almost planar geometry (Colombo et al. 2006). The C6-C7 double bond constricts the DYG molecule into a rigid conformation suitable for a selective interaction with the PG receptor, while PG is less selective as it exists in different conformations and may adapt more easily to the requisites of different receptors (Colombo et al. 2006). We suggest that the C6-C7 double bond could be the reason behind the lower effects of DYG on fluidity and on its incorporation in the bilayers.

## Hydrophobicity

Compared 17-β-estradiol which proved weak effect on membrane fluidity, Liang et al. (2001) proved that E-3-ol and 17-β-estradiol octyl ether which are more hydrophobic are able to decrease DPPC liposomes fluidity. For the present series, the hydrophobicity is probably not the main factor as the logP values of PG, DYG, 17-OHPG, 21-OHPG, MP, and MPA are, respectively, 4.04 (Torres-Cartas et al. 2000), 3.58 (Torres-Cartas et al. 2000), 3.17, 2.88 (Alvarez Nunez and Yalkowsky 1997) 3.38, and 4.11 (Torres-Cartas et al. 2000). Compared to PG, MPA is more hydrophobic and had less effect. 21OHPG, which is the most hydrophilic derivative, had the strongest effect on membrane integrity.

## Conclusion

Depending on their geometry, the presence of intramolecular hydrogen bond and the presence of an acetyl function at C21, PG derivatives interact differently with DPPC lipid membranes. The presence of a flat shape and free polar groups as substituents of the ring D seem to be the main factors favoring the destabilization of lipid membranes.

**Acknowledgments** Authors thank the Doctoral School of Sciences and Technologies at the Lebanese University for supporting the Bioactive Molecules Research Group.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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### **1.3 Tetra- and penta- cyclic triterpenes interaction with lipid bilayer membrane: a structural comparative study**

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**Journal of Membrane Biology (2016) 249: 327-338**



## Tetra- and Penta-Cyclic Triterpenes Interaction with Lipid Bilayer Membrane: A Structural Comparative Study

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Received: 13 October 2015 / Accepted: 1 January 2016  
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**Abstract** The effect of tetracyclic (cortisol, prednisolone, and 9-fluorocortisol acetate) and pentacyclic (uvaol and erythrodiol) triterpenes (TTPs) on the fluidity of dipalmitoyl phosphatidyl choline (DPPC) liposome membrane was investigated by differential scanning calorimetry, Raman spectroscopy, and fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). Liposomes were prepared in the absence and presence of TTPs at molar ratios DPPC:TTP 100:1, 100:2.5, and 100:10. All the studied TTPs abolished the pre-transition and modified the intensity of the Raman peak at  $715\text{ cm}^{-1}$  proving the interaction of TTP molecules with the choline head group of phospholipids. An increase in the Raman height intensity ratios of the peaks  $I_{2935/2880}$ ,  $I_{2844/2880}$ , and  $I_{1090/1130}$ , giving information about the ratio disorder/order of the alkyl chains, and a decrease of the main transition temperature demonstrated the interaction of TTPs with the alkyl chains. The tetracyclic TTPs produced broadening of the phase transition profile. Besides, a scarcely splitting of the main transition peak was obtained with prednisolone and 9-fluorocortisol acetate. The results of fluorescence depolarization of DPH showed that the studied molecules fluidized the liposomal membrane at 25, 41, and 50 °C. Pentacyclic TTPs, being more hydrophobic than tetracyclic ones, demonstrated higher fluidizing effect than tetracyclic TTPs

in the liquid crystalline phase suggesting a deeper incorporation in the lipid bilayer. The presence of a free polar head group at the ring D seems to control the TTP incorporation in the bilayer and consequently its effect on the membrane fluidity.

**Keywords** DPPC membrane · DSC · Fluidity · Fluorescence polarization · Raman spectroscopy · Triterpenes

### Introduction

The triterpenoids are a large and structurally diverse group of natural products derived from squalene or related acyclic 30-carbon precursors (Connolly and Hill 2002). The triterpenoids group displays well over 100 distinct skeletons (Xu et al. 2004). Triterpenoids (TTPs) with well-characterized biological activities include steroids and saponins.

Squalene is oxidized to oxidosqualene, which is the common starting point for cyclization reactions in triterpenoid biosynthesis (Vincken et al. 2007). Oxidosqualene is converted to cyclic derivatives via protonation and epoxide ring opening, which creates a carbocation that can undergo several types of cyclization reactions. After these cyclizations, subsequent rearrangements can proceed in different ways by a series of hydride shifts and/or methyl migrations, which lead to the formation of new carbocations. The transformation of dammarenyl C20 carbocation leads to the synthesis of pentacyclic lupane, hopane, oleanane, taraxasteranes, and ursane triterpenes (Vincken et al. 2007), while the protosteryl C20 carbocation is transformed to cucurbitanes, cycloartenes, and lanostanes. Lanosterol can also undergo demethylation and

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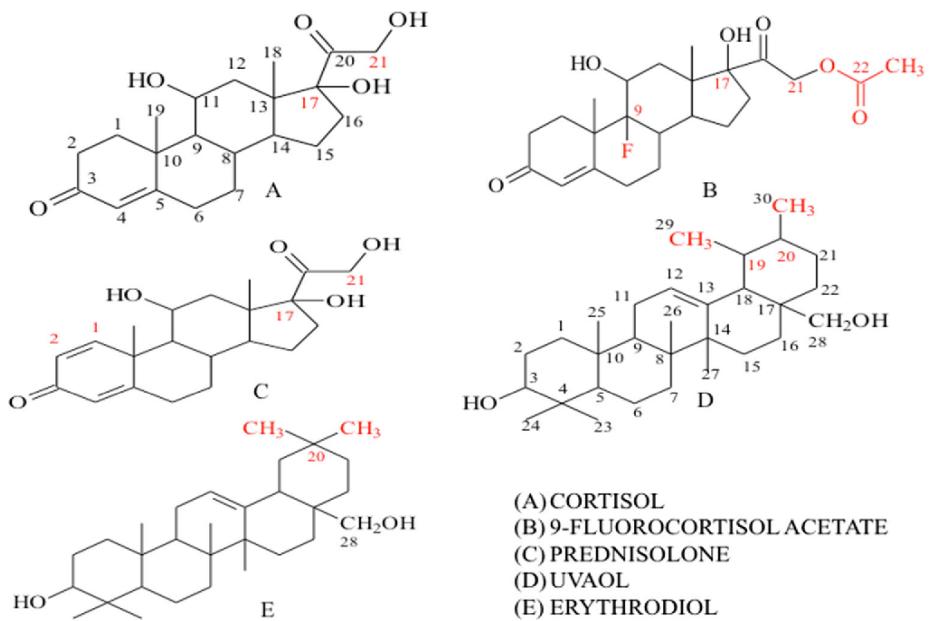
isomerisation of the double bond leading to cholesterol then to steroids (Vincken et al. 2007).

Glucocorticosteroids are a group of steroid hormones, which are structurally related to, and bio-chemically derived from, cholesterol. Corticosteroids influence the intermediary metabolism of carbohydrates, fats, and proteins as well as the function of different tissues. They proved anti-inflammatory effect and used to treat rheumatoid arthritis, asthma, etc. (Ghosh et al. 1996). Cortisol (Co) (Fig. 1a) is produced by the adrenal gland and the released amount is regulated by the pituitary gland inside the brain (Kumar et al. 2007). In a healthy person, 10 % of the Co is present in the free form in plasma, 70 % is bound to cortisol binding globulin ( $\alpha$ -globulin, transcortin), and 20 % is bound to albumin (Dubey and Boujoukos 2004). Free Co is the physiologically active form of the hormone (Dubey and Boujoukos 2004). Co and its synthetic analogues, prednisolone (PD), methylprednisolone, triamcinolone, dexamethasone, betamethasone, and paramethasone are known to exhibit many important physiological and biochemical effects (Cevc and Blume 2004). Structurally speaking, PD differs from Co by an additional double bond between C1 and C2; 9-Fluorocortisol acetate (9-FA) (Fig. 1b) is a Co with an acetate group at C21 and a fluorine atom at C9. PD (Fig. 1c) is administered orally in doses of the order of a few milligrams per tablet (Mazurek and Szostak 2006). PD binds to both corticosteroid binding globulin and albumin (Shibasaki et al. 2008), with 2.5 times stronger to transcortin and more than 300 times stronger to albumin

compared to Co (Rocci et al. 1982). These differences in binding affinities between Co and PD are due to the 1–2 double bond in the A-ring of the PD molecule, which favors the binding of PD to these proteins (Rocci et al. 1982). 9-FA is a powerful synthetic adrenocortical steroid (Ast and Abdou 1979), and it is commercially available in compressed tablets only (Cisternino et al. 2003). All of the synthetic analogues of Co are metabolized more slowly than Co by the liver (Cevc and Blume 2004).

Naturally occurring pentacyclic triterpenic compounds in the form of dialcohols (uvaol (UV) (Fig. 1d) and erythrodiol (ER) (Fig. 1e) or acids (oleanolic and maslinic acids) are composed of 30-carbon atom skeletons structured as 5-membered rings (Lopez et al. 2014). ER and UV are widely distributed in the plant kingdom, either in free form or esterified with fatty acids (Lopez et al. 2014). They are present in the leaves, fruit of olive tree, and in virgin olive oil (Allouche et al. 2009). In olive fruit or leaves, ER and UV account for approximately 60 and 40 % of triterpenic dialcohols, respectively. The concentrations of these triterpenic compounds in virgin olive oil are approximately 75 mg/kg ER and 20 mg/kg UV. Together with sterols, ER and UV are relevant markers for authenticating the genuine virgin olive oil and detecting adulteration (Lopez et al. 2014). UV and ER possess pharmacological properties including anticancer activities against various cancer cell lines (Allouche et al. 2011; Martin et al. 2009; Juan et al. 2008; Es-Saady et al. 1994), antioxidant activity against lipid peroxidation in rats (Perona et al. 2005),

**Fig. 1** Chemical Structure of Cortisol (a), 9-Fluorocortisol acetate (b), Prednisolone (c), Uvaol (d), and Erythrodiol (e)



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antithrombotic and anti-inflammatory effects (Allouche et al. 2010; Dzubak et al. 2006).

TTPs show structural similarities to cholesterol, which is an important constituent of cell membranes and a modulator of membrane properties. Interaction of TTPs with membranes has been widely investigated, and some TTPs were shown to affect the properties of lipid membranes (Han et al. 1997). Recently, we published studies on the interaction of cucurbitacin E (Habib et al. 2013), erythrodiol (Habib et al. 2015), progesterone, and its natural and synthetic derivatives (Abboud et al. 2015) with lipid membranes.

It has been reported that TTPs and beyond the well-known genomic effects affect cell function via non-genomic mechanisms. The latter operate in many systems and organs including the cardiovascular, immune, endocrine, and nervous systems, smooth and skeletal muscles, and liver and fat cells (Haller et al. 2008). The cell membrane constitutes the target where TTPs can modify its properties. In order to understand the effect of the presence of an additional cycle in a triterpenic structure on the modulation of lipid membrane properties, the interaction of both tetra- and penta-cyclic TTPs molecules with liposomal membranes is investigated in this study.

Liposomes, defined as microscopic spherical-shaped vesicles, consist of an internal aqueous compartment entrapped by one or multiple concentric lipidic bilayers were discovered in the 60s by Bangham (Laouini et al. 2012). They are considered as substantial models for the study of membrane surface characteristics and biological membrane properties such as permeability, osmotic activity, and interaction with various solutes (Demetzos 2008).

In this work, liposomes were prepared in the absence and presence of tetra-TTPs (Co, 9-FA and PD) and penta-TTPs (UV and ER) by the thin-film hydration method. The obtained batches were characterized by DSC, Raman, and fluorescence anisotropy.

## Materials and Methods

### Chemicals

DPPC was from Lipoid GmbH, Germany. Prednisolone was purchased from Sigma-Aldrich, China. 9-Fluorocortisol acetate was purchased from Sigma-Aldrich, Italy. Uvaol, the buffer reagent Trizma base and the organic solvents: chloroform, methanol, were from Sigma-Aldrich, France. Cortisol and tetrahydrofuran were from Sigma-Aldrich, USA. Erythrodiol, from Genay Cedex, France, 1,6-diphenyl-1,3,5-hexatriene, from Acros organics, New Jersey USA, and HCl from VWR, France.

## Methods

### Preparation of Multilamellar Vesicles

MLVs were prepared by the thin-film hydration technique described by Bangham et al. (1965). DPPC and the drug were mixed in molar ratios of 100:0, 100:1, 100:2.5, and 100:10. DPPC (25 mg/ml) and the drug were dissolved in a solvent mixture of chloroform/methanol 2:1 (v/v). The preparation was then evaporated in a rotary evaporator (BÜCHI Rotavapor R-124) at a temperature above the phase transition temperature of the lipid (55 °C) to form a thin lipid film. The latter was then hydrated with Tris–HCl (pH 7.4, 0.1 M), 2 ml. The preparations were alternatively vortexed for 5 min and warmed in a water bath at 55 °C for 5 min. The cycle was repeated three times. The preparations in which DPPC and the drug were at a molar ratio of 100:0 were considered as controls. For each molar ratio, three MLV batches were prepared.

### Differential Scanning Calorimetry

Thermal scans were obtained using a DSC Q200 scanning calorimeter (TA Instruments, France). The samples (10 µl) were encapsulated in hermetically sealed standard aluminum DSC pans. An empty pan was used for the reference during the analysis. Samples were run at a scan rate of 1 °C/min in a temperature range of 20–55 °C. DSC data were analyzed with the instrument software package.

### Raman Spectroscopy

The Raman spectra were obtained with 1 cm<sup>-1</sup> resolution from 3100 to 600 cm<sup>-1</sup>, recorded on a LabRAM HR 800 (Horiba Jobin Yvon, Villeneuve d'Ascq, France) at Ecole Centrale de Lyon (IVTV ANR-10-EQPX-06-01) equipped with a detector 1024 × 256 pixel and a LabSpec software. The laser power was constant within 12mW during the experiments, with a wavelength of 633 nm. Samples of 10–15 µl from the liposomal suspensions were used for the Raman spectroscopy. An integrated camera was employed to acquire images of trapped vesicles (L × H = 760 × 628 pixels).

### Fluorescence Anisotropy Measurements

Changes in lipid fluidity of liposomes were measured by steady-state fluorescence polarization using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies). 1, 6-diphenyl-1, 3, 5-hexatriene (DHP) was dissolved in tetrahydrofuran (THF). An aliquot of DHP solution ( $4.30 \times 10^{-3}$  mol/L) was then added to the membrane

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suspension prepared as described above to obtain a molar ratio DPH:phospholipid 1:500. The mixture was incubated in the dark for 1 h to allow the intercalation of the DPH molecules into the lipid bilayer. The final concentration of THF did not exceed 0.01 % and showed to have no effect on the fluorescence anisotropy. The experiments were performed at 28, 41, and 50 °C. The probe was excited with a manual polarizer accessory (Malaysia), using vertically polarized light at 360 nm and the emission intensities measured at 450 nm both parallel and perpendicular to the plane of the excitation beam yielding components  $I_{Vv}$  and  $I_{VH}$ , respectively. The Cary eclipse Bio software, delivered with the spectrophotometer, gives the fluorescence anisotropy ( $r$ ), which is defined as

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}},$$

where  $I_{\parallel}$  and  $I_{\perp}$  are the fluorescence intensities of the light emitted with its polarization plane parallel ( $\parallel$ ) and perpendicular ( $\perp$ ) to that of the exciting beam.

### Statistics

Values are expressed as mean  $\pm$  SD. The differences between the means of parameters determined for drug-loaded liposomes and their corresponding controls were

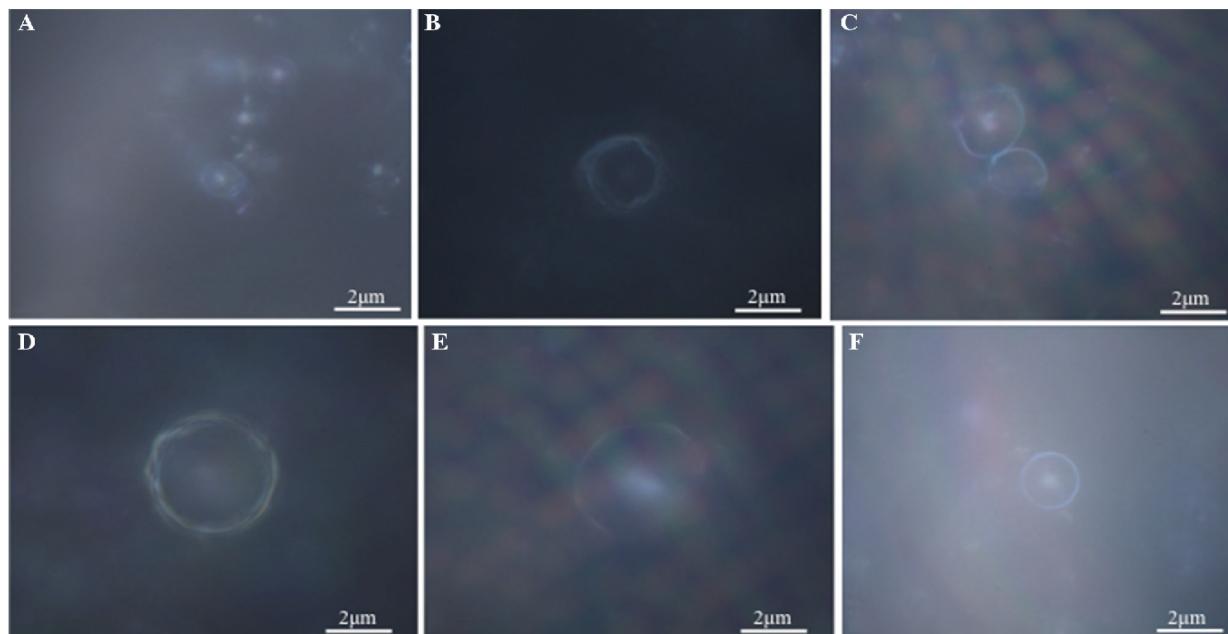
tested with t test. A  $P$  value  $<0.05$  was accepted as the level of significance.

## Results

### Raman Spectroscopy

The confocal Raman microscope allows lipid vesicles to be optically trapped in the laser focus and their spectra can be acquired. The Raman microscopy allowed to visualize blank and TTP-loaded liposomes. The images revealed the formation of nano- and micrometric spherical vesicles in all batches. Figure 2 shows the images of blank liposomes (A) and TTP-loaded liposomes prepared at molar ratio DPPC:TTP 100: 10 (B, C, D, E, and F). Similar images were obtained at lowest molar ratios.

By monitoring changes in the peak intensities of Raman scattering from phospholipid vesicles, differences in acyl chain conformation and the corresponding intra- and intermolecular membrane order can be determined (Fox et al. 2007). The pure DPPC spectrum exhibits three characteristic areas from which useful information can be derived regarding the conformation of the molecule. These areas are 2800–3000 cm<sup>-1</sup> (area a), 1000–1200 cm<sup>-1</sup> (area b), and 700–800 cm<sup>-1</sup> (area c).



**Fig. 2** DPPC liposome trapping images for Blank (a), Cortisol- (b), 9-Fluorocortisol acetate- (c), Prednisolone- (d), Uvaol- (e), and Erythrodiol-loaded liposomes (f) obtained at molar ratio DPPC:TTP 100:10

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Area a contains the peaks at 2844 and 2880  $\text{cm}^{-1}$ , which correspond to the symmetrical and asymmetrical vibration, respectively, of stretching of the C–H bond of the methylene groups. It contains also the peak at 2935  $\text{cm}^{-1}$  which is attributed to the symmetrical vibration of stretching of the C–H bond of the final methyl group of the alkyl chain. The bands of area a are commonly used to monitor changes in the lateral packing properties and mobility of the lipid chains for the bilayer system (Potamitis et al. 2011).

Of interest are also the height intensity ratios  $I_{2935/2880}$  and  $I_{2844/2880}$  (Gardikis et al. 2006a, b).  $I_{2935/2880}$  measures the effects originating from changes both in interchain and intrachain processes in the bilayer. It has been used as an overall indicator of both intramolecular (gauche/trans ratio) and intermolecular (packing order); as this ratio increases, more freedom of motion and rotational disorders are detected (Fox et al. 2007). In our study, an increment in the rotational disorder of DPPC membrane in the presence of the TTPs molecules was obtained (Table 1).

$I_{2844/2880}$  describes the main changes occurring in the hydrocarbon chain region of the lipids. It is sensitive to subtle changes in conformational order from rotations, kinks, twists, and bends of the lipid chains (Potamitis et al. 2011). The studied TTPs displayed a common effect, which is an increment of the ratio  $I_{2844/2880}$  (Table 1).

The area b includes the stretching vibrations of the C–C bonds of the alkyl chains of the phospholipids. The peak at 1130  $\text{cm}^{-1}$  is attributed to the stretching vibration of the C–C bond for the trans conformations of the alkyl chains, while the peak at 1090  $\text{cm}^{-1}$  is attributed to the stretching vibration of the C–C bond for the gauche conformations of the alkyl chains (Gardikis et al. 2006a, b). The height intensity ratio of these peaks ( $I_{1090/1130}$ ) allows the direct comparison of the order–disorder transitions between liposome preparations (Potamitis et al. 2011). The studied TTPs increased the *gauche/trans* ratio ( $I_{1090/1130}$ ) and disturbed the lipid chain order. The effect was significant for 9-FA at molar ratio DPPC:TTP 100:1, for ER at molar ratios 100:1 and 100:10, and for UV at molar ratio 100:2.5. Besides, the effect of Co, ER, and UV on the  $I_{1090/1130}$  value was non-monotonous; it was high for mini and maxi Co, ER, and UV concentrations but low for the medium concentration. Further studies can be conducted using a narrower DPPC:TTP molar ratio range between 100:2.5 and 100:10.

The area c contains the peak at 715  $\text{cm}^{-1}$  that represents the stretching vibration of the C–N bond of the choline group of DPPC (Gardikis et al. 2006a, b; Potamitis et al. 2011). The studied TTPs decreased in a significant manner the intensity of this peak (Table 1).

**Table 1** Height intensity for Raman peaks of DPPC vesicles incorporating TTPs

MLVs	$I_{2935/2880}$	$I_{2844/2880}$	$I_{1090/1130}$	$I_{\text{C}-\text{N}}$
DPPC (100/0)	0.43 ± 0.00	1.02 ± 0.02	0.61 ± 0.13	261.69 ± 29.14
DPPC/Co				
DPPC/Co (100/1)	0.44 ± 0.05	1.14 ± 0.01*	0.74 ± 0.06	107.27 ± 1.10*
DPPC/Co (100/2.5)	0.49 ± 0.08	1.12 ± 0.07	0.61 ± 0.05	102.55 ± 8.60*
DPPC/Co (100/10)	0.58 ± 0.14	1.13 ± 0.03*	0.86 ± 0.27	117.9 ± 1.27*
DPPC/PD				
DPPC/PD (100/1)	0.45 ± 0.02	1.07 ± 0.02*	0.62 ± 0.08	120.54 ± 17.36*
DPPC/PD (100/2.5)	0.50 ± 0.06	1.08 ± 0.03*	0.71 ± 0.11	144.46 ± 4.89*
DPPC/PD (100/10)	0.52 ± 0.03*	1.13 ± 0.01*	0.75 ± 0.10	71.63 ± 10.03*
DPPC/9-FA				
DPPC/FA (100/1)	0.47 ± 0.07*	1.10 ± 0.06	0.83 ± 0.11*	59.98 ± 8.90*
DPPC/FA (100/2.5)	0.50 ± 1.11	1.12 ± 0.01*	0.81 ± 0.13	56.56 ± 6.36*
DPPC/FA (100/10)	0.51 ± 0.01*	1.20 ± 0.02*	0.86 ± 0.20	75.19 ± 12.53*
DPPC/ER				
DPPC/ER (100/1)	0.62 ± 0.06*	1.05 ± 0.03	0.81 ± 0.03*	48.13 ± 7.79*
DPPC/ER (100/2.5)	0.41 ± 0.04	1.13 ± 0.01*	0.76 ± 0.13	71.86 ± 26.58*
DPPC/ER (100/10)	0.60 ± 0.09*	1.71 ± 0.03*	0.87 ± 0.18*	75.42 ± 13.30*
DPPC/UV				
DPPC/UV (100/1)	0.46 ± 0.01*	1.08 ± 0.01*	0.77 ± 0.21	68.07 ± 13.57*
DPPC/UV (100/2.5)	0.55 ± 0.07	1.10 ± 0.04	0.93 ± 0.07*	60.93 ± 8.69*
DPPC/UV (100/10)	0.56 ± 0.00*	1.05 ± 0.00	0.84 ± 0.20	120.90 ± 34.13*

Values are expressed as the means of three repetitions ± SD

\*  $P < 0.05$  compared with the control value

### Differential Scanning Calorimetry

Figures 3, 4, 5, 6, and 7 show the DSC curves of liposomes containing, respectively, Co, PD, 9-FA, ER, and UV at molar ratios DPPC:TTP of 100:0, 100:1, 100:2.5, and 100:10.

Table 2 summarizes the calorimetric parameters, which display the average of three independent experiments. Blank liposomes, displayed a low enthalpic pre-transition ( $L_{\beta'}$  to  $P_{\beta'}$ ) at  $34.94 \pm 0.79$  °C and a sharp main transition ( $P_{\beta'}$  to  $L_{\alpha}$ ) at  $40.48 \pm 0.21$  °C. The enthalpy of the main transition was much higher ( $\approx 43.63$  J/g) than that of the pre-transition ( $\approx 3.75$  J/g). The sharpness of the main transition peak obtained for DPPC liposomes with  $\Delta T_{1/2}$  value of 0.29 °C is an indication of the purity of the system and a highly cooperative transition.

Compared to blank liposomes, the main phase transition temperature shifted to lower values and the pre-transition completely abolished for Co-, PD-, and 9-FA-loaded liposomes prepared at the various molar ratios DPPC:TTP (Figs. 3, 4, 5). Furthermore, the transition peak broadened and  $\Delta T_{1/2}$  values increased for Co- (at three various molar ratios), PD- (at molar ratios 100:2.5 and 100:10) and 9-FA (at molar ratio 100:10)-loaded liposomes (Table 2). Besides, a shoulder of the main calorimetric peak was observed for PD and 9-FA at molar ratio DPPC:TTP (100:10) (Figs. 4, 5). The  $\Delta H_m$  values increased significantly at the various molar ratios DPPC:TTP for Co and 9-FA and at 100:1 for PD (Table 2).

Compared to blank liposomes, the addition of ER and UV abolished the pre-transition temperature and decreased

significantly the  $T_m$  and  $\Delta H_m$  at the two highest molar ratios DPPC:TTP (Table 2). The presence of ER or UV in liposomes broadened the transition peak at all molar ratios (Figs. 6, 7), with a significant increment in  $\Delta T_{1/2}$  values in a concentration-dependent manner (Table 2).

### Fluorescence Anisotropy

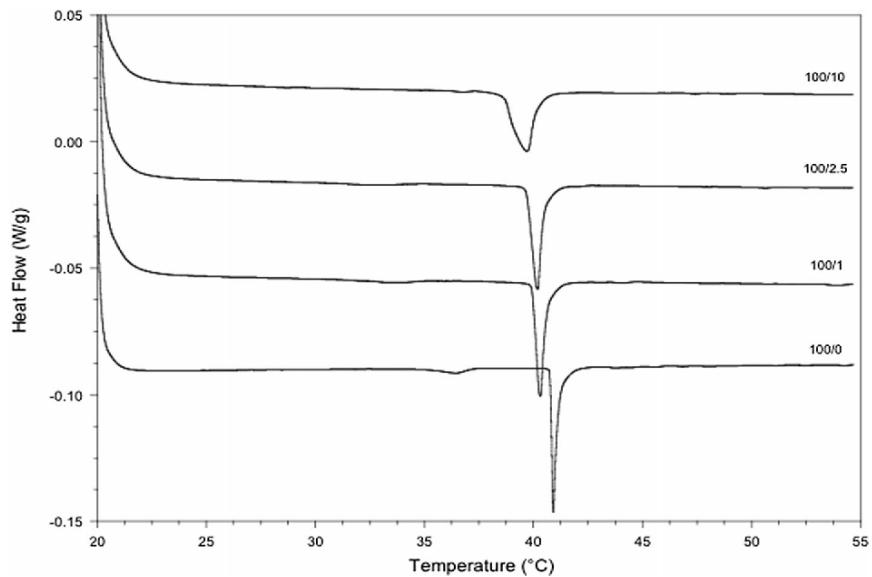
Lipid membranes typically do not display intrinsic fluorescence. For this reason, it is common to label them with probes, which spontaneously partition into the non-polar side chain region of the membranes. One of the most commonly used membrane probes is DPH. Because of its low solubility and quenched emission in water, DPH emission is only seen from membrane-bound DPH (Lakowicz 2006; Yengo and Berger 2010).

Table 3 shows the DPH anisotropy values of blank and TTP-loaded liposomes. Compared to blank vesicles, the presence of TTPs in vesicles reduced the anisotropy values significantly in the gel state at the various molar ratios.

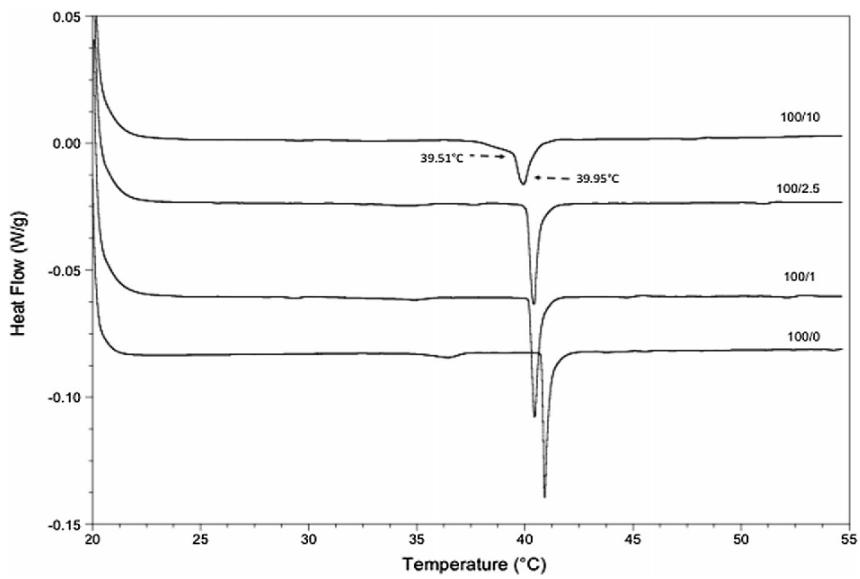
At the transition phase, the DPH anisotropy value decreased significantly at the highest molar ratio DPPC:TTP (100:10). Similar result was obtained for Co-loaded liposomes at molar ratios 100:1 and 100:2.5 and for UV at molar ratio 100:2.5.

As for the liquid crystalline phase, pentacyclic TTPs at the various molar ratios and Co at the highest molar ratio 100:10 reduced significantly the anisotropy.

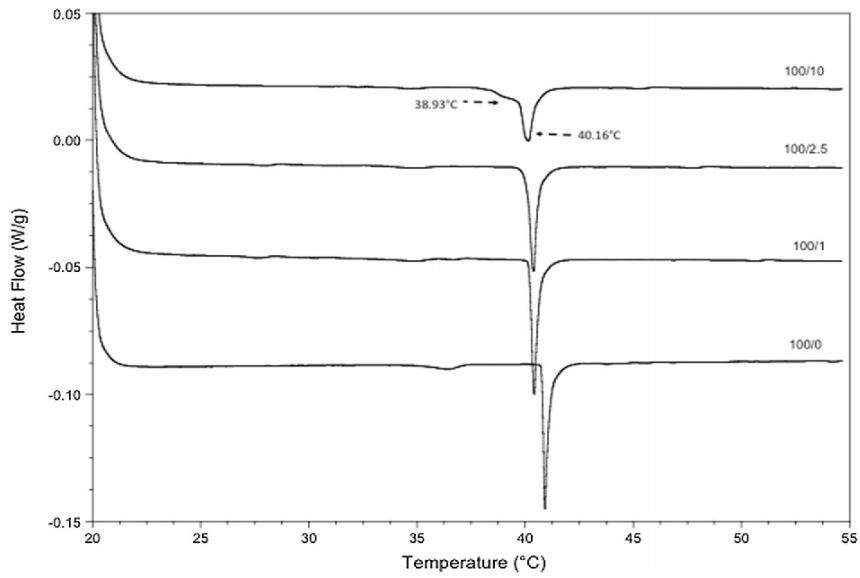
**Fig. 3** DSC scans of DPPC:Co liposomes at molar ratios of 100:0, 100:1, 100:2.5, and 100:10



**Fig. 4** DSC scans of DPPC:PD liposomes at molar ratios of 100:0, 100:1, 100:2.5, and 100:10



**Fig. 5** DSC scans of DPPC and DPPC:9-FA liposomes at molar ratios of 100:0, 100:1, 100:2.5, and 100:10

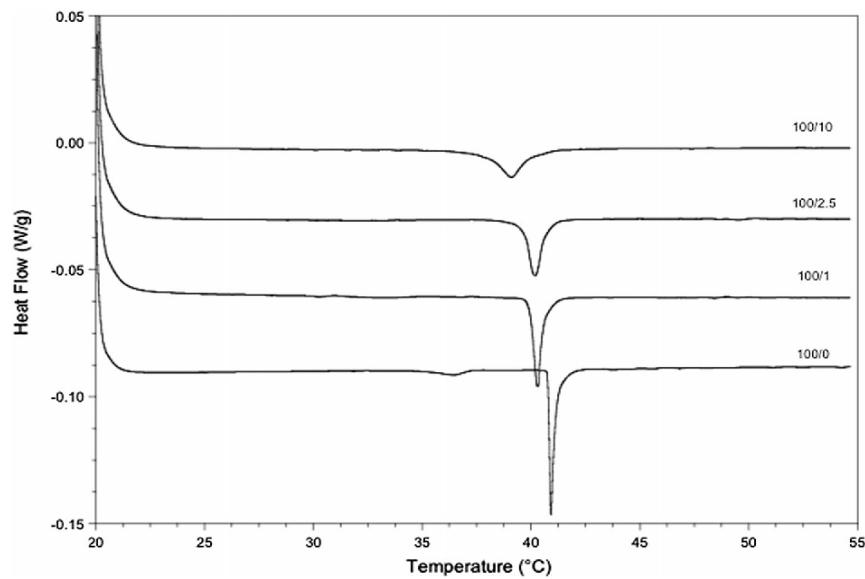


## Discussion

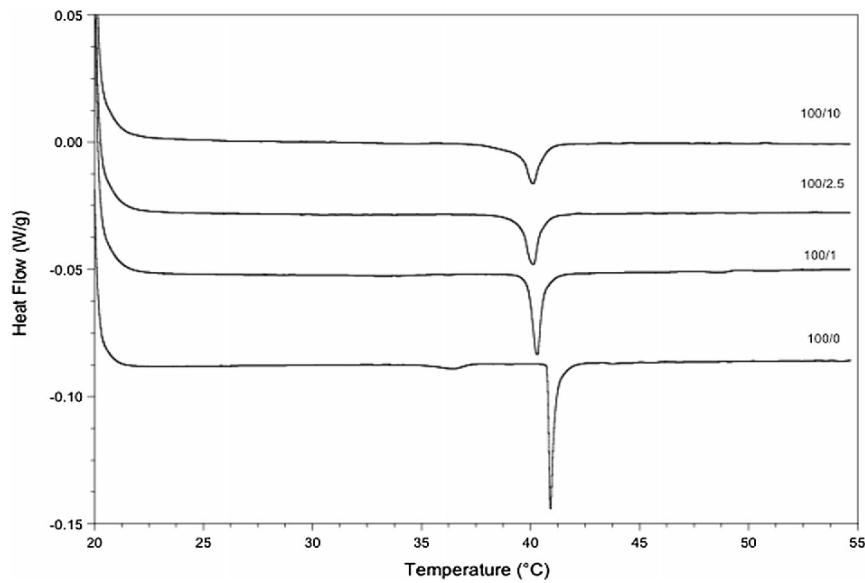
The pre- and main phase transitions are often observed in lipid bilayers. The pre-transition corresponds to the conversion of a lamellar gel phase to a rippled gel phase and is mainly related to the polar region of phosphatidylcholine bilayers. The disappearance of the pre-transition (Table 2) peak upon addition of the drugs even at low concentration (100:1) suggests an interaction of these molecules with the choline head group of phospholipids. This interaction is

also proved by Raman spectroscopy where a decrease of the intensity of the peak at  $715\text{ cm}^{-1}$  (Table 1), which corresponds to C–N stretch vibrations of choline molecules (Gardikis et al. 2006a), was obtained. Similar results were reported for other triterpene molecules such as cucurbitacin E (Habib et al. 2013), oleanolic acid and its derivatives maslinic and ursolic acids (Prades et al. 2011), progesterone derivatives (Korkmaz and Severcan 2005, Abboud et al. 2015), and for some corticosteroids like dexamethasone (Bhardwaj and Burgess 2010), beclomethasone

**Fig. 6** DSC scans of DPPC and DPPC:ER liposomes at molar ratios of 100:0, 100:1, 100:2.5, and 100:10



**Fig. 7** DSC scans of DPPC and DPPC:UV liposomes at molar ratios of 100:0, 100:1, 100:2.5, and 100:10



dipropionate (Elhissi et al. 2006), and hydrocortisone (Ganesan et al. 1984).

The main phase transition reflects the change from a rippled gel phase to a liquid crystal phase and is closely related to the acyl chains of phosphatidylcholine bilayers (Cong et al. 2009). The main phase transition of phosphatidylcholines can be used to probe the interaction between the acyl chains of phosphatidylcholine and exogenous substances.  $T_m$ , the transition temperature, is

determined from the maximum of the recorded heat capacity,  $\Delta H_m$ , the enthalpy of main phase transition, is obtained from the area under the peak, and  $\Delta T_{1/2}$ , the half-height width, is an index of the cooperativity of this conversion: the narrower the peak, the higher the cooperativity (Cong et al. 2009). The changes of these calorimetric parameters can be attributed to the packing of exogenous molecules within the hydrophobic interior of phospholipids array (Cong et al. 2009, Rui-guang et al. 2012). Tetra- and

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**Table 2** Thermodynamics parameters for the interaction of TTPs with DPPC vesicles obtained by DSC measurements

MLVs	$T_m$ (°C)	$\Delta H_m$ (J/g)	$\Delta T_{1/2}$ (°C)
DPPC (100/0)	40.48 ± 0.21	43.63 ± 0.74	0.29 ± 0.07
DPPC/Co			
DPPC/Co (100/1)	40.19 ± 0.16*	55.74 ± 0.38*	0.37 ± 0.01*
DPPC/Co (100/2.5)	40.19 ± 0.00*	55.92 ± 0.11*	0.46 ± 0.00*
DPPC/Co (100/10)	39.67 ± 0.02*	62.58 ± 1.07*	0.95 ± 0.07*
DPPC/PD			
DPPC/PD (100/1)	40.4 ± 0.11	45.79 ± 1.23*	0.30 ± 0.03
DPPC/PD (100/2.5)	40.31 ± 0.02	43.92 ± 0.71	0.49 ± 0.03*
DPPC/PD (100/10)	39.95 ± 0.02*	43.17 ± 0.53	0.65 ± 0.02*
DPPC/9-FA			
DPPC/FA (100/1)	40.40 ± 0.00	47.36 ± 0.41*	0.27 ± 0.01
DPPC/FA (100/2.5)	40.38 ± 0.01	46.82 ± 0.46*	0.31 ± 0.02
DPPC/FA (100/10)	40.17 ± 0.04*	45.03 ± 0.34*	0.45 ± 0.09*
DPPC/ER			
DPPC/ER (100/1)	40.12 ± 0.23	40.76 ± 1.66	0.37 ± 0.00*
DPPC/ER (100/2.5)	39.92 ± 0.26*	40.08 ± 0.91*	0.69 ± 0.18*
DPPC/ER (100/10)	38.24 ± 0.87*	36.09 ± 3.49*	1.30 ± 0.12*
DPPC/UV			
DPPC/UV (100/1)	40.32 ± 0.00	43.73 ± 1.45	0.40 ± 0.05*
DPPC/UV (100/2.5)	40.17 ± 0.04*	40.44 ± 1.66*	0.60 ± 0.02*
DPPC/UV (100/10)	40.13 ± 0.00*	38.47 ± 0.30*	0.64 ± 0.04*

Calorimetric data of the main transition peak are mean of three independent experiments ± SD

\*  $P < 0.05$  compared with the control value

penta-TTPs fluidized the membrane as their presence in liposomes produced a clear decrease of the  $T_m$  value. Also, the DPH anisotropy results confirmed their fluidizing effect since a decrease of anisotropy values was obtained at the various temperatures when compared to blank liposomes. Moreover, the increment of *gauche/trans* ratio ( $I_{1090}/I_{130}$ ) determined by Raman spectroscopy, suggest that the incorporation of TTPs molecules in the bilayers leads to an increase in the chain disorder giving rise in the bilayer fluidity.

Tetra- and penta-TTPs reduced the cooperativity between the acyl chains since an increase of  $\Delta T_{1/2}$  was noticed (Table 2). The peak height intensity ratios  $I_{2844}/I_{2880}$  and  $I_{2935}/I_{2880}$  (Table 1) increased with increasing TTPs concentrations, suggesting that the TTPs incorporation in liposomes perturbed the carbon-chain and the terminal methyl group of DPPC (Fotakis et al. 2010). The findings were in accordance with DPH anisotropy results, where the fluidizing effect resulting from the disorder was proved. Also, the broadening of the main transition peak was clearly observed for penta-TTPs, for PD, and 9-FA at highest molar ratio DPPC:TTP (100:10). For these tetra-TTPs, a scarcely splitting of the peak was also noticed (Figs. 4, 5). And this can be attributed to the formation of drug-rich and -poor domains. The drug-rich domain possesses the lowest  $T_m$  values.

Compared to blank liposomes, tetracyclic TTPs increased the  $\Delta H_m$  values, while the contrary effect was obtained with pentacyclic TTPs (Table 2). The studied tetracyclic TTPs having more polar groups than the penta-TTP ones (Fig. 1) can be rather localized at the upper chain/glycerol/head group region of the lipid bilayer. This location may enhance the van der Waals interactions between the alkyl chains (Fotakis et al. 2010). The penta-TTPs weaken the van der Waals forces between the alkyl chains, may introduce empty spaces in the deep interior of the bilayer, and provide the phospholipid acyl chain a higher degree of freedom (Gardikis et al. 2006a). They may incorporate deeply the bilayer. This is also in accordance with their highest effect on the anisotropy values at the liquid crystalline phase (50 °C) (Table 3), as pentacyclic TTPs were more potent to decrease the anisotropy values than the tetra-TTPs.

The presence of two or three neighbor polar groups at D-ring (Co, PD, and 9-FA) might favor the formation of intramolecular hydrogen bond, rendering the molecules less potent to affect the fluidity when compared to structures with a free polar group at D-ring like the pentacyclic TTPs, UV, and ER with a hydroxyl group at C28. Also, it has been reported that the presence of the hydroxyl group at C<sub>17</sub> of estradiol in contact with the hydrophobic chains of phospholipids induces an important disorder and

**Table 3** Fluorescence anisotropy of DPPC vesicles incorporating TTPs at 28, 41, and 50°

MLVs	Anisotropy r		
	28 °C	41 °C	50 °C
DPPC (100/0)	0.32 ± 0.00	0.28 ± 0.00	0.12 ± 0.00
DPPC/Co			
DPPC/Co (100/1)	0.29 ± 0.00*	0.26 ± 0.00*	0.11 ± 0.01
DPPC/Co (100/2.5)	0.25 ± 0.00*	0.25 ± 0.00*	0.11 ± 0.00
DPPC/Co (100/10)	0.23 ± 0.01*	0.25 ± 0.01*	0.09 ± 0.00*
DPPC/PD			
DPPC/PD (100/1)	0.30 ± 0.01*	0.27 ± 0.00	0.12 ± 0.02
DPPC/PD (100/2.5)	0.28 ± 0.01*	0.27 ± 0.00	0.11 ± 0.00
DPPC/PD (100/10)	0.28 ± 0.01*	0.26 ± 0.00*	0.09 ± 0.01
DPPC/9-FA			
DPPC/FA (100/1)	0.30 ± 0.00*	0.27 ± 0.00	0.12 ± 0.00
DPPC/FA (100/2.5)	0.28 ± 0.00*	0.26 ± 0.01	0.11 ± 0.00
DPPC/FA (100/10)	0.25 ± 0.01*	0.25 ± 0.00*	0.11 ± 0.00
DPPC/ER			
DPPC/ER (100/1)	0.30 ± 0.00*	0.28 ± 0.01	0.1 ± 0.01*
DPPC/ER (100/2.5)	0.26 ± 0.01*	0.26 ± 0.02	0.09 ± 0.00*
DPPC/ER (100/10)	0.25 ± 0.00*	0.24 ± 0.00*	0.10 ± 0.00*
DPPC/UV			
DPPC/UV (100/1)	0.30 ± 0.00*	0.27 ± 0.00	0.09 ± 0.05*
DPPC/UV (100/2.5)	0.30 ± 0.00*	0.25 ± 0.00*	0.08 ± 0.01*
DPPC/UV (100/10)	0.27 ± 0.00*	0.23 ± 0.00*	0.06 ± 0.00*

Values are expressed as the means of three repetitions ± SD

\*  $P < 0.05$  compared with the control value

perturbations in liposomes membrane and thus an increase in membrane fluidity (Whiting et al. 2000). Recently, we demonstrated that PG derivatives increase the membrane fluidity in the order: progesterone = 21-hydroxyprogesterone = medroxyprogesterone acetate > 17-hydroxyprogesterone > medroxyprogesterone = dydrogesterone (Abboud et al. 2015). On the overall, the presence of free polar group in D-ring seems to control the fluidizing effect of tetra- or penta-TTPs since the best fluidizing and perturbing effects appeared with molecules having this feature.

The hydrophobicity is also another factor which affects the fluidizing effect of a TTP molecule. Indeed, the  $\log P$  values of Co, PD, and 9-FA are, respectively, 1.61 (Alvarez Nunez and Yalkowsky 1997), 1.62 (Alvarez Nunez and Yalkowsky 1997), and 1.7, being much lower than those of UV and ER, 7.4, and 7.6, respectively. Being more hydrophobic, the penta-TTPs have tendency to enter deeply in the lipid bilayers.

Cyclic triterpenes are structurally related to cholesterol, as both possess the same basic steroid nucleus and are

lipophilic. Using the fluorescence polarization (Parasassi et al. 1995; Rodriguez et al. 1997) and DSC techniques (Kyrikou et al. 2004), many studies were reported that cholesterol fluidizes the membrane in the gel phase ( $T < T_m$ ) and rigidifies it in the liquid crystalline phase ( $T > T_m$ ). The studied tetra- and penta-cyclic TTPs fluidize the membrane throughout the temperature range (Table 3). This can be due to the presence of the aliphatic chain at C<sub>17</sub>, which is a characteristic of the cholesterol molecule only. Also, the rigid tetracyclic skeleton and the lateral chain in C<sub>17</sub> of cholesterol interact through Van der Waals interactions with the phospholipid acyl chains, restricting the movement of the fatty acyl chain in the hydrophobic region and thus rigidify the membrane for  $T > T_m$  (Rodriguez et al. 1997). The absence of lateral chain in both tetra- and penta-cyclic and of planarity in the pentacyclic skeleton (Rodriguez et al. 1997) introduce empty spaces in the bilayer deep interior and might explain the non-condensing effect of the studied TTPs compared to cholesterol.

It is worthy to mention that, among the tetra-TTPs, PD and 9-FA increased the  $\Delta H_m$  values in a weaker manner compared to Co (Table 2). The additional C1-C2 double bond for PD and the presence of an acetate function and the fluorine (C<sub>9</sub>) for 9-FA are able to modulate the TTP effect on the bilayer. Moreover, compared to blank liposomes, the  $\Delta T_{1/2}$  values (Table 2) increased for penta-TTP-loaded liposomes and the effect was more pronounced for ER. This can be due to the different localization of the methyl group at C<sub>19</sub> for UV and C<sub>20</sub> for ER. It has been reported that different effects of ER and UV in normal and breast cancer cells are due to the difference of location of a methyl group (Quesada and Gaforio 2015).

## Conclusion

The present study proved that penta- and tetra-cyclic TTPs fluidize the lipid membranes. Tetracyclic TTPs can be rather localized at the upper chain/glycerol/head group region of the lipid bilayer, while pentacyclic ones may incorporate deeply the bilayer. The presence of a free polar group in D-ring controls the fluidizing effect of both tetra- and penta-TTPs.

**Acknowledgments** Authors thank the Research Funding Program at the Lebanese University for supporting the Bioactive Molecules Research Group.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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## *Chapitre 2:*

*Interaction des triterpènes avec la sérum albumine*

## Introduction

La sérum albumine est de loin la protéine la plus abondante dans le plasma (~35-50 mg/mL) (Kratz 2008). L'albumine est synthétisée dans le foie et est utilisée cliniquement pour maintenir la pression osmotique sanguine (Kratz, 2008). Elle est très soluble dans l'eau et sa charge globale à pH physiologique est de -15. La sérum albumine humaine (SAH) est composée de 585 acides aminés. Elle est caractérisée par un nombre élevé de cystéine (35), d'acides aminés chargés (lysine, arginine, acides aspartique et glutamique) (Nicholson et al., 2000) et par la présence d'un seul tryptophane (Trp) en position 214 (Sugio et al., 1999). L'ordre des acides aminés de la SAH montre l'occurrence de trois domaines structuralement homologues; le domaine I, II et III (Sugio et al., 1999; Fasano et al., 2005). Chaque domaine de la SAH est formé de deux sous-domaines A et B (IA, IB, IIA, IIB et IIIA, IIIB) (Fasano et al., 2005). Les régions terminales des domaines de la sérum albumine contribuent à la formation des spirales d'inter-domaine liant le domaine I à II et II à III, respectivement (Fasano et al., 2005). La structure secondaire de la SAH est formée principalement d'hélices- $\alpha$ .

Les ligands se fixent principalement sur la SAH au niveau de deux sites, le site I le site II (Sudlow et al., 1975). Le site I, est une poche hydrophobe localisée dans le noyau du sous-domaine IIA. Ce site comporte les six hélices de ce sous-domaine et une partie du sous-domaine IB (Ghuman et al., 2005). Le mur interne de la poche est majoritairement apolaire mais contient deux groupes d'acides aminés polaires (Ghuman et al., 2005). Les ligands du site I sont typiquement de gros anions hétérocycliques comme la bilirubine (Ascenzi et al., 2009), l'acide salicylique (Ni et al., 2006), la warfarine (Yang F et al., 2014) et d'autres. Le site II est également une poche hydrophobe, localisée dans le noyau du sous-domaine IIIA. Ce site comporte les six hélices de ce sous-domaine et il est similaire au site I (Ghuman et al., 2005). La poche du site II contient des chaînes latérales hydrophobes et les ponts disulfures de la spirale IIIA-h3. Il est privilégié pour les ligands aromatiques qui sont soit neutres soit des anions tel que l'ibuprofène, le diflunisal, le diazepam et d'autres (Peng et al., 2014).

Malgré l'importance physiologique et thérapeutique des progestatifs et des glucocorticoïdes, peu d'études ont été menées sur leur interaction avec l'albumine. Il a été rapporté que la PG (Abu Teir et al., 2011), le Co (Rocci et al., 1982), la Pd, la Pn (Boudinot and Jusko 1984) et la dexaméthasone (Naik et al., 2010) se lient à l'albumine. Les constantes de liaison à l'albumine

ont été déterminées par la spectroscopie de fluorescence ainsi la spectroscopie infra rouge de fluorescence démontrait un changement au niveau de la structure secondaire de l'albumine.

Les méthodes spectroscopiques sont largement utilisées pour étudier l'interaction ligand-protéine. La séparation des fractions liées et libres du ligand n'est pas nécessaire. La spectroscopie UV visible, le dichroïsme circulaire, la spectroscopie de fluorescence, la spectroscopie infrarouge (FTIR) et la résonnance magnétique nucléaire (RMN) visent à détecter un changement d'une propriété physicochimique particulière du ligand ou de la protéine suite à la liaison d'un ligand sur la protéine.

La spectroscopie de fluorescence et infrarouge sont les techniques utilisées pourachever l'étude de l'interaction des TTPs avec la sérum albumine humaine.

La fluorescence intrinsèque de l'albumine provient principalement des résidus Trp (Sulkowska, 2002; Molina bolivar et al., 2015; Samanta et al., 2010). Une longueur d'onde d'excitation de 295 nm est généralement employée pour enregistrer le spectre d'émission du seul résidu Trp de la SAH et la fluorescence maximale se montre entre 340 et 350 nm. D'autre part l'excitation de la protéine à 280 nm est employée pour obtenir le spectre d'émission des résidus tyrosine distribués tout au long de la protéine et la fluorescence maximale se montre entre 330 et 340 nm (Khan et al., 2001). La modification de la fluorescence de la SAH en présence d'un ligand est un moyen d'étude de l'interaction de ce ligand avec la protéine. Elle peut indiquer l'accessibilité du ligand aux acides aminés fluorescents de la SAH et aider à identifier le site de liaison du ligand à la protéine. En réponse à la liaison du ligand, un décalage de la longueur d'onde à laquelle se produit l'émission maximale reflétant les changements de polarité autour du Trp peut être signalé. « Red or blue shifts » peuvent être accordés à une diminution ou à une augmentation de l'hydrophobicité au voisinage du résidu Trp, respectivement.

Les paramètres de liaison sont aussi déterminés comme: la constante de Stern-Volmer ( $K_{sv}$ ) indiquant la sensibilité du fluorophore au ligand et la constante d'association ( $K_a$ ) (Lakowicz, 2006). Afin de savoir si les TTPs sont capables de modifier l'interaction des ligands spécifiques du site I de la SAH, les paramètres de liaison de la bilirubine, un ligand spécifique du site I, sont déterminés en absence et en présence des TTPs.

La spectroscopie infrarouge est une technique qui pourrait être utilisée pour étudier l'interaction entre la protéine et le ligand (Jackson and Mantsch, 2001). Elle fournit des informations

concernant la structure secondaire de la protéine (Pelton and McLean, 2000). Pour les protéines et les polypeptides, on peut noter la présence des deux bandes dues aux vibrations amide I et II, à environ 1700 et 1500 cm<sup>-1</sup>. Celles-ci proviennent des liaisons amide qui lient les acides aminés. Les bandes d'amide I et II sont formées des transitions dues à l'absorption des modes d'elongation des liaisons C=O et des modes de pliage des liaisons N-H des liaisons amide d'un peptide, respectivement (Garidel and Schott 2006). Comme les groupements C=O et N-H sont impliqués dans les liaisons hydrogènes qui ont lieu entre les différents éléments ( $\alpha$  et  $\beta$ ) de la structure secondaire, les emplacements des bandes des deux amide I et II sont sensibles au contenu de la structure secondaire d'une protéine. Les protéines et les polypeptides contiennent souvent plus qu'un motif structural secondaire, et par conséquent donnent lieu à plus qu'une bande d'amide I. Malheureusement, la largeur et la séparation de ces absorptions est difficile. Pour améliorer l'utilité de la spectroscopie infrarouge dans ce domaine, l'utilisateur doit tirer parti des techniques mathématiques comme Fourier auto- déconvolution et la dérivation, ce qui réduit mathématiquement la largeur des bandes d'absorption et permet la visualisation des bandes qui se chevauchent (Garidel and Schott 2006).

Notre expérience a été effectuée à pH 7,4 où l'albumine est sous sa forme neutre « N » (Fasano et al., 2005). Également nous avons utilisé la bilirubine pour évaluer le site de fixation des molécules d'intérêts.

En comparant les spectres d'émission de la SAH et des stéroïdes (progestatifs et glucocorticoïdes) obtenus à  $\lambda_{exc} = 295$  nm, on observe que les stéroïdes fluorescent très faiblement à la longueur d'onde d'émission maximale de la SAH. Ces résultats permettent ainsi d'étudier l'interaction des stéroïdes avec la SAH. L'addition des stéroïdes à la SAH, à des rapports variant de 0 à 10, induit une diminution de la fluorescence de la SAH. Ceci suggère que les molécules d'intérêts se lient à la SAH entraînant des modifications dans l'environnement du résidu Trp 214.

Nos résultats ont montré une représentation linéaire de « Stern-Volmer » indiquant la présence d'une molécule fluorescente accessible au ligand. Par la suite, les progestatifs et les glucocorticoïdes interagissent d'une façon modérée avec la SAH vu que la constante d'association ( $K_a$ ) est de l'ordre de 10<sup>5</sup>. La liaison de ces molécules au site I est maintenue par des interactions hydrophobes.

L'analyse des résultats spectroscopiques en présence du ligand spécifique du site I (bilirubine) montre que l'interaction de ce dernier avec la SAH n'est pas affectée par la présence des stéroïdes. De plus, l'emplacement des bandes des deux amide I et II de la SAH a décalé ainsi qu'une variation est observée dans la région amide I, suggérant la formation des liaisons hydrogènes entre les stéroïdes d'intérêts et la SAH. Également, la structure secondaire de l'albumine a été faiblement altérée en présence des progestatifs et des glucocorticoïdes.

Ce chapitre est présenté sous forme d'une revue bibliographique et d'un article.

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## 2.1 Interaction of triterpenoids with Human Serum Albumin: a review

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**Submitted (2017) “Chemistry and Physics of Lipids”**

**Abstract**

Triterpenoids are a large group of natural and synthetic products. This review deals with the current state of knowledge on their interaction with serum albumin. The binding of drugs to albumin may control their distribution in tissues. In literature, different techniques were used to investigate the albumin-triterpenoid interaction and include fluorescence spectroscopy, Fourier transform infrared spectroscopy, circular dichroism, calorimetric techniques and molecular modeling. Changes in fluorescence intensity of albumin were observed upon triterpenoid-albumin complex formation. Thermodynamic analyses proved that hydrophobic interactions and hydrogen bonds were the mainly binding forces in triterpenoid-albumin systems. Molecular docking and site marker competitive experimental results revealed that triterpenoids bound to Sudlow's site I of albumin. Furthermore, Fourier transform infrared spectroscopy and circular dichroism spectra analysis indicated that the native conformation of the protein is affected upon binding to triterpenoids.

**Keywords:** albumin; binding; triterpenoids.

**Abbreviations:**

BSA: bovine serum albumin; CD: circular dichroism; FTIR: Fourier transform infrared spectroscopy; HSA: human serum albumin.

## Introduction

Any drug, whether applied orally, intravenously, sublingual, subcutaneous or intramuscularly, is transported by the blood and its first encounter is not the various cellular components but the plasma proteins (Kratz and Elsadek 2012). The complexity of the human plasma proteome is immense and contains about 100,000 proteins (Kratz and Elsadek 2012). Albumin is by far the most abundant protein with a concentration of ~35-50 mg/mL (Kratz 2008). The concentration of other major plasma proteins is much lower (Kratz and Elsadek 2012). Drugs can bind to albumin and other plasma proteins, such as  $\alpha_1$ - acid glycoprotein, lipoproteins and immunoglobulins, but due to the large excess and small size of albumin, this protein is predominantly involved in drug-protein interaction (Kratz and Elsadek 2012). Albumin is responsible for the colloid osmotic pressure of the blood (Kratz 2008) and has an anticoagulant effect (Mulvihill et al., 1990).

Terpenoids represent the largest class of secondary metabolites. Many terpenoids have pronounced pharmacological activities and are therefore interesting for medicine and biotechnology (Ashour et al., 2010). Hence, it would be valuable to determine their interaction with human serum albumin (HSA) because albumin modulates distribution and bioavailability of the drugs, decreases their toxicity and control their final therapeutic efficiency (Sleep et al., 2013; Lee and Wu 2015).

In this review we focused on the interaction between albumin and triterpenoids (TTPs). The binding constants of TTPs to albumin determined in literature figure in a recapitulative table. The effect of TTPs on the albumin secondary structure is also presented. Moreover, the role of TTPs structure and the hydrophobicity on the interaction binding mode is analyzed.

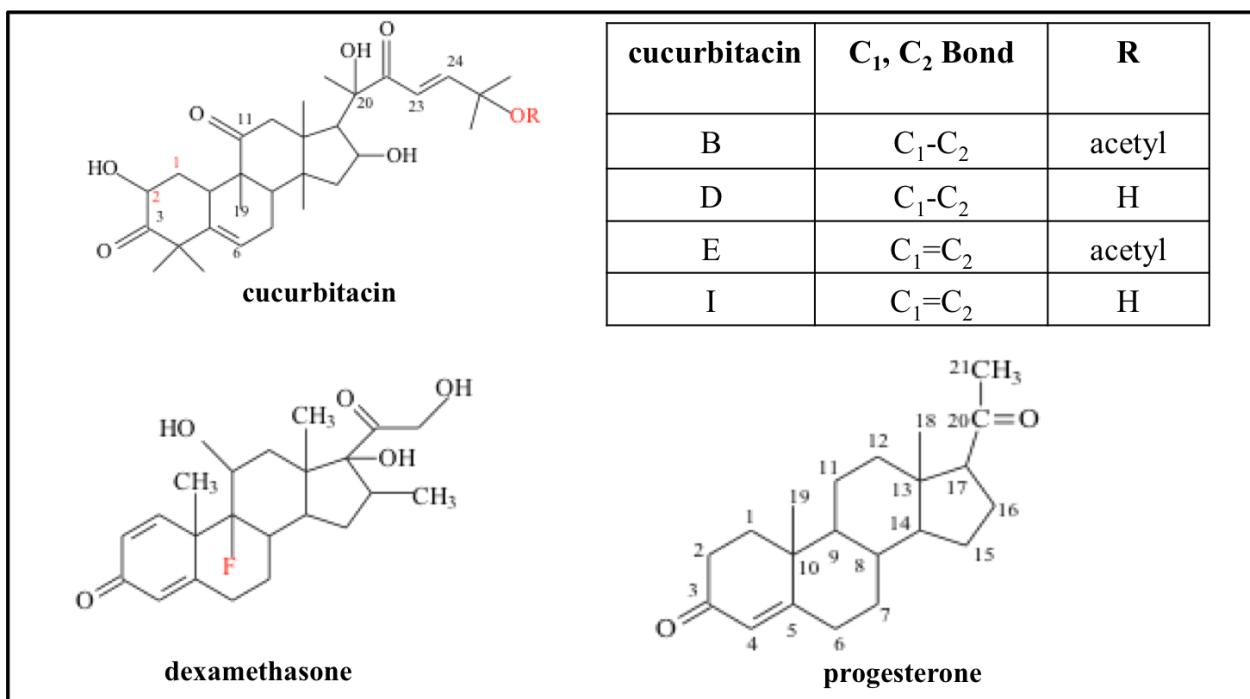
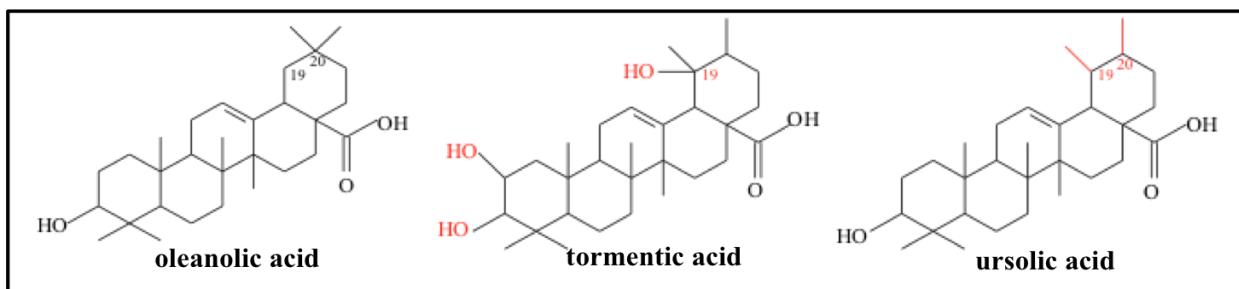
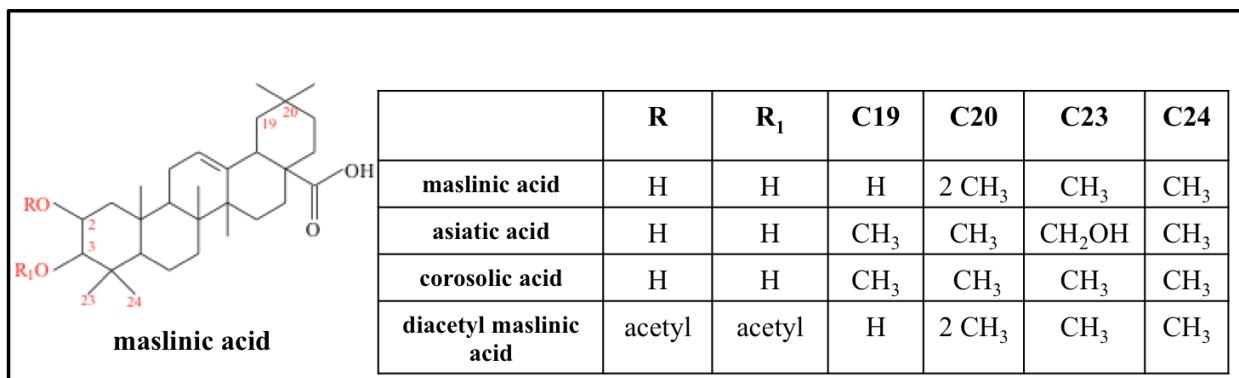
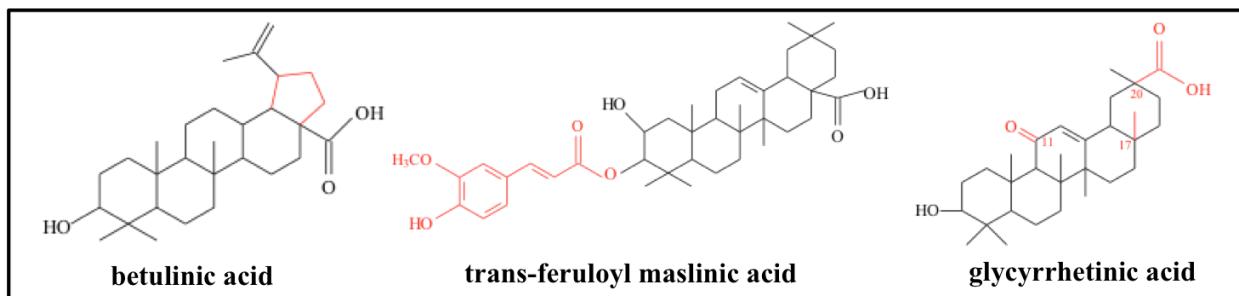
## Triterpenoids

TTPs are a large group of natural products derived from C<sub>30</sub> precursors. The TTP group displays well over 100 distinct skeletons (Xu et al., 2004). The first step in TTP biosynthesis is the cyclization of 2,3-oxidosqualene catalyzed by oxidosqualene cyclase (Vincken et al., 2007; Sawai and Saito 2011). After these cyclizations, subsequent rearrangements can proceed in different ways by a series of hydride shifts and/or methyl migrations, which lead to the formation of new carbocations. The type of cyclase that is involved in the cyclization reaction primarily determines the skeleton that is formed. Different kinds of cyclases (cycloartenol synthase, lanosterol synthase,  $\beta$ -amyrin synthase) have been described (Vincken et al., 2007; Sawai and Saito 2011).

The cyclization of oxidosqualene to saponins can progress in two ways, either via the ‘*chair-chair-chair*’ or via the ‘*chair-boat-chair*’ conformation. The cyclization of the ‘*chair-chair-chair*’ conformation results in the tetracyclic dammarenyl C20 carbocation, which leads to the 5-membered ring and produces the pentacyclic lupane, hopane, oleanane, taraxasteranes and ursane triterpenes (Vincken et al., 2007). Whereas if oxidosqualene is in a ‘*chair-boat-chair*’ conformation, cyclization leads to a tetracyclic protosteryl C20 carbocation, which produces cucurbitanes, cycloartenes and lanostanes. Lanosterol can also undergo demethylation and isomerisation of the double bond, leading to cholesterol then to steroids (Vincken et al., 2007). TTPs with well-characterized biological activities include sterols, steroids and saponins (Xu et al., 2004).

TTPs are known for their anti-inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory and tonic effects (Dzubak et al., 2006). Steroid hormones control many developmental and physiological processes (Miller and Auchus 2011) and are involved in the intrauterine fetal development, bone maturation and other functions (Falkenstein et al., 2000). They affect many systems such as cardiovascular and central nervous systems (Olive 2002).

Figure 1 represents the chemical structures of TTPs that appear throughout the review. They are classified into groups according to their structural similarity. Figure 1(a) contains the structures of tetracyclic TTPs and Figure 1(b) those of pentacyclic ones.

**Figure 1(a)** Chemical structures of tetracyclic TTPs.**Figure 1(b)** Chemical structures of pentacyclic TTPs.

## Albumin

Albumin is synthesized and secreted from the liver, where it is produced at a rate of approximately 0.7 mg/h for every gram of liver (10-15 g daily) (Kratz 2008). Albumin is not stored by the liver but is secreted into the portal circulation as soon as it is manufactured (Nicholson et al., 2000).

HSA is characterized by an abundance of charged residues, such as Lys, Arg, Glu and Asp (Nicholson et al., 2000) and has a negative charge at normal blood pH (Busher 1990) with a molecular weight of 66.5 kDa (Soni 2004; Lee and Wu 2015). It exhibits an average half-life of 19 days (Kratz 2008).

Structurally, HSA is mostly composed of  $\alpha$ -helices with an overall structure that resembles a heart shape. It is a single non-glycosylated polypeptide of 585 amino acids. It contains 35 cysteine residues, and all of them except one, Cys34, are involved in disulfide bonds stabilizing the structure of HSA (Sugio et al., 1999; Yang F et al., 2014). Albumin has nine double loops spanning three homologous domains, named I (residues 1-195), II (196-383) and III (384-585) (Fasano et al., 2005). Each domain is made up by two separate helical subdomains (named A and B), connected by random coil. Thus, the protein has subdomain IA and IB in domain I, subdomain IIA and IIB in domain II and subdomain IIIA and IIIB in domain III. Terminal regions of sequential domains contribute to the formation of interdomain helices linking domain IB to IIA, and IIB to IIIA, respectively (Fasano et al., 2005). Subdomains with separate helical structures mediate HSA binding with various endogenous and exogenous ligands (Lee and Wu 2015). HSA possesses a single tryptophan (Trp 214) located in subdomain IIA.

Bovine serum albumin (BSA) is also used to study albumin-drug interactions (Cheng and Zhang 2008 and Peng et al., 2014). It shares a 76% sequence homology with HSA (Samanta et al., 2010). BSA consists of 583 amino acid residues (Samanta et al., 2010) and has two Trp residues. Trp-212 is located in subdomain IIA and Trp-134 lies in the subdomain IA (Samanta et al., 2010).

Albumin is able to transport numerous endogenous compounds such as fatty acids, hormones, bile acids, amino acids, metals and many metabolites. Additionally, there is a wide variety of drugs that are delivered to their targeting organs/tissues by binding with albumin (Yang F et al., 2014). While the albumin domains have similar structure, each domain has particular ligand-binding properties. Albumin possesses six binding sites (I to VI) distributed along the molecule. Two principal hydrophobic binding sites are known: site I and site II located respectively in

subdomain IIA and IIIA (Sudlow et al., 1975). These sites are presented in this review as being the most concerned in the binding of TTPs.

### Site I

Site I is a big hydrophobic cavity that can hold several drugs at the same time (Ghuman et al., 2005). It is a pre-formed binding pocket within the core of subdomain IIA that comprises all six helices of the subdomain and a loop-helix feature (residues 148-154) contributed by IB (Ghuman et al., 2005). The interior of the pocket is predominantly apolar formed by hydrophobic side chains but contains two clusters of polar residues, an inner one towards the bottom of the pocket (Tyr150, His242, Arg257) and an outer cluster at the pocket entrance (Arg222, Arg218, Lys199, Lys195) (Ghuman et al., 2005). The large binding cavity is comprised of a central zone from which extend three distinct compartments. The back end of the pocket is divided by Ile264 into left and right hydrophobic subchambers, whereas a third subchamber protrudes from the front of the pocket, delineated by Phe211, Trp214, Ala215, Leu238 and aliphatic portions of Lys199 and Arg218 (Ghuman et al., 2005).

Site I is known to bind a bulky heterocyclic anion with a negative charge localized in the middle of the molecule, such as warfarin, phenylbutazone, 3,5-diiodosalicylic acid and azapropazone (Fasano et al., 2005; Peng et al., 2014; Lee and Wu 2015). Salicylic acid primarily binds in site I but in a different location from the high-affinity binding site of warfarin, and its interaction with albumin partially overlaps with the low-affinity binding site for warfarin (Ni et al., 2006). Bilirubin, a catabolic product of hemoglobin, binds to albumin with high affinity at a site located at or near the loop 4 in domain IIA (Khan et al., 2000). Chloroform binds also in domain IIA in the close vicinity of single Trp214 and has a site at or near the bilirubin-binding site (Khan and Tayyab 2001; Ahmad et al., 2005).

### Site II

Site II corresponds to the hole of subdomain IIIA (composed of all six helices) and is well known as the indole-benzodiazepine site (Ghuman et al., 2005). The pocket is lined by hydrophobic side chains and the double disulfide bridges of helix IIIa-h3. The side chain of Arg410 is located at the mouth of the pocket while the hydroxyl of Tyr411 faces toward the inside of the pocket (Sugio et al., 1999). Ligands of site II are aromatic carboxylic acids with a negatively charged acidic group at the end of the molecule, like ibuprofen, flufenamic acid, diflunisal and diazepam (Peng et al., 2014).

## **Triterpenoid-albumin binding**

Many methods are used for the analysis of TTPs-albumin interaction; each one provides unique and/or complementary information. The list includes fluorescence, infrared and circular dichroism spectroscopies and molecular modeling.

### **Binding constants determination**

The fluorescence of HSA and BSA comes from Trp, Tyr and Phe residues. Actually, the intrinsic fluorescence of HSA and BSA is almost contributed by Trp alone, because Phe has a very low quantum yield and the fluorescence of Tyr is almost totally quenched if it is ionized or near an amino group, a carboxyl group or a Trp residue (Sulkowska 2002; Samanta et al., 2010; Molina bolivar et al., 2015). The Trp residues (Trp 214 of HSA and Trp 212 of BSA) are located in site I and commonly used to study the binding of molecules to albumin by fluorimetric titration (Abou-Khalil et al., 2009; Samanta et al., 2010). All the studied TTPs had no intrinsic fluorescence at the maximal excitation wavelengths of albumin. Emission spectra of albumin recorded after excitation at 280 nm are attributed to Trp and Tyr residues while those recorded after excitation at 295 nm are attributed to the Trp residue (s) of albumin.

Table 1 resumes the parameters of the binding of TTPs to albumin (Stern-Volmer constant  $K_{sv}$ , quenching rate constant  $K_q$ , binding constant  $K_a$  and number of binding site n) published in literature.

Increase in Trp fluorescence intensity in response to TTPs binding has been reported in the literature but was less common than the quenching phenomenon. TTPs that induce increase in Trp fluorescence include betulinic acid, trans-feruloyl maslinic acid and cucurbitacins. The other TTPs quenched the fluorescence intensity of albumin. Decrease or increase of the fluorescence intensity of human albumin indicate that drugs bind to albumin and the binding site on albumin is adjacent to the sole Trp residue of the protein.

The maximum emission shift reflects polarity changes around fluorophore molecules. The TTPs molecules that induce red or blue shifts of the maximum emission of the protein are mentioned in Table 1 (\* and \*\*, respectively for blue and red shifting). The blue shift may be ascribed to increased hydrophobicity in the vicinity of the fluorophore residues as a consequence of drug binding (Naik et al., 2010; Yang H et al., 2014; Molina-Bolivar et al., 2015). However, the red-shift effect suggests that TTP bind to the hydrophobic cavity of HSA or BSA and this results in

loose structure of albumin and an increase in the polarity around the Trp residues (Tang et al., 2006). Whereas, when the maximum emission wavelength remains constant, the local dielectric environment of Trp residue is unchanged (Cheng and Zhang 2008).

Titration method consists on the addition of the drug to the protein so that various emission spectra can be obtained at various TTPs to albumin molar ratios. The Stern-Volmer quenching constant  $K_{sv}$  indicates the sensitivity of the fluorophore to a quencher. A linear Stern-Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to quencher.  $K_{sv}$  is obtained from the slope of the plot between  $F_0/F$  versus  $[Q]$  (Lakowicz 2006). The quenching rate constant ( $K_q$ ) is also calculated using the fluorescence lifetime of  $10^{-8}$  s for HSA or  $0.56 \times 10^{-8}$  s for BSA. Besides, the binding constant ( $K_a$ ) and the number of binding sites ( $n$ ) are generally determined by Scatchard method or the modified Stern-Volmer equation (Lakowicz 2006).

Stern-Volmer curves for the most of TTPs-albumin systems demonstrated a linear behavior whatever the molar ratio used, indicating the existence of a single binding site. Different patterns were showed for diacetyl maslinic acid and glycyrrhetic acid at high TTP/albumin molar ratios. For diacetyl maslinic acid, a downward curvature was obtained between 100 and 140  $\mu\text{M}$  (Molina Bolivar et al., 2015) while an upward curvature was described for glycyrrhetic acid above 15.6  $\mu\text{M}$  (Tang et al., 2006). The curvature suggested the existence of additional binding sites. It is worthy nothing that these concentrations are much higher than the physiological plasma concentrations of TTPs.

The determined  $K_{sv}$  values are presented in Table 1. When  $K_{sv}$  is inversely correlated with temperature, this suggests that the fluorescence quenching process is due to a static interaction between drugs and albumin rather than a dynamic mechanism. Both static and dynamic quenching require molecular contact between fluorophore and quencher. In the case of collisional quenching, the quencher must diffuse to the fluorophore during the lifetime of the excited state. Upon contact, the fluorophore returns to the ground state, without emission of a photon. In static quenching a complex is formed between the fluorophore and the quencher and this complex is non fluorescent (Lakowicz 2006). For all the TTP-albumin complexes, the fluorescence quenching process is proved to be static (Table 1).

In many studies, the authors reported that dynamic and static quenching can be distinguished not only by their different dependence on temperature, but also from the values of  $K_q$ . The obtained

$K_q$  values (Table 1) for TTPs-albumin complexes are much larger than the maximum dynamic quenching constant for various quenchers with biopolymer ( $2 \times 10^{10}$  Lmol $^{-1}$ s $^{-1}$ ) (Lakowicz 2006). These findings support the idea that the quenching process between TTPs and albumin is not the result of dynamic collisions but is due to the formation of a complex (Table 1). However, Van de Weert and Stella mentioned that static and dynamic quenching can be distinguished by measuring fluorescence lifetimes. Only if the lifetime decreases, dynamic quenching is present (Van de Weert and Stella 2011).

The binding constant values of the most TTPs to albumin were in the range of  $10^4$  M $^{-1}$  and  $10^5$  M $^{-1}$  indicating a moderate attachment to albumin. Highest binding constant values were reported for trans-feruloyl maslinic acid, betulinic acid and cucurbitacins, while a lowest value was determined for progesterone in a single study (Abu Teir et al., 2011) (Table 1). In addition, the values of binding sites were close to unity assuming the presence of one binding site of TTPs to albumin (Table 1).

**Table 1:** Interaction of TTPs with albumin: Stern-Volmer constant ( $K_{sv}$ ), fluorescence quenching mechanism, quenching rate constant ( $K_q$ ), binding constant ( $K_a$ ) and number of binding sites (n), and type of bonds proposed in aqueous buffered medium (pH 7.4).

[Drug] ( $\mu\text{M}$ )	[Albumin] ( $\mu\text{M}$ )	$K_{sv} (\text{M}^{-1})$	Mechanism and $K_q (\text{M}^{-1}\text{s}^{-1})$	$K_a (\text{M}^{-1})$ and n	Bonds and binding sites
[Asiatic acid] <sup>(4)</sup>	[HSA]			$3.86 \times 10^4$	
[Betulinic acid] = 10 to 100 <sup>(5; 10)</sup>	[HSA] = 25 <sup>(10)</sup>			$1.68 \times 10^6$ <sup>(10)</sup>	Hydrophobic forces <sup>(5)</sup> ; subdomains IIA and IIB <sup>(5)</sup>
[Corosolic acid] = 10 to 60 <sup>(13)</sup>	[HSA] = 2	(298K: $1.32 \times 10^4$ ) (310K: $1.21 \times 10^4$ ) (318K: $1.06 \times 10^4$ )	Static		Hydrogen bonds; site I
[Cucurbitacins] (B, D, E and I) = 0.01 to 4 <sup>(1)</sup>	[HSA] = 1			Cucurbitacin B (288K: $2.46 \times 10^6$ ; n = 1.07) (293K: $2.33 \times 10^6$ ; n = 1.02) (298K: $2.13 \times 10^6$ ; n = 1.21) (303K: $2.02 \times 10^6$ ; n = 1.17) Cucurbitacin E (288K: $2.18 \times 10^6$ ; n = 1.32) (293K: $2.00 \times 10^6$ ; n = 1.26) (298K: $1.73 \times 10^6$ ; n = 1.55) (303K: $1.54 \times 10^6$ ; n = 1.45) Cucurbitacin D (288K: $1.58 \times 10^6$ ; n = 1.31) (293K: $1.31 \times 10^6$ ; n = 1.39) (298K: $1.09 \times 10^6$ ; n = 1.35) (303K: $0.96 \times 10^6$ ; n = 1.11)	Mainly hydrophobic forces; site I and II
[Diacetyl maslinic acid] <sup>*</sup> = 6 to 140 <sup>(7)</sup>	[BSA] = 25	(298K: $2.56 \times 10^3$ ) (305K: $2.40 \times 10^3$ ) (310K: $2.07 \times 10^3$ )	Static $4.6 \times 10^{11}$	(298K: $2.2 \times 10^4$ ; n = 1.18) (305K: $3.4 \times 10^4$ ; n = 1.22) (310K: $4.5 \times 10^4$ ; n = 1.32)	Hydrophobic forces, site I
[Dexamethasone] <sup>*</sup> = 2.5 to 20 <sup>(8)</sup>	[Albumin] = 5	BSA-Dexamethasone (288K: $1.63 \times 10^4$ ) (298K: $1.41 \times 10^4$ ) (308K: $1.18 \times 10^4$ )	Static HSA-Dexamethasone $2.44 \times 10^{12}$	HSA-Dexamethasone (288K: $3.80 \times 10^4$ ; n = 1.00) (298K: $1.70 \times 10^4$ ; n = 0.99) (308K: $0.71 \times 10^4$ ; n = 1.05)	Mainly hydrophobic forces for BSA-dexamethasone.

		HSA-Dexamethasone (288K; $2.44 \times 10^4$ ) (298K; $1.83 \times 10^4$ ) (308K; $1.46 \times 10^4$ )	BSA- Dexamethasone $1.63 \times 10^{12}$	BSA-Dexamethasone (288K: $3.78 \times 10^4$ ; n = 1.07) (298K: $3.37 \times 10^4$ ; n = 1.06) (308K: $2.63 \times 10^4$ ; n = 1.06)	Hydrogen bonds and weak van der Waals forces for HSA-dexamethasone.  Site I of BSA and HSA.
[trans-feruloyl maslinic acid] = 10 to 150 <sup>(11)</sup>	[HSA] = 25			$1.42 \times 10^8$ ; n = 0.6	Three hydrogen bonds; subdomain IB
[Glycyrrhetic acid] ** = 4 to 45 <sup>(12)</sup>	[HSA] = 3		Static	[Glycyrrhetic acid] < 15.6 $\mu\text{M}$ (291K: $2.74 \times 10^5$ ; n = 0.44) (301K: $1.65 \times 10^5$ ; n = 0.43) (310K: $1.18 \times 10^5$ ; n = 0.46) (318K: $0.74 \times 10^5$ ; n = 0.54)  [Glycyrrhetic acid] > 15.6 $\mu\text{M}$ (291K: $7.32 \times 10^4$ ; n = 0.61) (301K: $4.58 \times 10^4$ ; n = 0.73) (310K: $3.92 \times 10^4$ ; n = 0.79) (318K: $3.23 \times 10^4$ ; n = 0.89)	Hydrogen bonds for the first binding site; site I.  Mainly hydrophobic forces and electrostatic interaction for the second binding site.
[Maslinic acid] = 10 to 200 <sup>(6)</sup>	[HSA] = 25	(298K: $7.12 \times 10^2$ ) (304K: $3.82 \times 10^2$ ) (310K: $3.09 \times 10^2$ )	Static $1.3 \times 10^{11}$	$5.40 \times 10^4$	Hydrogen bonds and van der-Waals forces
[Maslinic acid] = 10 to 60 <sup>(13)</sup>	[BSA] = 2	(298K: $0.56 \times 10^4$ ) (310K: $0.45 \times 10^4$ ) (318K: $0.41 \times 10^4$ )	Static		Hydrogen bonds; site I
[Oleanolic acid] * = 1 to 6 <sup>(14)</sup>	[Albumin] = 2	HSA-Oleanolic acid (298K: $5.63 \times 10^4$ ) (303K: $4.6 \times 10^4$ ) (310K: $3.1 \times 10^4$ )  BSA-Oleanolic acid (298K: $3.12 \times 10^4$ ) (305K: $1.57 \times 10^4$ )	Static	HSA-Oleanolic acid (298K: $3.48 \times 10^5$ ) (303K: $2.17 \times 10^5$ ) (310K: $0.63 \times 10^5$ )  BSA-Oleanolic acid (298K: $1.15 \times 10^5$ ) (305K: $1.05 \times 10^5$ )	Hydrophobic interactions

		(310K: $1.40 \times 10^4$ )		(310K: $0.97 \times 10^5$ )	
[Oleanolic acid] = 5 to 45 <sup>(9)</sup>	[BSA] = 1	(298K: $1.89 \times 10^4$ ) (305K: $1.78 \times 10^4$ ) (310K: $1.70 \times 10^4$ )	Static $3.3 \times 10^{12}$	(298K: $1.73 \times 10^4$ ; n = 0.99) (305K: $1.18 \times 10^4$ ; n = 0.96) (310K: $0.98 \times 10^4$ ; n = 0.96)	Hydrogen bonds and and van der Waals; site I
[Oleanolic acid] = 1.67 to 20 <sup>(3)</sup>	[BSA] = 5	(298K: $4.72 \times 10^4$ ) (305K: $3.50 \times 10^4$ ) (310K: $3.44 \times 10^4$ )	Static	(298K: $6.53 \times 10^4$ ; n = 1.06) (305K: $3.51 \times 10^4$ ; n = 0.99) (310K: $2.91 \times 10^4$ ; n = 0.89)	Hydrogen bonds and van der Waals forces
[Progesterone] = 120 to 1082 <sup>(2)</sup>	[HSA] = 601	$6.26 \times 10^2$	Static $6.20 \times 10^{10}$	$6.35 \times 10^2$	
[Tomentic acid] = 10 to 60 <sup>(13)</sup>	[HSA] = 2	(298K: $0.16 \times 10^4$ ) (310K: $0.14 \times 10^4$ ) (318K: $0.13 \times 10^4$ )	Static		Hydrogen bonds; site I
[Ursolic acid] = 5 to 45 <sup>(9)</sup>	[BSA] = 1	(298K: $1.56 \times 10^4$ ) (304K: $1.44 \times 10^4$ ) (310K: $1.37 \times 10^4$ )	Static $2.7 \times 10^{12}$	(298K: $1.50 \times 10^4$ ; n = 0.99) (304K: $1.04 \times 10^4$ ; n = 0.97) (310K: $0.83 \times 10^4$ ; n = 0.96)	Hydrogen bonds and van der Waals; site I

[\*]: Blue shift

[\*\*]: Red shift

[1] Abou Khalil et al., 2009; [2] Abou teir et al., 2011; [3] Cheng and Zhang 2008; [4] Gokara et al., 2014; [5] Malleda et al., 2012; [6] Molina-Bolivar et al., 2014; [7] Molina-Bolivar et al., 2015; [8] Naik et al., 2010; [9] Peng et al., 2014; [10] Subramanyam et al., 2009 a; [11] Subramanyam et al., 2009 b; [12] Tang et al., 2006; [13] Wang et al., 2015; [14] Yang H et al., 2014.

## Forces involved in the binding of TTPs to albumin and identification of binding sites

The interaction between drugs and biomolecules commonly proceed through multiple hydrogen bonds, van der Waals, electrostatic and hydrophobic forces (Min He and Carter 1992). The thermodynamic parameters (enthalpy, entropy and free energy change) are fundamental to estimate the binding mode and are evaluated using Van't Hoff's equation (Toneatto and Argüello 2011). Ross and Subramanian 1981 generalized the signs and magnitudes of the thermodynamic parameters associated with different solitary kinds of interactions that might settle in protein association processes. The positive entropy and the positive enthalpy are frequently taken as evidence for the hydrophobic interaction, while they are negative for van der Waals forces and hydrogen bonds shaping in low dielectric medium. Furthermore, concrete electrostatic interactions between ionic species in aqueous solution were defined by a positive value of entropy and a negative enthalpy (almost zero). A negative enthalpy is observed whenever there is a hydrogen bond in the process. Moreover, a negative value of free energy change indicates that the formation of the complex is a spontaneous process. The determination of binding constants at various temperatures followed by the calculation of thermodynamic parameters were used to study the interaction of cucurbitacins, dexamethasone, glycyrrhetic acid, maslinic acid, oleanolic acid and ursolic acid with albumin. Hydrophobic, hydrogen, electrostatic and van der Waals bonds are involved in the interaction of TTPs with the protein. The last column of table 1 resumes the literature data concerning the forces involved in the binding of TTPs to albumin.

Molecular Modeling has been used as a complementary method to investigate the interaction of betulinic acid, corosolic acid, trans-feruloyl maslinic acid, glycyrrhetic acid, maslinic acid, oleanolic acid, tormentic acid and ursolic acid with albumin (Tang et al., 2006; Subramanyam et al., 2009 b; Malleda et al., 2012; Peng et al., 2014; Wang et al., 2015). Molecular docking allows the determination of the principal binding sites of ligands to the protein. Displacement experiments using site markers allow also the identification of the binding sites. The binding parameters of ligands were determined in the absence and presence of a TTP molecule. Generally the experiments were conducted at TTP/albumin molar ratio equal to 1. Site I is involved in the binding of corosolic acid, diacetyl maslinic acid, dexamethasone, glycyrrhetic acid, maslinic acid, oleanolic acid, tormentic acid and ursolic acid to albumin (Table 1). The interaction between corosolic acid, maslinic acid or tormentic acid and HSA was dominated by hydrogen bonds (Wang et al., 2015). Arg-257, Ala-261, Lys-199 and Arg-222 residues of HSA formed intermolecular hydrogen bonds with the hydroxyl and carboxyl groups of the TTPs (Wang et al., 2015). Carbonyl groups of oleanolic acid and ursolic acid generate a hydrogen-bonds network

with the Arg-194 and Ser-201. Also, another hydrogen bond could be formed between oleanolic acid and Arg-435 (Peng et al., 2014). Many studies proved that the interaction of oleanolic acid with albumin involves hydrophobic forces (Cheng and Zhang 2008; Yang H et al., 2009 and Peng et al., 2014) where Tyr180 residue is involved in oleanolic acid-BSA interaction. The methyl groups of glycyrrhetic acid were proved to be adjacent to the hydrophobic residues Leu-219, Leu-238, Val-241, Val-216, Trp-214, etc., of subdomain IIA of HSA, suggesting that hydrophobic interactions are involved in glycyrrhetic acid -HSA interaction.

The electrostatic interaction between the COOH of oleanolic acid and the Arg-222 residue of BSA cannot be denied. Similarly, electrostatic interactions are proposed for the interaction of glycyrrhetic acid with HSA (Tang et al., 2006).

Betulinic acid is proved to interact with albumin in a region located between sub-domains IIA and IIB. The cyclohexyl groups of betulinic acid interacts with a hydrophobic binding pocket involving Phe-206, Arg-209, Ala-210, Ala-213, Leu-327, Gly-328, Leu-331, Ala-350 and Lys-351. Furthermore, hydrogen bonding interactions were observed between the hydroxyl groups of betulinic acid and Phe-206 and Glu-354 of HSA (Malleda et al., 2012).

Trans-feruloyl maslinic acid binds to HSA at the domain IB *via* three hydrogen bonds where the side chain of the feruloyl ring binds to Arg-114 and the main part of maslinic acid interacts with Leu-115 and Asp-173. This interaction produced fluorescence changes in HSA molecule even the site of interaction is not near the Trp residue (Subramanyam et al., 2009 b).

The binding of a series of cucurbitacins to albumin was proved at site I. The binding constants of warfarin, bilirubin and salicylic acid were affected upon binding of cucurbitacins (Greige-Gerges et al., 2007 a, b and Abou-Khalil et al., 2009). Moreover, Fabini et al., 2016 demonstrated that cucurbitacins were able to modulate the binding of biliverdin, a subdomain IB specific marker.

On the overall, literature data confirmed that the hydrophobic cyclic rings of TTPs interact with the apolar amino acid residues of albumin through hydrophobic interactions. Furthermore, the binding of TTPs to albumin can be stabilized by hydrogen bonds involving their carbonyl and hydroxyl groups. Site I and subdomain IB are preferentially concerned.

## Modulation of albumin secondary structure by TTPs

The effect of TTPs on albumin secondary structure has been studied in literature by Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD). The characteristic bands found in the infrared spectra of proteins and polypeptides include the amide I and amide II. The amide I ( $1700\text{--}1600\text{ cm}^{-1}$  region) absorption band arises predominantly from stretching vibrations of the C=O moieties of polypeptide amide groups. The amide II ( $1600\text{--}1480\text{ cm}^{-1}$  region) arises from the bending vibration of amide N–H groups strongly coupled to the stretching of amide C–N stretching groups. Both the C=O and the N–H bonds are involved in the hydrogen bonding that takes place between the different elements of the protein secondary structure. The locations of amide I and amide II bands are sensitive to the secondary structure content of a protein (Garidel and Schott 2006).

CD provides also a powerful method to assess the global change in protein folding as the result of ligand interaction (Drake 2001; Kelly et al., 2005). The secondary structure composition (% helix, sheets, turns...) can be obtained from FTIR and CD studies of proteins.

Table 2 resumes the amide I and II bands assignment in the absorbance spectra of albumin in the absence and presence of TTPs. The amide I and amide II peak positions shifted to lower and higher wavenumbers, indicating that TTPs interact with the CO, CN and NH groups in the protein through hydrogen-bonds.

**Table 2:** Amide I and II bands assignment in the absorbance spectra of albumin in the absence and presence of TTPs measured by FTIR.

[Drug] ( $\mu\text{M}$ )	[Albumin] ( $\mu\text{M}$ )	Amide I ( $\text{cm}^{-1}$ )	Amide II ( $\text{cm}^{-1}$ )
[Corosolic acid] = 200 <sup>(13)</sup>	[HSA] = 200	1653 → 1654	1541 → 1544
[Dexamethasone] = 5 <sup>(8)</sup>	[HSA]= 5	1659 → 1653	1549 → 1553
	[BSA]=5	1660 → 1653	1540 → 1534
[Diacetyl maslinic acid] = 80 <sup>(7)</sup>	[BSA]= 25	1654 → 1650	
[Glycyrrhetic acid] = 9 <sup>(12)</sup>	[HSA]= 3	1656 → 1637	1540 → 1522
[Maslinic acid] = 100 <sup>(6)</sup>	[BSA]= 25 <sup>(6)</sup>	1652 → 1645 <sup>(6)</sup>	
[Maslinic acid] = 200 <sup>(13)</sup>	[HSA]= 200 <sup>(13)</sup>	1653 → 1658 <sup>(13)</sup>	1541 → 1546 <sup>(13)</sup>
[Oleanolic acid] = 100 <sup>(3)</sup>	[BSA]= 50	1644 → 1641	1553 → 1541
[Progesterone] = 1081.8 <sup>(2)</sup>	[HSA]=601	1656 → 1658	1543 → 1548
[Tormentic acid] = 200 <sup>(13)</sup>	[HSA] = 200	1653 → 1657	1541 → 1545

[2] Abu teir et al., 2011; [3] Cheng and Zhang 2008; [6] Molina-Bolivar et al., 2014; [7] Molina-Bolivar et al., 2015; [8] Naik et al., 2010; [12] Tang et al., 2006; [13] Wang et al., 2015.

The CD spectra of albumin exhibits two negative ellipticities in the UV region at 208 and 220 nm, characteristic of  $\alpha$ -helical structure of protein (Cheng and Zhang 2008; Subramanyam et al., 2009 a, b; Wang et al., 2015; Peng et al., 2014). When various concentrations of TTPs (asiatic acid, betulinic acid, Corosolic acid, trans-feruloyl maslinic acid, maslinic acid, oleanolic acid and tormentic acid) were added to the protein, the intensity at 208 and 220 nm decreased, proving changes in the protein secondary structure and some loss of helical stability. TTPs bound to the amino acid residues of the main polypeptide chain of the protein producing disruption of the hydrogen bond networks (Wang et al., 2015; Subramanyam et al., 2009 a, b; Cheng and Zhang 2008; Gokara et al., 2014).

The percentage values for the components of amide I of albumin determined by FTIR and CD in the absence and presence of TTPs are summarized in Table 3. Variations of the  $\beta$ -turn, the random coils, the  $\alpha$ -helix and  $\beta$ -sheets were obtained upon TTP-albumin interaction which are indicative of an alteration of the protein secondary structure.

**Table 3:** The percentage values for the components of amide I of albumin in the absence and presence of TTPs determined by FTIR and CD.

[Drug] ( $\mu\text{M}$ )	[Albumin] ( $\mu\text{M}$ )	Methods	Amide I ( $\text{cm}^{-1}$ )			
			$\alpha$ -helix (%)	$\beta$ -sheets (%)	$\beta$ -turn (%)	Random coils (%)
[Corosolic acid] = 200 <sup>(13)</sup>	[HSA] = 200	FTIR	52 → 37	11 → 12	19 → 32	18 → 19
[Dexamethasone] = 5 <sup>(8)</sup>	[HSA] = 5	FTIR	51.4 → 37.4	40.5 → 35.8	8.1 → 26.8	
[Glycyrrhetic acid] = 9 <sup>(12)</sup>	[HSA] = 3	FTIR	50.93 → 24.73	25.46 → 25.27	23.61 → 13.98	
[Maslinic acid] = 200 <sup>(13)</sup>	[HSA] = 200	FTIR	52 → 39	11 → 11	19 → 35	18 → 15
[Progesterone] = 1081.8 <sup>(2)</sup>	[HSA] = 601	FTIR	56 → 52	13 → 10	12 → 13	14 → 12
[Tormentic acid] = 200 <sup>(13)</sup>	[HSA] = 200	FTIR	52 → 40	11 → 14	19 → 25	18 → 21
[Diacetyl maslinic acid] = 80 <sup>(7)</sup>	[BSA] = 25	FTIR	49.1 → 44.6	24.9 → 22.3	26 → 33.1	
[Dexamethasone] = 5 <sup>(8)</sup>	[BSA] = 5	FTIR	44.48 → 57.79	28.80 → 22.24	26.72 → 19.97	
[Asiatic acid] <sup>(4)</sup>	[HSA]	CD	57.50 → 50		25 → 29	
[Betulinic acid] = 100 <sup>(10)</sup>	[HSA] = 25	CD	57.90 → 53.10	12.1 → 14	13.30 → 13.70	16.70 → 19.20
[Corosolic acid] = 800 <sup>(13)</sup>	[HSA] = 2	CD	54.17 → 50.11			
[trans-feruloyl maslinic acid] = 100 <sup>(10)</sup>	[HSA] = 25	CD	57.90 → 38.9	12.1 → 15.5	13.30 → 16.1	16.70 → 29.5
[Maslinic acid] = 800 <sup>(13)</sup>	[HSA] = 2	CD	54.17 → 51.97			
[Tormentic acid] = 800 <sup>(13)</sup>	[HSA] = 2	CD	54.17 → 52.09			
[Oleanolic acid] = 16 <sup>(3)</sup>	[BSA] = 5	CD	45.1 → 36.4			
[Oleanolic acid] = 4 <sup>(9)</sup>	[BSA] = 1	CD	61.3 → 49.2	8.1 → 10.1	11.9 → 14.4	18.7 → 26.3
[Ursolic acid] = 4 <sup>(9)</sup>	[BSA] = 1	CD	61.3 → 52.1	8.1 → 9.9	11.9 → 14.2	18.7 → 23.8

[2] Abou teir et al., 2011; [3] Cheng and Zhang 2008; [4] Gokara et al., 2014; [7] Molina-Bolivar et al., 2015; [8] Naik et al., 2010; [9] Peng et al., 2014; [10] Subramanyam et al., 2009 a; [12] Tang et al., 2006; [13] Wang et al., 2015.

## Role of the chemical structure of TTPs

The analysis of the binding constants of various TTPs to albumin proved that the hydrophobicity is not the only factor that controls the interaction of tetra- and penta-cyclic TTPs with albumin (Table 4). Oleanolic acid and ursolic acid, the most hydrophobic in the series, did not prove the highest binding constant values. The addition of a hydrophobic moiety (feruloyl group) to maslinic acid improved the interaction with albumin, while diacetyl maslinic acid did not prove a higher binding constant value when compared to maslinic acid (Table 1). Also, the number of hydrogen bonds that a TTP may create is not the only element that affects the TTP-albumin interaction. Compared to many other TTPs, betulinic acid with a single hydroxyl group has a higher binding constant to albumin (Table 1).

**Table 4:** log P and binding constant ( $K_a$ ) values of TTPs.

TTPs	log P	$K_a (M^{-1})$	References
Asiatic acid	5.32	$3.86 \times 10^4$	Kartasasmita et al., 2014
Betulinic acid	6.61	$1.68 \times 10^6$	Elusiany et al., 2011
Cucurbitacin B	2.6	$2.46 \times 10^6$	Pubchem
Cucurbitacin E	3.2	$2.18 \times 10^6$	Pubchem
Cucurbitacin D	2.1	$1.58 \times 10^6$	Pubchem
Dexamethasone	1.83	$3.78 \times 10^4$	Alvarez Nunez and Yalkowsky 1997
Diacetyl maslinic acid		$2.2 \times 10^4$	
Glycyrrhetic acid	6.4	$2.74 \times 10^5$	Pubchem
Maslinic acid	6.55	$5.40 \times 10^4$	Putz et al., 2011
Oleanolic acid	7.32	$3.48 \times 10^5$ <sup>(14)</sup> $1.73 \times 10^4$ <sup>(9)</sup> $6.53 \times 10^4$ <sup>(3)</sup>	Putz et al., 2011
Progesterone	4.04	$6.35 \times 10^2$	Torres-Cartas et al., 2000
Ursolic acid	7.32	$1.50 \times 10^4$	Pubchem

[3] Cheng and Zhang 2008; [9] Peng et al., 2014; [14] Yang H et al., 2014.

**Conclusion**

The binding of penta- and tetra-cyclic TTPs to albumin is reviewed in this work. The published studies revealed for the majority of TTPs the presence of a single class of binding site. TTPs bind moderately to the protein mainly at site I and alter the protein secondary structure. Small changes in TTP structure did not produce remarkable variation in TTP-albumin interaction. Moreover, hydrophobicity is not only the main factor that controls TTP-albumin binding.

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## 2.2 Interaction of glucocorticoids and progesterone derivatives with human serum albumin

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**Submitted (2017) “Chemistry and Physics of Lipids”**

**Abstract**

Glucocorticoids (GCs) and progesterone derivatives (PGDs) are steroid hormones with well-known biological activities. Their interaction with human serum albumin (HSA) may control their distribution. Their binding to albumin is poorly studied in literature. This paper deals with the interaction of a series of GCs (cortisol, cortisone, prednisolone, prednisone, 6-methylprednisolone and 9-fluorocortisol acetate) and PGDs (progesterone, hydroxylated PGDs, methylated PGDs and dydrogesterone) with HSA solution (pH 7.4) at molar ratios steroid to HSA varying from 0 to 10. Similar titrations were conducted using Trp aqueous solution. Fluorescence titration method and Fourier transform infrared spectroscopy (FTIR) are used. PGDs (except dydrogesterone), cortisone and 9-fluorocortisol acetate affected weakly the fluorescence of Trp in buffer solution while they decreased in a dose-dependent manner that of HSA. Their binding constants to HSA were then calculated and found to be of the order of  $10^5 \text{ M}^{-1}$  indicating moderate attachment to the protein. Moreover, displacement experiment was performed using bilirubin as a site marker. The binding constant of bilirubin to albumin was determined in the absence and presence of a steroid at a molar ratio steroid to HSA of 1. The results indicate that the steroids bind to HSA at site I in a pocket different from that of bilirubin. Furthermore, the peak positions of amide I and amide II bands of HSA were shifted in the presence of progesterone, dydrogesterone and GCs. Also a variation was observed in amide I region indicating the formation of hydrogen bonding between albumin and steroids.

**Keywords:** bilirubin, glucocorticoids, Human Serum Albumin, progesterone derivatives.

**List of abbreviations:**

Bil: Bilirubin; Co: cortisol; Cn: cortisone; DYG: dydrogesterone; 9-FA: 9-fluorocortisol acetate; FTIR: Fourier Transform Infrared Spectroscopy; GC: glucocorticoid; HSA: Human Serum Albumin; 17-OHPG: 17-hydroxyprogesterone; 21-OHPG: 21-hydroxyprogesterone; MP: medroxyprogesterone; MPA: medroxyprogesterone acetate; 6-MPd: 6-methylprednisolone; Pd: prednisolone; Pn: prednisone; PG: progesterone; PGD: progesterone derivative; Trp: tryptophan.

## Introduction

Glucocorticoids (GCs) (Fig. 1) and progesterone derivatives (PGD) (Fig. 2) are a group of steroid hormones that affect many tissues in the human body (Olive 2002; Patel et al., 2014; Christiansen et al., 2007; Gupta and Bhatia 2008; Sun et al., 2012; Zhao et al., 2012). They are oxygenated tetracyclic triterpenes characterized by their basic 21-carbon skeleton called a pregnane skeleton (C21).

GCs influence the intermediary metabolism of carbohydrates (Patel et al., 2014; Christiansen et al., 2007), fats (Christiansen et al., 2007) and proteins and prove anti-inflammatory and immunosuppressive effects (Gupta and Bhatia 2008). The presence of a hydroxyl group at carbon-11 of the steroid molecule is crucial for GC activity (Gupta and Bhatia 2008). Cortisol (Co) or hydrocortisone and cortisone (Cn) are the natural human GCs. Co (Fig. 1) is released by the adrenal glands and regulated by the pituitary gland inside the brain (Kumar et al., 2007). Cn (Fig. 1) is a 11-Keto compound, derived from the peripheral metabolism of Co and lacks GC activity (Gupta and Bhatia 2008; Kumar et al., 2007).

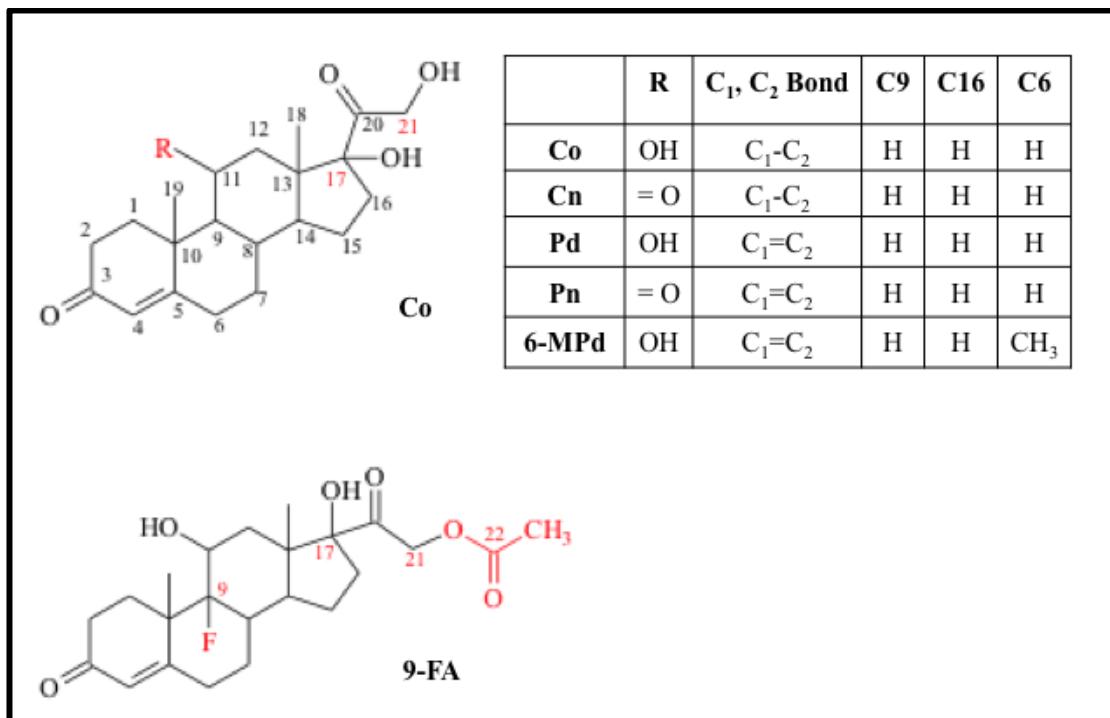
PGDs are named for their function in maintaining pregnancy. They are involved in the menstrual cycle (Olive 2002; Zhao et al., 2012) and may affect many systems such as cardiovascular and central nervous systems (Olive 2002; Sun et al., 2012). The natural human progestogens are progesterone (PG) (Fig. 2), 17-hydroxyprogesterone (17-OHPG) (Fig. 2) and 21-hydroxyprogesterone (21-OHP) (Fig. 2). 17-OHPG is obtained by enzymatic reactions with as substrates PG and 17-hydroxypregnenolone via 17-hydroxylase or  $3\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5-4}$  isomerase, respectively (Dunn et al., 1981). 21-OHPG also known as deoxycorticosterone is produced from PG by  $21\beta$ -hydroxylase (Wood 2006).

Orally administered, Co and natural progestogens show low plasma levels as they are readily inactivated by the liver (De Ziegler and Fanchin 2000; Gupta and Bhatia 2008). Especially for this reason, synthetic GCs (prednisolone (Pd), prednisone (Pn), methylprednisolone (MPd), betamethasone, dexamethasone (DEX) and fluorocortisol acetate (FA)) and progestins (medroxyprogesterone (MP), medroxyprogesterone acetate (MPA) and dydrogesterone (DYG)) were developed.

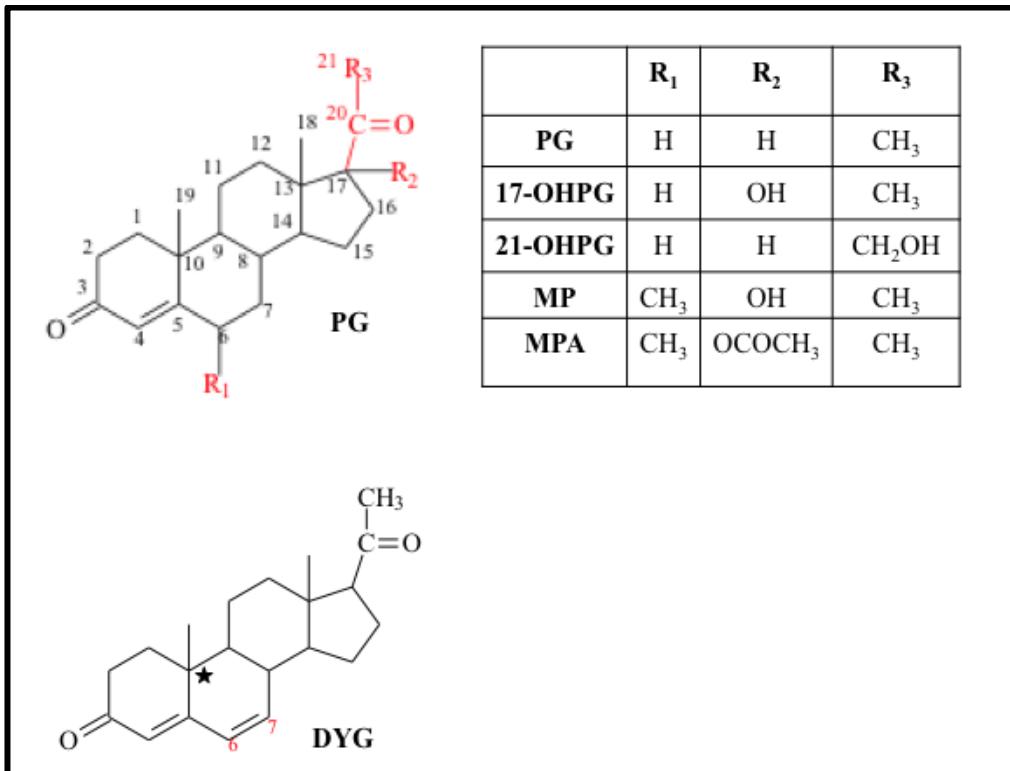
Pd (Fig. 1) differs from natural Co by the presence of an additional double bond between C1 and C2. Cn and Pn (Fig. 1) are inactive prohormones that must be reduced respectively to Co and Pd

by hepatic 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (Gupta and Bhatia 2008; Miller and Auchus 2011). 6-MPd (Fig. 1) is the methylated form of Pd at C6. 9- FA (Fig. 1) is a Co with an acetate group at C21 and a fluorine atom at C9. It is a synthetic corticosteroid with moderate GC potency and much greater mineralocorticoid activity. It is used in aldosterone replacement therapy (Gupta and Bhatia 2008; Cisternino et al., 2003).

MP (Fig. 2) is a 17-OHPG with a methyl function at C6 and MPA (Fig. 2) is an acetylated form of MP. DYG (Fig. 2) is a retroprogesterone, a stereoisomer of PG, with an additional double bond between C6 and C7. Moreover the methyl group at C10 and the hydrogen atom at C9 have inverted configurations than those in PG molecule. Consequently, the PG molecule is almost flat, whereas, the retroprogesterone molecule is bent (Schindler et al., 2003).



**Figure 1.** Chemical structure of the studied corticosteroids.



**Figure 2.** Chemical structure of the studied progesterone derivatives.

It is well known that the distribution and the metabolism of drugs are affected by drug–protein interactions in the blood stream (Hu et al., 2005; Kamat et al., 2005; Naik et al., 2009; Naik et al., 2010; Yang H et al., 2014). Moreover, this type of interaction can influence the drug stability and toxicity during therapeutic process (Naik et al., 2009). Human serum albumin (HSA) is the most abundant protein in blood plasma, accounts for about 60% of the plasma's total protein content (Cater and Ho 1994) and shows a typical blood concentration of 5g/100ml (Sugio et al., 1999). The amounts of other major plasma proteins are much lower (Kratz 2008). HSA is one of the smallest proteins present in blood plasma (Kratz 2008). It can play a dominant role in drug disposition and transport, by binding reversibly to a wide variety of endogenous and exogenous substances. Its ready availability in plasma, its biodegradability and its lack of toxicity and immunogenicity led albumin to be an integral part of drug delivery system (Kratz 2008). HSA, a heart-shaped molecule, consists of 585 amino acids that form into three structurally helical domains (referred domains I, II and III), containing 17 pairs of disulfide bridges and one free cysteine (Sugio et al., 1999). Each domain contains ten helices and is divided into six-helix and four-helix subdomains (A and B). The principal binding sites of HSA are referred to as site I and site II, and are located in the hydrophobic cavities of sub-domains IIA and IIIA, respectively. HSA possesses a single tryptophan residue (Trp 214) located in site I that is commonly used to

study the binding of molecules to albumin by fluorimetric titration (Greige-gerges et al., 2007; Abou Khalil et al., 2009; Patra et al., 2012). The fluorescence of HSA comes from Trp, tyrosine and phenylalanine residues. Actually, the intrinsic fluorescence of HSA is mainly due to the Trp residue, because phenylalanine has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group or a Trp (Sulkowska 2002). Furthermore and in order to purchase a general fundamental perception into drug-protein binding, drug-albumin interaction was considered as a model in many studies (Naik et al., 2009; Subramanyam et al., 2009a; Subramanyam et al., 2009b; Naik et al., 2010; Molina-Bolivar et al., 2014; Yang H et al., 2014).

Plasma protein binding of drugs may cause interference with the binding of other endogenous and/or exogenous ligands as a result of overlap of binding sites and/or conformational changes. Despite, the physiological and therapeutic importance of GCs and PGDs, the studies on their interaction with albumin are very limited. The previous studies concerned Co, DEX, Pd, Pn and PG (Naik et al., 2010; Rocci et al., 1982; Boudinot and Jusko 1984; Abu teir et al., 2011). To our knowledge no data are available concerning the binding of other GCs and PGDs to albumin.

In this study, fluorescence spectroscopy is used to investigate the binding of series of GCs and PGDs to albumin. Studies are conducted by direct titration method and the data are used to determine the binding constants. Also, the binding of bilirubin (Bil) (site I ligand) to albumin is studied in the absence and presence of the steroids. Besides, FTIR is used to evaluate their effect on the albumin secondary structure.

## Materials and Methods

### *Chemicals*

HSA (99%), Co, Pn, potassium phosphate monobasic ( $\text{KH}_2\text{PO}_4$ ) and sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ ) were purchased from Sigma-Aldrich, USA. Cn, Pd and 6-MPd were purchased from Sigma-Aldrich, China. 9-FA and Bil were purchased from Sigma-Aldrich, Italy and Switzerland, respectively. PG, 21-OHPG, 17-OHPG, MP, MPA, DYG and dimethylsulfoxide (DMSO) were from Sigma-Aldrich, France. Ethylenediaminetetraacetic acid and NaOH were from Merck, Germany.

### *Preparation of solutions*

Stock standard solutions of the steroids (1 mg/ml) were prepared in DMSO. HSA and Trp

solutions (5.0  $\mu\text{M}$ ) were prepared in 0.1 M phosphate buffer (pH 7.4). The Bil solution was prepared by dissolving 10 mg of Bil in 10 ml of 5 mM NaOH solution containing 1 mM ethylenediaminetetraacetic acid and completed to 100 ml with 0.1 M phosphate buffer (pH 7.4).

#### *Fluorescence spectroscopy measurements*

The fluorescence emission spectra were carried out on a spectrofluorimeter (Hitachi, F-7000 Fluorescence Spectrophotometer, Japan). The slits were set at 2.5 nm for excitation and emission. The path length of the sample quartz cuvette was 1 cm.

The intrinsic fluorescence of Trp was measured by exciting the solutions (Trp and albumin) at 295 nm and emission spectra were recorded between 200–450 nm.

#### *Fluorimetric titration*

Aliquots from a steroid stock solution were removed and added to 3 ml of 0.1 M phosphate buffer (pH 7.4), Trp (5.0  $\mu\text{M}$ ) or HSA solution (5.0  $\mu\text{M}$ ). The emission spectra were recorded. The molar ratios of steroid/Trp or steroid/HSA varied from 0 to 10 (0; 0.1; 0.15; 0.18; 0.22; 0.25; 0.5; 0.75; 1; 2.5; 5; 7.5 and 10).

For correction of the inner filter effect, a spectrophotometer (Thermo Electron Corporation, England) with a 1cm path length cuvette was used.

$$F_{corrected} = F_{measured} \times 10^{\frac{[A_{\lambda_{exc}} + A_{\lambda_{em}}]}{2}}$$

$$F_{corrected} = F_{measured} \times H(1)$$

In this equation,  $A_{\lambda_{exc}}$  is the absorbance of the drug at the protein excitation wavelength ( $\lambda_{exc}$  295 nm) and  $A_{\lambda_{em}}$  ( $\lambda_{em}$  342 nm) is the absorbance of the drug at the protein emission wavelength. Factor 2 assumes that the absorption and emission take place at the center of the cell. Measurements of absorbance were done at steroid concentrations ranging from 0 to 50  $\mu\text{M}$  in 0.1 M phosphate buffer (pH 7.4).

#### *Determination of the binding parameters of Bil to albumin in the absence and presence of steroids*

The protein concentration was 5.0  $\mu\text{M}$ . The fluorescence quenching titration was used to determine the binding constant of Bil–HSA in the absence and presence of a steroid at a molar ratio steroid/HSA of 1. For bilirubin, aliquots were removed and added to HSA solution (5.0  $\mu\text{M}$ ) with and without a steroid. The molar ratio of Bil to HSA varied between 0 and 2.

### *Fourier Transform Infrared Spectroscopy*

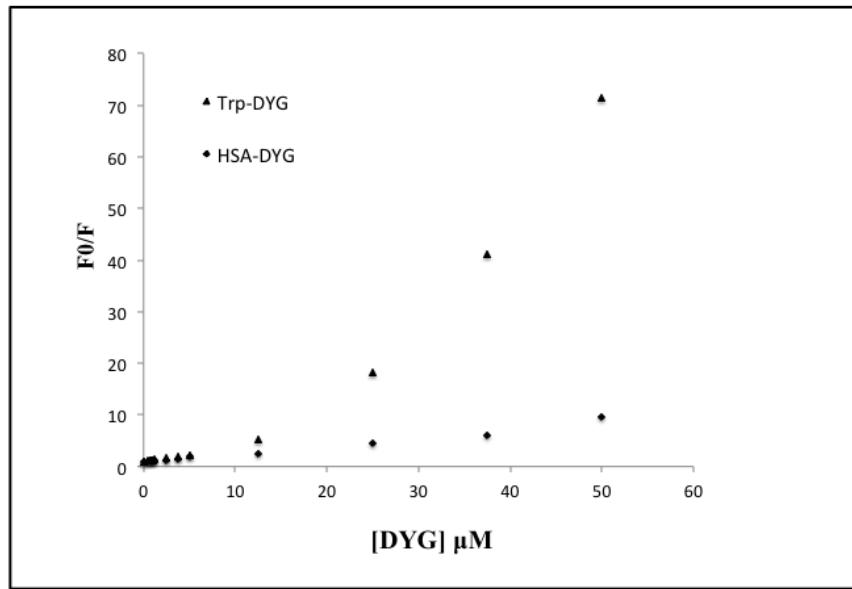
FTIR spectra were obtained in the frequency range between 4000 and 400  $\text{cm}^{-1}$  and recorded on a JASCO 6300 spectrophotometer, Japan. FTIR samples were prepared in the form of potassium bromide pellets. Interferograms were averaged for 60 scans at 4  $\text{cm}^{-1}$  resolutions. The water and the  $\text{CO}_2$  bands were subtracted. Baseline correction, normalization and peak areas calculations were performed for all the spectra by ORIGIN software.

## **Results and discussion**

### *Analysis of fluorescence quenching of Trp and albumin solutions by steroids*

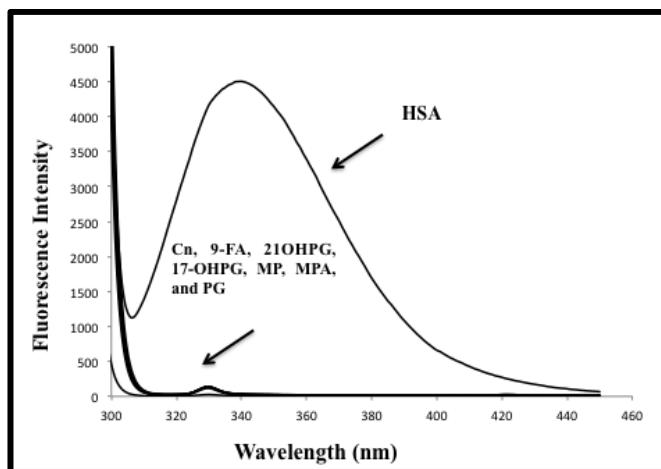
For the determination of all emission spectra,  $\lambda_{\text{exc}}$  was fixed at 295 nm. The emission spectra of Trp and albumin solutions demonstrated maximum fluorescence values at 354 and 342 nm, respectively. The difference can be attributed to the increased hydrophobicity in the vicinity of Trp residue in the albumin molecule. The absorption by Tyr is practically negligible above 295 nm while Trp absorption is still significant at this wavelength. Therefore, excitation at 295 nm provides an emission spectrum due to Trp residues only (Bobone et al., 2014). The single Trp residue of HSA is located at position 214.

Except DYG, all the studied molecules showed, at concentrations below 50  $\mu\text{M}$ , low absorbance values at excitation and emission maximal wavelengths of albumin such that the H factor (Eq. 1) was negligible. Therefore titrations were performed between 0 and 50  $\mu\text{M}$ . The H factor of DYG was not negligible above 12.5  $\mu\text{M}$  due to a high absorbance at the excitation maximum wavelength of albumin. Besides, Co, DYG, Pd, Pn and 6-MPd decreased the fluorescence of Trp solution in a dose-dependent manner. Consequently, the study of their interaction with albumin was abandoned by fluorimetric titration method. Fig. 3 showed the variation of  $F_0/F$  as a function of DYG concentration chosen as example.



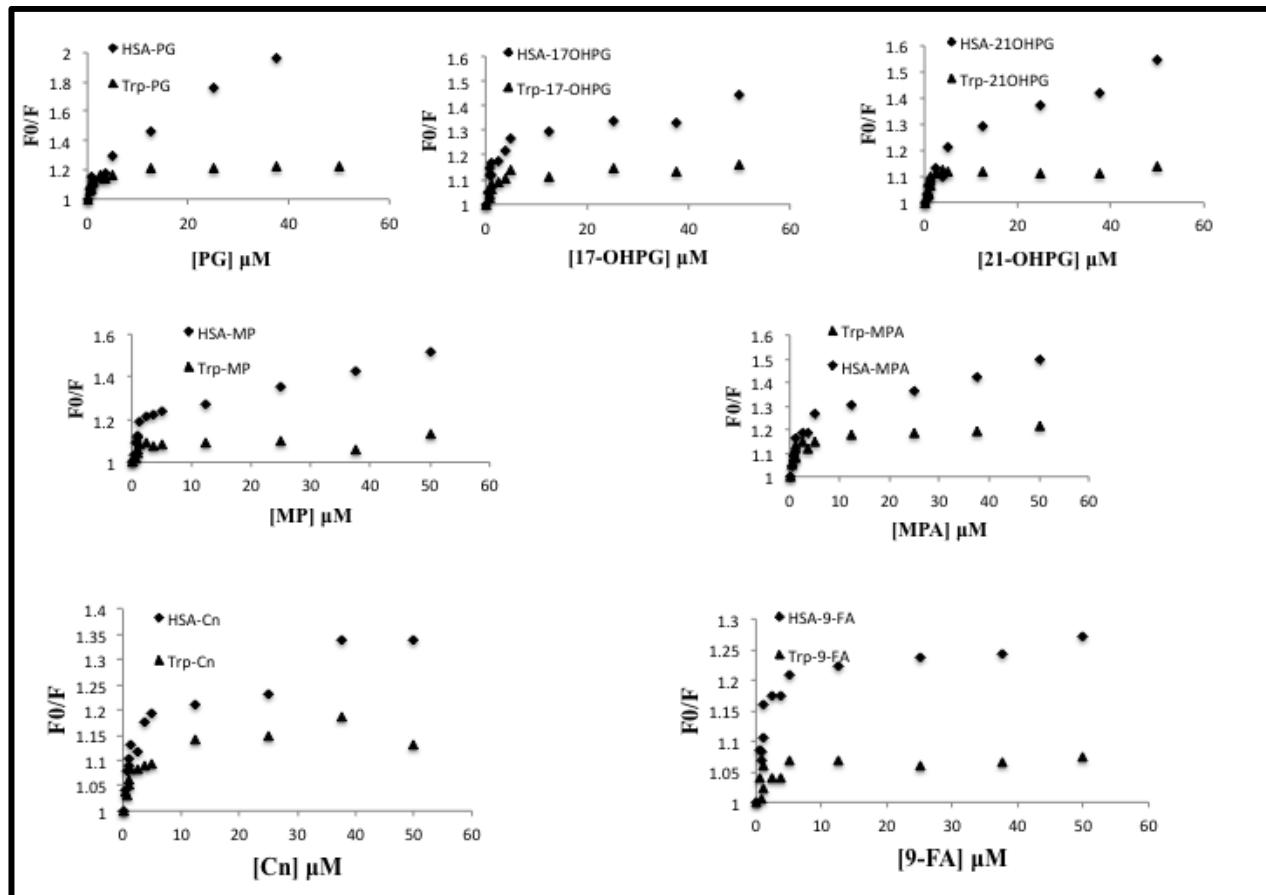
**Figure 3.** Variation of  $F_0/F$  as a function of DYG concentration using Trp and albumin solutions ( $5.0 \mu M$ ).

Fig. 4 represents the emission spectra of remaining steroids (Cn, 9-FA, 21-OHPG, 17-OHPG, MP, MPA and PG) at a concentration of  $50 \mu M$  with respect to that of HSA ( $5.0 \mu M$ ). These steroids did not demonstrate any fluorescence at  $342 \text{ nm}$  indicating that the fluorescence spectroscopy is a suitable technique for the study of their interaction with albumin. Cn, 9-FA, 21-OHPG, 17-OHPG, MP, MPA and PG showed weak effect on the fluorescence of Trp in buffer whatever was the steroid to Trp molar ratio, while they demonstrated a pronounced effect on albumin fluorescence.



**Figure 4.** Emission spectra of HSA ( $5.0 \mu M$ ) and steroids ( $50 \mu M$ ) in phosphate buffer (pH 7.4),  $\lambda_{\text{exc}} = 295 \text{ nm}$ .

Figure 5 represents the variation of  $F_0/F$  as a function of steroids concentration using Trp or albumin solution. The fluorescence intensity of albumin decreased regularly with the increase of steroids concentration indicating that these steroids bind to HSA. The results are in accordance with literature concerning the binding of DEX (Naik et al., 2010) and PG (Abu teir et al., 2011) to albumin. Also, the maximum emission wavelength of HSA was not shifted upon the addition of the studied molecules suggesting that their binding did not modify the hydrophobicity in the vicinity of Trp residue.



**Figure 5.** Variation of  $F_0/F$  as a function of Cn, 9-FA, 17-OHPG, 21-OHPG, MP, MPA and PG concentration using Trp and albumin solutions (5.0  $\mu M$ ).

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample (Lakowicz 2006). The decrease in intensity is described by the Stern-Volmer equation (2):

$$F_0/F = 1 + K_{sv} [Q] \quad (2)$$

where  $F_0$  and  $F$  are the fluorescence intensity before and after the addition of the quencher, respectively.  $Q$  is the concentration of the quencher. The Stern-Volmer quenching constant  $K_{sv}$  indicates the sensitivity of the fluorophore to a quencher. Linear curves were plotted according to

the Stern-Volmer equation for steroid concentration ranging between 12.5 and 50  $\mu\text{M}$ . The value of  $K_{sv}$  was obtained from the slope of the plot between  $F_0/F$  versus  $[Q]$ . We can notice from Table 1, that the Stern-Volmer constant for the studied GCs and PGDs is of the order of  $10^3$ .

Our results are consistent with the literature since the values of  $K_{sv}$  determined for DEX and PG are  $1.83 \times 10^4 \text{ M}^{-1}$  and  $6.26 \times 10^2 \text{ M}^{-1}$ , respectively (Naik et al., 2010; Abu teir et al., 2011).

### **Determination of the binding constant**

The binding constants ( $K_a$ ) of GCs and PGDs to HSA were determined according to the following equation (Van de Weert and Stella 2011):

$$\frac{F_0 - F}{F_0 - F_c} = \frac{[P]_t + [L]_a + K_d - \sqrt{([P]_t + [L]_a + K_d)^2 - 4 * [P]_t * [L]_a}}{2 * [P]_t} \quad (3)$$

where  $F$  is the measured fluorescence,  $F_0$  the starting fluorescence,  $F_c$  the fluorescence of the fully complexed protein,  $K_d$  the dissociation constant,  $[P]_t$  the concentration of protein, and  $[L]_a$  the concentration of added ligand.

As  $F_c$  and  $K_d$  are two unknowns, a mathematical software program was used to determine  $K_d$ ; the  $K_a$  was then calculated and the values are presented in Table 1. The binding constants values of the studied steroids to albumin are of the same magnitude ( $10^5 \text{ M}^{-1}$ ), suggesting that the hydroxylation, methylation or acetylation of the steroid core doesn't affect strongly the steroid-albumin interaction. GCs and PGDs are largely unionized under the experimental conditions. Hence, electrostatic interaction could be excluded from the binding process. In addition, site I and II are the principal regions of ligand binding to HSA and are located in hydrophobic cavities. The studied steroids involve four hydrophobic rings that may interact with apolar amino acid residues of albumin through hydrophobic interactions. Furthermore, hydrogen bonds may also be involved in the binding process. Moreover, the  $K_a$  values were found in accordance with those reported in the literature for DEX (Naik et al., 2010) and pentacyclic triterpenes like the gycyrrhetic acid (Tang et al., 2006) and oleanolic acid (Yang H et al., 2014), where their  $K_a$  values to albumin are in the order of  $10^4$  and  $10^5 \text{ M}^{-1}$ , respectively at 298K. Using equilibrium dialysis, weaker interaction of Co, Pd and Pn to HSA was reported. The  $K_a$  values were  $2.79 \text{ M}^{-1}$  for Co-HSA (Rocci et al., 1982),  $4.32 \times 10^2 \text{ M}^{-1}$  for Pd-HSA (Rocci et al., 1982),  $10^3 \text{ M}^{-1}$  for Pd-HSA (Boudinot and Jusko 1984) and  $10^3 \text{ M}^{-1}$  for Pn-HSA (Boudinot and Jusko 1984).

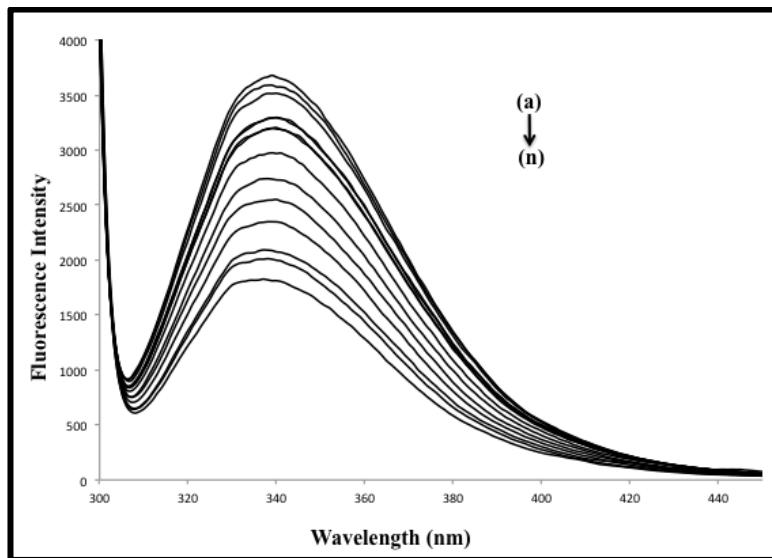
**Table 1** Stern-Volmer and binding constants of the complex HSA–steroid at pH 7.40. Values are the means of triplicate experiments  $\pm$  S.D.

<b>Drugs</b>	<b>K<sub>sv</sub> (<math>\times 10^3</math> M<sup>-1</sup>)</b>	<b>K<sub>a</sub> (<math>\times 10^5</math> M<sup>-1</sup>)</b>
<b>Cn</b>	3.13 $\pm$ 0.78	8.98 $\pm$ 1.70
<b>9-FA</b>	1.20 $\pm$ 0.34	3.19 $\pm$ 0.40
<b>17-OHPG</b>	3.75 $\pm$ 0.34	1.25 $\pm$ 0.31
<b>21-OHPG</b>	7.44 $\pm$ 1.22	1.96 $\pm$ 0.60
<b>MP</b>	5.79 $\pm$ 0.61	2.13 $\pm$ 0.25
<b>MPA</b>	5.89 $\pm$ 1.86	1.20 $\pm$ 0.16
<b>PG</b>	16.3 $\pm$ 2.45	2.86 $\pm$ 0.12

### ***Effect of steroids on the Bil binding to HSA***

Site 1 is a pre-formed binding pocket within the core of subdomain IIA that comprises all six helices of the subdomain and a loop-helix feature (residues 148–154) contributed by IB. The interior of the pocket is predominantly apolar but contains two clusters of polar residues, an inner one towards the bottom of the pocket (Y150, H242, R257) and an outer cluster at the pocket entrance (K195, K199, R218, R222) (Ghuman et al., 2005). Hence, site I contains three sub-chambers that can be occupied more specifically by salicylic acid (Ni et al., 2006), bilirubin (Ascenzi and Fasano 2009) and warfarin (Yang F et al., 2014). Some drugs may interact with one of the three pockets more than with the others.

The fluorescence spectra of HSA obtained in the absence and presence of various concentrations of Bil are shown in Fig. 6. The K<sub>a</sub> value of Bil-HSA determined in our experiment in the absence of steroid (Table 2) is in accordance with literature (Berde et al., 1979; Greige-Gerges et al., 2013). GCs and PGDs did not affect the binding of Bil to albumin suggesting that they did not share the same sub-chamber. We probably propose that the tested steroid molecules might bind to warfarin or salicylic acid pocket.



**Figure 6.** Fluorescence emission spectra of HSA in the absence and presence of Bil at molar ratios Bil to HSA ranging from 0 to 2: (a) 0, (b) 0.01, (c) 0.025, (d) 0.05, (e) 0.075, (f) 0.10, (g) 0.25, (h) 0.50, (i) 0.75, (j) 1, (k) 1.25, (l) 1.50, (m) 1.75 and (n) 2.

**Table 2** Binding constants for the interaction of bilirubin with HSA in the absence and presence of the steroids measured by fluorimetric titration at pH 7.4. Values are the means of triplicate experiments  $\pm$  S.D. Molar ratio steroid/HSA = 1; [HSA] = 5  $\mu$ M.

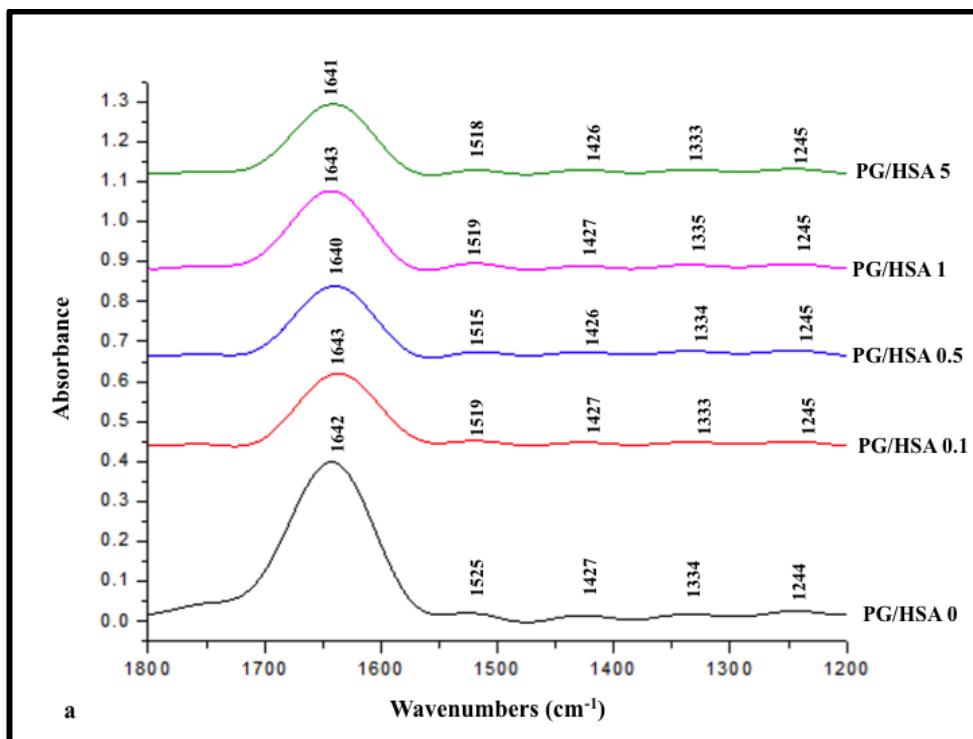
Drugs	$K_a (\times 10^6 \text{ M}^{-1})$
<b>Bil-HSA</b>	$2.16 \pm 0.72$
<b>Bil-HSA-Cn</b>	$2.56 \pm 0.09$
<b>Bil-HSA-9-FA</b>	$4.51 \pm 0.21$
<b>Bil-HSA-17OHPG</b>	$2.19 \pm 0.18$
<b>Bil-HSA-21OHPG</b>	$2.21 \pm 0.45$
<b>Bil-HSA-MP</b>	$2.19 \pm 0.52$
<b>Bil-HSA-MPA</b>	$2.34 \pm 0.09$
<b>Bil-HSA-PG</b>	$2.11 \pm 0.87$

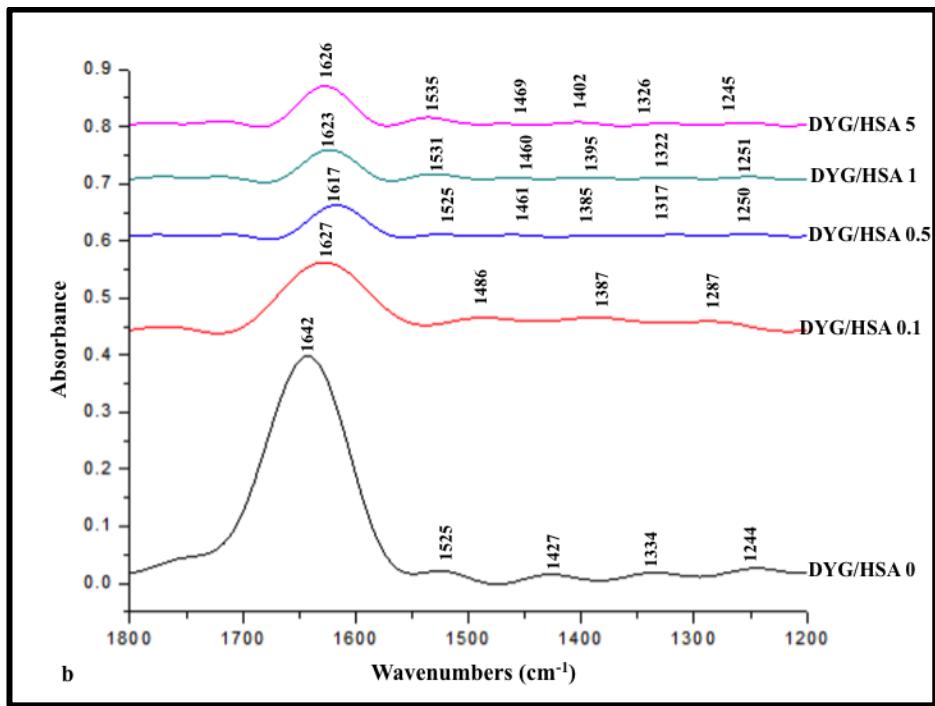
### FTIR spectroscopy

Infrared spectra of proteins exhibit a number of amide bands which result from the vibrations of the peptide groups of proteins. The modes most widely used in protein structural studies are amide I, II and III (Barth 2007). The amide I ( $1700\text{--}1600 \text{ cm}^{-1}$  region) absorption band arises predominantly from stretching vibrations of the C=O moieties of polypeptide amide groups. The amide II ( $1600\text{--}1480 \text{ cm}^{-1}$  region) arises from the bending vibration of amide N–H groups

strongly coupled to the stretching of amide C–N stretching groups, and Amide III (1330–1220 cm<sup>-1</sup> region) to the C–N groups coupled to the stretching of amide N–H stretching groups (Jackson and Mantsch 2001). The amide I band is more sensitive than amide II, and is generally employed to gather information on protein secondary structure (Barth 2007).

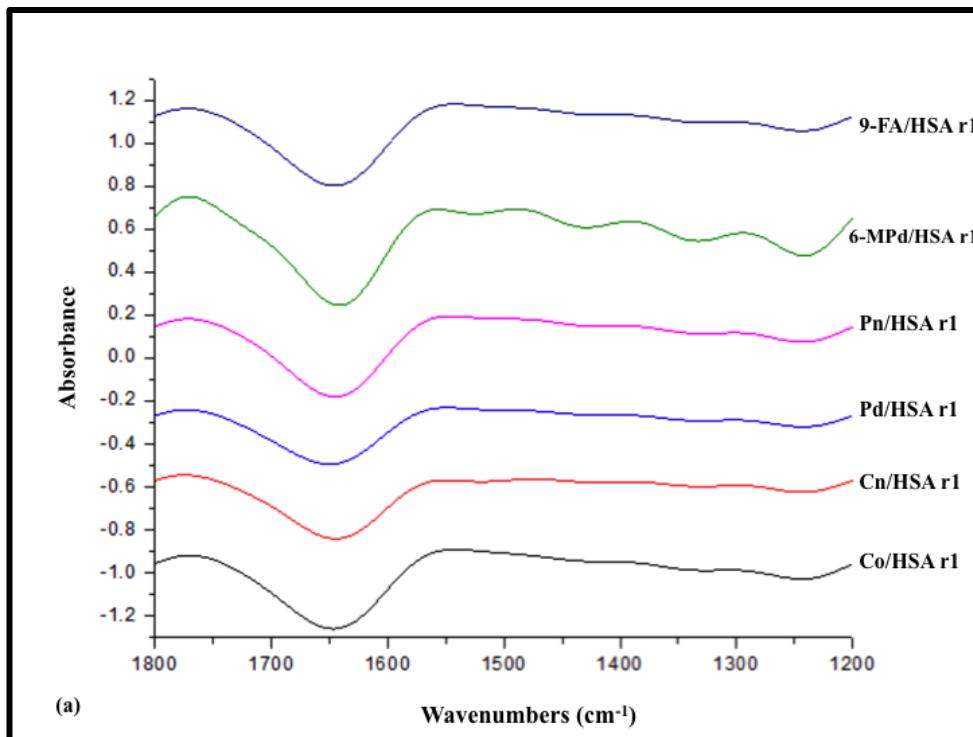
The spectra for HSA in the absence and presence of various concentrations of PG and DYG are presented in Fig. 7. The spectra are dominated by absorbance bands of amide I and amide II at peak positions 1642 cm<sup>-1</sup> and 1525 cm<sup>-1</sup>, respectively. It is obviously seen that the intensities of amide I and II decreased when PG or DYG to albumin molar ratios increased. The result is in accordance with a previous study (Abu Teir et al., 2011) regarding the effect of PG on albumin FTIR spectrum. Furthermore, for HSA-PG system, the amide I band was shifted from 1642 to 1641 cm<sup>-1</sup> while the amide II peak was shifted from 1525 to 1518 cm<sup>-1</sup> (Fig. 7a). For HSA-DYG system, the amide I band was shifted from 1642 to 1626 cm<sup>-1</sup> while the amide II peak was shifted from 1525 to 1535 cm<sup>-1</sup> (Fig. 7b). These results indicated that DYG and PG interacted with the both CO and CN groups of the protein. The other steroids affected weakly the intensities of amide I and amide II (data not shown).

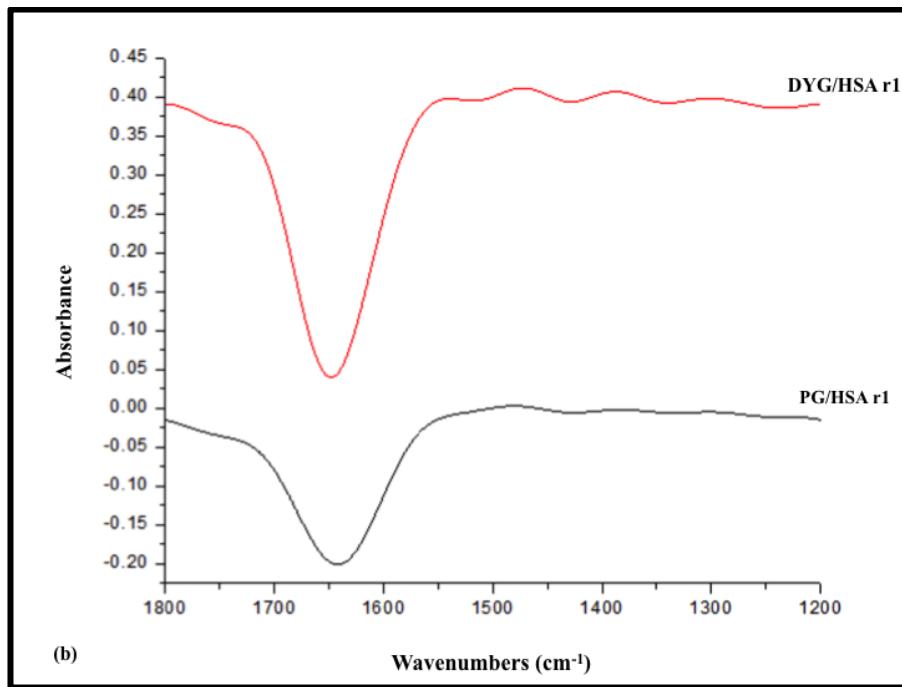




**Figure 7.** The FTIR spectra of HSA-PG (7a) and HSA-DYG (7b). The molar ratio steroid/HSA varied from 0 to 5.

The difference spectra for  $[(\text{HSA+steroid}) - (\text{HSA})]$  are shown in Fig. 8. The variation observed in the amide I band suggests that steroid molecules could be hydrogen bonded to C=O groups of the protein polypeptide chain.





**Figure 8.** Difference FTIR spectra of HSA and the GCs-HSA complexes (a) and PG/DYG-HSA complexes (b) at steroid/albumin molar ratio of 1.

The decrease and the shifts of amide I and II were more potent for DYG compared to PG suggesting a partial alteration of the protein secondary structure in the presence of DYG. The structural difference between PG and DYG is important and concerns the geometry of the molecule. Moreover, it has been reported that the interaction of PG and DYG with PG receptors (Colombo et al., 2006) and lipid membranes (Abboud et al., 2015) is different.

### Conclusion

In the present work, the binding of GCs and PGDs to HSA is studied. GCs and PGDs have been proven to bind to albumin. The association between steroids and HSA may involve hydrophobic interactions and hydrogen bonds. The binding site is located in the sub-domain IIA in a pocket different from that of Bil. This study allows a better understanding of the pharmacokinetics of steroids.

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## *Conclusion et perspectives*

L'objectif de ce travail consiste à étudier l'interaction des TPPs avec les membranes des vésicules lipidiques et la sérum albumine humaine dans le but de mieux comprendre leurs interactions moléculaires et leurs propriétés pharmacologiques.

La première partie expérimentale de notre thèse aborde la préparation des liposomes (MLV) par la méthode d'hydratation du film lipidique, en absence et en présence des molécules triterpéniques.

Les analyses réalisées par DSC, FTIR et Raman ont montré que tous les TPPs interagissent avec les parties polaires de la bicouche lipidique du fait de la disparition du pic de pré-transition, un changement d'intensité du pic à  $715\text{ cm}^{-1}$  et une modification des fréquences des groupements fonctionnels C=O et PO<sub>2</sub><sup>-</sup> des têtes polaires, en présence des molécules. Cependant, l'effet des molécules sur la transition de phase des phospholipides a été différent. En présence de toutes les molécules, la température de transition a diminué et la largeur de la transition à mi-hauteur du pic principal ( $\Delta T_{1/2}$ ) a augmenté. De plus, les courbes thermiques ont révélé une diminution notable de l'enthalpie de transition pour les liposomes incorporant les progestatifs, l'érythrodiol et l'uvaol et une augmentation en présence du cortisol, de la prednisolone et du 9-fluorocortisol acétate par rapport aux liposomes témoins, ce qui reflète une perturbation de l'arrangement des phospholipides. Par ailleurs, l'apparition du pic supplémentaire avec un taux de 10% de la 21-hydroxyprogestérone, de la prednisolone et du 9-fluorocortisol acétate montre que ces molécules induisent la formation de domaines hétérogènes et une séparation de phase.

Le comportement thermique de la bicouche lipidique, permet de conclure sur la localisation des molécules triterpéniques au niveau membranaire. Il apparaît que la progestérone, la 21-hydroxyprogestérone, l'érythrodiol et l'uvaol interagissent avec le cœur lipophile de la bicouche. Elles agissent comme des molécules substitutionnelles (substitutional impurities) de la membrane, en prenant la place des molécules lipidiques tout en causant des variations au niveau de la  $T_m$  et de l'enthalpie de transition. Elles s'insèrent au sein de la bicouche perturbant ainsi les fortes interactions hydrophobes entre les molécules de lipides. Alors que le cortisol, la prednisolone, le 9-fluorocortisol, la 17-hydroxyprogestérone, la medroxyprogestérone, la medroxyprogestérone acétate et la dydrogestérone agissent comme des "molécules interstitielles" (interstitial impurities) en s'intercalant entre les chaînes acyles des phospholipides comme elles provoquent seulement des changements au niveau de la  $T_m$ . Ceci suggère que ces molécules interagissent avec la partie

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hydrophobe des phospholipides mais sans s'insérer profondément dans la bicoche.

Les résultats obtenus par DSC, FTIR, Raman et polarisation de fluorescence ont montré que l'incorporation des molécules triterpéniques au niveau membranaire tend à augmenter le désordre des phospholipides, particulièrement au niveau des chaînes hydrocarbonées et par conséquent produit un effet fluidifiant. Cet effet est important pour la progestérone, la 21-hydroxyprogestérone, l'érythrodiol et l'uvaol, modéré pour la 17-hydroxyprogestérone, la medroxyprogestérone acétate, la medroxyprogestérone et la dydrogestérone et faible pour le cortisol, la prednisolone et le 9-fluorocortisol acétate.

Différents motifs structuraux ont mené à cette localisation et par la suite à leur effet fluidifiant. Lorsque les groupements hydroxyl et carbonyl de la 17-OHPG, la 21-OHPG, la MP, le Co, la Pd et le 9-FA se situent d'une façon à former une liaison hydrogène intramoléculaire, ceci diminue l'effet des TTPs sur la fluidité membranaire. Ainsi, la présence de plusieurs groupements polaires au niveau des corticostéroïdes induit la localisation de ces molécules à l'interface polaire-apolaire de la bicoche rendant les molécules moins puissantes pour affecter la fluidité. Ceci explique l'augmentation de l'enthalpie de transition en présence de ces molécules. Également, la présence d'une fonction acétyle (MPA et 9-FA) et d'une double liaison entre les carbones 6 et 7 (DYG) empêchent l'insertion des molécules au sein de la bicoche et par la suite produisent un effet minime sur la fluidité membranaire. Par ailleurs, la présence d'un groupement polaire libre (cycle D) pour la PG, l'UV et l'ER semble être un facteur essentiel dans la déstabilisation des membranes, produisant un effet significatif sur la fluidité. Au sein de la série des progestatifs, l'hydrophobicité ne semble pas être un facteur principal dans leur interaction avec la membrane. Cependant, l'hydrophobicité de l'UV et de l'ER favorise leur intégration profonde au niveau de la bicoche lipidique.

La deuxième partie expérimentale de notre thèse porte sur l'interaction des progestatifs et des glucocorticoïdes avec la sérum albumine humaine, une protéine essentielle du plasma. Pour les rapports molaires utilisés variant de 0 à 1, les stéroïdes diminuent la fluorescence du résidu Trp de l'albumine et une liaison stéroïde-albumine est ainsi démontrée. Les stéroïdes se lient à l'albumine avec des valeurs de  $K_a$  de l'ordre de  $10^5$ , suggérant un attachement modéré à la protéine. On constate que l'hydroxylation (17-OHPG et 21-OHPG), la méthylation (MP) et l'acétylation (MPA, 9-FA) n'affecte pas la liaison à la SAH. De plus, les stéroïdes se localisent au niveau du site I de

l'albumine humaine dans une sous-poche différente de celle de la bilirubine stabilisée par des interactions hydrophobes et des liaisons hydrogènes. Par ailleurs, la configuration inversée en C9 et en C10, la présence d'une double liaison entre les carbones 6 et 7 et la forme géométrique différente de la DYG induisent une altération plus prononcée de la structure secondaire de l'albumine par rapport aux autres progestatifs et glucocorticoïdes.

En conclusion, les motifs structuraux affectant la fluidité membranaire ne semblent pas être les mêmes qui contrôlent l'interaction des TTPs avec la membrane.

Les perspectives de ce travail comportent une détermination des effets des stéroïdes sur des membranes biologiques dont leur fluidité est essentielle pour leur fonction. On cite les membranes plasmatiques des spermatozoïdes dont leur fluidité dépend de leur taux en cholestérol. Ce dernier est impliqué dans le contrôle de la réaction acrosomique. Les érythrocytes peuvent constituer aussi un modèle des cellules biologiques, étant donné qu'elles sont les plus exposées aux agents externes. Des changements de la composition de la membrane érythrocytaire sont démontrés dans des cas pathologiques. De plus, tout changement dans les propriétés physico-chimiques de la membrane peut influencer les fonctions cellulaires en affectant la conformation, l'exposition et la diffusion des composants membranaires.

Autres techniques spectroscopiques et calorimétriques pourraient aussi aider à la compréhension des interactions moléculaires TTPs-albumine.

## **Communications**

### **Orale**

1. Progesterone derivatives effects on lipid bilayers: A structure-activity relationship. Greige-Gerges H., Khreich N., Abboud R., Shaaban R., Abi-Saad G., Charcosset C. BioBeirut 4 International Meeting- Cancer Biology and Stem Cells Meeting 2014-Hadath Liban.
2. Interaction Of Triterpenes Molecules With Lipid Bilayer Membrane. Abboud, R., Charcosset, C. & Greige-Gerges, H. Forum Doctoral-UL- EDST 2016 Hadath-Liban.

### **Par affiche**

1. Effets des triterpènes tétra- et penta- cycliques sur la membrane des bicouches lipidiques. Abboud R., Charcosset, C., Greige-Gerges H. Les Troisièmes Journées Franco-libanaises 3-EDST 2015, Hadath-Liban.
2. Effect of Progesterone, its Hydroxylated and Methylated Derivatives, and Dydrogesterone on Lipid Bilayer Membranes. Abboud, R., Greige-Gerges, H. & Charcosset, C. Graduate School Meeting, 7 mai 2015, Lyon France.
3. Progestins and Corticoids interaction with Human Serum Albumin. Abboud, R., Charcosset, C. & Greige-Gerges, H. 6<sup>th</sup> ENOR Symposium, Current Trends in Oxysterols & Related Sterols : Biological and Medical Aspects 29-30 September 2016, Paris, France.
4. Effect of tetra- and penta-cyclic triterpenes on lipid membrane fluidity : studies by DSC, FTIR, RAMAN and fluorescence anisotropy Abboud, R., Charcosset, C. & Greige-Gerges, H. 6<sup>th</sup> ENOR Symposium, Current Trends in Oxysterols & Related Sterols: Biological and Medical Aspects 29-30 September 2016, Paris, France.