UNIVERSITÉ LAVAL

Évaluation biologique in vitro d'inhibiteurs de la stéroïde sulfatase ayant un effet SERM

Mémoire

Charles Ouellet

Maîtrise en physiologie-endocrinologie
Maître ès sciences (M.Sc.)

Québec, Canada

Résumé

Initialement, les cancers du sein sont majoritairement hormonodépendants, c'est-à-dire qu'ils sont stimulés par les hormones estrogéniques endogènes. Une première approche pour traiter ce type de cancer consiste à utiliser un antiestrogène pour bloquer l'activation du récepteur des estrogènes dans le tissu mammaire. Une seconde approche consiste à inhiber la synthèse des estrogènes par l'utilisation d'inhibiteurs spécifiques pour certaines enzymes clés de la stéroïdogénèse. La stéroïde sulfatase (STS), une enzyme impliquée dans la biosynthèse des estrogènes, constituerait une importante cible thérapeutique.

Dans le cadre de ce mémoire, je discuterai d'un nouveau projet de recherche qui consiste à développer des composés à double-action combinant les deux types d'approches nommées précédemment. Nous cherchons à développer une molécule non-stéroïdienne qui serait en mesure d'inhiber la STS mais également de bloquer le récepteur des estrogènes dans le tissu mammaire. Notre composé doit donc posséder un effet SERM (Selective Estrogen Receptor Modulator), c'est-à-dire de jouer le rôle d'un antagoniste dans le tissu mammaire tout en jouant le rôle d'un agoniste dans d'autres tissus (i.e. tissu osseux) où l'action des estrogènes est importante.

Dans ce mémoire, j'aborderai rapidement la synthèse chimique des deux générations de composés pour m'attarder davantage sur les tests biologiques *in vitro* nécessaires à l'évaluation de leur potentiel à double-action. Les composés ont d'abord été testés sur la STS pour évaluer leur potentiel d'inhibition de l'enzyme. Ensuite, les composés ont été testés sur des cellules cancéreuses du sein sensibles aux estrogènes pour s'assurer qu'ils ne stimulent pas leur prolifération. Afin de vérifier l'effet SERM désiré, les composés furent testés sur des ostéoblastes pour déterminer s'ils sont en mesure d'induire leur prolifération et leur différentiation. Finalement, l'affinité pour le récepteur des estrogènes de type alpha (REα) fut également évaluée. La 2^{e} génération de composés a produit de bons résultats car certains composés inhibent bien la STS et possèdent l'effet SERM désiré. Ce projet a donc permis de développer des composés à double-action qui pourraient être utilisés lors de futures études *in vivo*.

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17β-HSD	17β-Hydroxystéroïde déshydrogénase
^{1}H NMR	Résonance magnétique nucléaire du proton
^{13}C NMR	Résonance magnétique nucléaire du carbone
Δ^4-dione	4-Androstène-3,17-dione
Δ^5-diol	5-Androstène-3β,17β-diol
AF-2	Fonction d'activation de la transcription 2
ALP	Alkaline Phosphatase
Arg	Arginine
BSA	*Bovine serum albumin*
°C	Degré Celsius
Ci	Curie
Ctrl	*Control*
DBD	*DNA binding domain*
DCM	Dichlorométhane
DEA	Diéthanolamine
DHEA	Déhydroépiandrostérone
DHEAS	Déhydroépiandrostérone sulfate
DIPEA	*N*,*N*-Diisopropyléthylamine
DMF	Diméthylformamide
DMSO	Diméthylsulfoxyde
DXA	*Dual-energy X-ray absorptiometry*
E1	Estrone
E1S	Estrone sulfate
E2	Estradiol
EMATE	Estrone sulfamate
ERE	Élément de réponse aux estrogènes
FasL	Ligand Fas
FBS	*Fetal bovine serum*
Glu	Acide glutamique
h	Heure
HEK	*Human embryonic kidney*
HER2	*Human epidermal growth factor receptor 2*
His	Histidine
HPLC	*High performance liquid chromatography*
IC_{50}	Concentration d'un produit nécessaire à inhiber 50% d'une activité enzymatique ou à bloquer 50% de la liaison d'un ligand à son récepteur
IR	Infrarouge
IU	Unité internationale
kDa	Kilodalton
K_m	Constante de Michaelis
LBD	*Ligand binding domain*
M	Molaire
min	Minute

mg	Milligramme
mL	Millilitre
mM	Millimolaire
MPP	1,3-bis(4-hydroxyphényl)-4-méthyl-5-[4-(2-pipéridinyléthoxy)phénol]-1H pyrazole
MTS	3-(4,5-Diméthylthiazol-2-yl)-5-(3-carboxyméthoxyphényl)-2-(4-sulfophényl)-2H-tetrazolium
NAD^+	Nicotinamide adénine dinucléotide
NADH	Nicotinamide adénine dinucléotide (forme réduite)
$NADP^+$	Nicotinamide adénine dinucléotide phosphate
NADPH	Nicotinamide adénine dinucléotide phosphate (forme réduite)
ng	Nanogramme
nm	Nanomètre
nM	Nanomolaire
NMP	N-méthyl-2-pyrrolidone
NTD	*N-terminal domain*
OPG	Ostéoprotégérine
PHTPP	4-[2-phényl-5,7-bis(trifluorométhyl)pyrazolo[1,5-a]pyrimidin-3-yl]phénol
pNPP	*Para*-nitrophénylphosphate
PREG	Prégnénolone
PREGS	Prégnénolone sulfate
PTH	*Parathyroid hormone*
RANK	*Receptor activator of nuclear factor κB*
RANKL	*Receptor activator of nuclear factor κB ligand*
RBA	*Relative binding affinity*
RE	Récepteur des estrogènes
REα	Récepteur des estrogènes alpha
REβ	Récepteur des estrogènes bêta
RP	Récepteur de la progestérone
SD	*Standard deviation*
SDR	*Short chain dehydrogenases/reductases*
SEM	*Standard error of the mean*
SERD	*Selective estrogen receptor down-regulator*
SERM	*Selective estrogen receptor modulator*
STS	Stéroïde sulfatase
T	Testostérone
TLC	*Thin layer chromatography*
THF	Tétrahydrofurane
µg	Microgramme
µL	Microlitre
µM	Micromolaire

Remerciements

Je désire tout d'abord remercier mon directeur de recherche, le professeur Donald Poirier, pour m'avoir accueilli dans son laboratoire. Sa grande disponibilité, son encadrement, son écoute ainsi que ses nombreuses connaissances furent grandement appréciés et essentiels au bon déroulement de ma maîtrise. Je le remercie également pour m'avoir offert un projet de recherche original et stimulant.

Je désire aussi remercier les autres membres du laboratoire : René, Marie-Claude, Jean-Yves, Amélie, Alexandre et Guy-Bertrand. Je remercie plus particulièrement Étienne, pour son implication importante dans mon projet, ainsi que Jenny, Diana et Lucie-Carolle pour leur aide avec les tests biologiques lorsque j'en avais besoin.

Je souhaite également remercier ma famille et mes proches pour m'avoir encouragé et soutenu tout au long de ma maîtrise. Je veux surtout remercier Jeanne qui durant ces deux dernières années m'a témoigné son amour et son soutien, ainsi que sa compréhension pour les fois où je dus m'absenter pour mes cellules.

Avant-propos

Ce mémoire de maîtrise, intitulé « *Évaluation biologique in vitro d'inhibiteurs de la stéroïde sulfatase ayant un effet SERM* » et présenté à la Faculté des études supérieures et postdoctorales de l'Université Laval pour l'obtention du grade de maître ès sciences, est rédigé sous la forme d'insertion d'articles.

Le premier article, intitulé « *Investigation of a tetrahydroisoquinoline scaffold as dual-action steroid sulfatase inhibitors generated by parallel solid-phase synthesis* » dont je suis troisième auteur, a été soumis au journal Medicinal Chemistry Communications. La synthèse chimique des composés a été réalisée par Étienne Ouellet, étudiant à la maîtrise dans l'équipe du Dr Donald Poirier, ainsi qu'avec l'aide du Dr René Maltais. Ma contribution à cet article a été d'effectuer les tests *in vitro* pour faire le criblage initial des composés à double-action ayant été synthétisés. J'ai testé les molécules pour leur potentiel d'inhibition de la stéroïde sulfatase, leur estrogénicité sur les cellules cancéreuses du sein MCF-7 ainsi que leur effet sur la prolifération et la différentiation des cellules ostéoblastiques Saos-2. J'ai participé à l'écriture du manuscrit en ce qui concerne la partie expérimentale et les résultats des tests *in vitro*, avec l'aide de mon directeur de recherche, le Dr Donald Poirier.

Le second article, intitulé « *In vitro evaluation of a steroid sulfatase inhibitor with a potential selective estrogen receptor modulator (SERM) capacity* » et dont je suis le premier auteur, sera éventuellement soumis à un journal approprié. Les deux composés rapportés dans cet article ont été synthétisés par Étienne Ouellet, étudiant à la maîtrise. J'ai effectué tous les tests *in vitro*, c'est-à-dire pour évaluer l'inhibition de la stéroïde sulfatase, l'effet sur les cellules cancéreuses du sein MCF-7 et T-47D, l'effet sur les cellules ostéoblastiques Saos-2 ainsi que l'affinité pour le récepteur des estrogènes alpha. J'ai écrit le manuscrit de cet article avec l'aide de mon directeur de recherche, le Dr Donald Poirier.

Le troisième article, intitulé « *Development of sulfamate tetrahydroisoquinoline-N-substituted derivatives as potent dual-action compounds for the treatment of estrogen-*

dependent breast cancer » et dont je suis le premier auteur, sera éventuellement soumis au Journal of Medicinal Chemistry. La synthèse des composés rapportés dans cet article a été faite par Étienne Ouellet, étudiant à la maîtrise. Ma contribution a été d'effectuer tous les tests *in vitro*, soit le criblage initial des composés ainsi que l'évaluation plus approfondie de certains composés. Ces quelques composés furent testés pour l'inhibition de la stéroïde sulfatase et leur effet sur les cellules cancéreuses du sein T-47D et les cellules ostéoblastiques Saos-2. J'ai écrit le manuscrit de cet article avec l'aide du Dr René Maltais ainsi que mon directeur de recherche, le Dr Donald Poirier.

Introduction

1. Cancer : Statistiques et généralités

Dans les pays développés, le cancer est la 2^{e} cause de décès après les maladies cardiovasculaires [1]. Au Canada, en 2011, on estime que 177 800 nouveaux cas de cancer et que 75 000 décès causés par cette maladie sont survenus [2]. Parmi les plus fréquents, il y a le cancer du poumon, du côlon, de la prostate et du sein. Ces deux derniers sont les plus diagnostiqués chez l'homme et la femme respectivement. Il est estimé qu'en 2011 il y a eu 25 600 nouveaux cas de cancer de la prostate, représentant 27.5% de tous les cas de cancers. Quant au cancer du sein, il y a eu 23 400 nouveaux cas, représentant 27.6% de tous les cancers diagnostiqués chez la femme. En ce qui a trait à la mortalité en 2011, le cancer du sein se retrouve au 2^{e} rang chez la femme avec approximativement 5000 décès tandis que chez l'homme, le cancer de la prostate arrive au 3^{e} rang avec environ 4100 décès. Par contre, le cancer du poumon est le cancer qui cause le plus de décès autant chez l'homme avec 11 300 décès que chez la femme avec 9400 décès. Dans le cadre de ce projet de recherche, nous nous sommes concentrés plus particulièrement sur le cancer du sein.

2. Cancer du sein

Le cancer du sein est une tumeur maligne qui provient principalement des lobules (carcinome lobulaire) ou des canaux galactophores (carcinome canalaire) du tissu mammaire. Ensuite, les cellules cancéreuses se dédifférencient, deviennent invasives et commencent à se développer dans les tissus adjacents au tissu d'origine. Par la suite, les cellules s'introduisent dans les vaisseaux lymphatiques et commencent à se développer dans les ganglions lymphatiques. À ce stade, les cellules cancéreuses ont acquis la capacité à se développer hors de la tumeur primaire et peuvent former des métastases un peu partout dans le corps (e.g. os, poumons, cerveau, foie) en utilisant le système lymphatique et sanguin. Selon le type et l'agressivité du cancer, ce processus de développement peut s'étendre sur une période de quelques semaines à plusieurs années [3].

Chez la femme, le risque de développer un cancer du sein au cours de sa vie est de 1 chance sur 9 et le risque d'en mourir est de 1 chance sur 29 [2]. Plus de la moitié des cas de cancer du sein surviennent chez les femmes de 50 et 69 ans, mais les décès seront plus nombreux chez celles de 80 ans et plus. Depuis le milieu des années 1990, les taux d'incidence et de mortalité reliés au cancer du sein ont diminué de 0.7% et de 3.1% respectivement. Cela est probablement dû au recours croissant au dépistage par mammographie et à l'efficacité accrue des traitements reçus à la suite de la chirurgie [2]. Chez l'homme, le risque de développer un cancer du sein est beaucoup moins élevé. En effet, il est estimé que 190 hommes se feront diagnostiqués un cancer du sein en 2011, ce qui représente environ 0.8% de tous les cancers du sein diagnostiqués [2].

2.1 Causes

Les causes du cancer du sein sont nombreuses. Le cancer du sein, comme plusieurs autres types de cancer, peut être causé par des mutations génétiques héréditaires ou par divers facteurs environnementaux. En effet, de 5 à 10% des cancers sont dus à des mutations génétiques acquises héréditairement tandis que les facteurs externes, tels que l'alcool, l'obésité et le tabac, comptent pour 90 à 95% des causes de cancer (Fig. 1) [4].

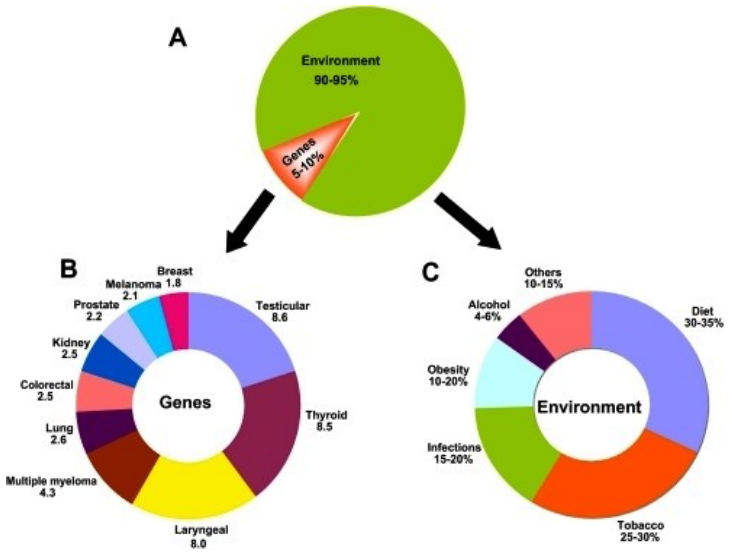


Figure 1. Le rôle des gènes et de l'environnement dans le développement du cancer [4].

Les mutations génétiques héréditaires ne sont responsables que d'une petite partie de tous les cancers mais augmentent considérablement le risque des porteurs de ces mutations de développer un cancer au cours de leur vie. Les mutations génétiques les plus souvent responsables du cancer du sein sont les mutations dans les gènes suppresseurs de tumeur *BRCA1* et *BRCA2*. En effet, les risques de développer un cancer du sein à l'âge de 70 ans est de 57% et 49% pour les femmes ayant une mutation dans les gènes *BRCA1* et *BRCA2* respectivement [5]. La mutation de ces deux gènes augmente également le risque de développer un cancer de l'ovaire, soit de 39% pour *BRCA1* et de 17% pour *BRCA2*. Le risque de développer un cancer du sein est aussi augmenté par des mutations génétiques dans d'autres gènes tels que *TP53*, *ATM* et *PTEN* [6] qui codent pour des protéines jouant un rôle dans la régulation du cycle cellulaire et la réparation de l'ADN.

Les facteurs externes, quant à eux, sont la cause majeure du développement des cancers. Plusieurs études ont démontré un lien entre le risque de développer un cancer du sein et certains facteurs externes non-héréditaires dont:

- La fumée de cigarette, qui contient de nombreux carcinogènes [7];
- La consommation régulière d'alcool [8];
- L'obésité et le surpoids [9];
- La densité des seins et la thérapie hormonale substitutive chez les femmes post-ménopausées [10];
- L'utilisation de contraceptifs oraux [11];
- Les menstruations précoces, la ménopause tardive et la première grossesse tardive [12];
- Une alimentation riche en gras saturés [13];
- Les virus, tel que le virus du papillome humain [14].

2.2 Diagnostique

À ses débuts, le cancer peut être difficilement détectable. L'auto-examen des seins est une bonne façon de détecter des anomalies, mais la mammographie s'est avérée être la plus efficace afin de dépister un cancer du sein précocement. Afin de donner un meilleur diagnostique, les médecins ont recours également à la biopsie et utilise diverses méthodes de classification:

- Détermination du grade (1 à 3) à la suite d'un examen microscopique des cellules:
    - le grade 1 correspond à des cellules bien différentiées qui ressemblent beaucoup aux cellules normales
    - le grade 3 correspond à des cellules peu différentiées qui prolifèrent beaucoup et qui sont invasives
- Détermination du stade (0 à 4) selon la grosseur et la distribution dans le corps des cellules cancéreuses:
    - Le stade 0 correspond à une petite tumeur *in situ* (carcinome lobulaire ou canalaire)
    - Le stade 4 correspond à un cancer du sein métastatique
- Détermination du statut des récepteurs des estrogènes (RE) et de la progestérone (RP) :
    - Les tumeurs RE+ et RP+ sont sensibles aux hormones et peuvent être traitées par l'hormonothérapie
    - Les tumeurs RE- et RP- sont insensibles aux hormones et doivent être traitées plus agressivement
- Détermination du niveau d'expression de la protéine HER2 :
    - Les tumeurs surexprimant HER2 peuvent être traitées avec l'anticorps monoclonal trastuzumab

À la suite de ces évaluations, les médecins sont en mesure de déterminer les meilleures options de traitement pour le patient.

2.3 Traitements

Selon les différentes caractéristiques du cancer du sein, divers choix de traitement sont offerts [15]:

- Résection chirurgicale partielle (lumpectomie) ou complète (mastectomie) du sein;
- Radiothérapie : irradiation des cellules cancéreuses;
- Chimiothérapie : utilisation de médicaments anticancéreux cytotoxiques;
- Thérapie biologique et ciblée: cible les cellules cancéreuses (e.g. Trastuzumab);
- Hormonothérapie : Bloque la biosynthèse ou l'action des estrogènes.

Plusieurs options de traitement s'offrent aux patients atteints de cancer mais ils entrainent souvent des effets secondaires importants et/ou indésirables tels que la perte de cheveux, la fatigue, les nausées et les vomissements [16]. Afin de trouver des alternatives thérapeutiques adaptées à chaque patient qui cibleraient plus spécifiquement les cellules cancéreuses et qui réduiraient les effets indésirables, la recherche de nouveaux traitements contre le cancer reste très active [17-19].

3. Hormonothérapie

L'hormonothérapie est une alternative plus douce à la radiothérapie et la chimiothérapie pour ralentir et arrêter le développement des cellules cancéreuses. Ce traitement est utilisé seulement si les tumeurs expriment le récepteur des estrogènes. Étant donné que la majorité des cancers du sein sont initialement estrogénodépendants, cette thérapie s'avère être une bonne alternative. En effet, approximativement 55% des femmes préménopausées et 75% des femmes postménopausées ayant un cancer du sein ont des tumeurs RE+ [20]. Selon le stade et les caractéristiques du cancer, l'hormonothérapie est utilisée pour inhiber les enzymes clés de la biosynthèse des estrogènes ou pour bloquer le récepteur des estrogènes.

3.1 Inhibiteurs de la biosynthèse des estrogènes

L'inhibition de la synthèse des estrogènes est une approche thérapeutique visant à diminuer la production de l'estrone et de l'estradiol. En bloquant leur synthèse, on empêche ainsi l'activation du récepteur des estrogènes et, par le fait même, la prolifération des cellules cancéreuses estrogénodépendantes. Pour le traitement du cancer du sein, les cibles enzymatiques principales, toutes impliquées dans la biosynthèse des estrogènes, sont la stéroïde sulfatase, l'aromatase et les 17β-hydroxystéroïdes déshydrogénases. Le rôle de ces enzymes et leurs inhibiteurs seront détaillés à la section 5.

3.2 Antiestrogènes et SERM

Une autre approche thérapeutique consiste à bloquer directement le récepteur des estrogènes dans les cellules cancéreuses du sein pour empêcher les principaux ligands endogènes, soit l'estrone (E1), l'estradiol (E2) et le 5-androstène-3β,17β-diol (Δ^5-diol), d'activer les gènes associés aux éléments de réponse des estrogènes (ERE). Un des premiers composés utilisés est le fulvestrant (ICI 182,780), un antiestrogène pur qui bloque l'action des estrogènes en se liant au récepteur des estrogènes (Fig. 2) [21]. Il induit également la régulation négative du récepteur (SERD en anglais), résultant ainsi en une diminution du nombre de récepteur des estrogènes dans les cellules [22]. Le fulvestrant est utilisé pour traiter les patientes postménopausées ayant un cancer du sein RE+ métastatique chez lesquelles on observe une progression de la maladie suite à l'utilisation d'autres inhibiteurs utilisés en thérapie hormonale. Le fulvestrant s'est avéré être un bon composé dans le traitement des cancers du sein métastatiques. Par contre, son action ne se limite pas au tissu mammaire et il entraine des effets secondaires. En effet, le fulvestrant peut avoir des effets néfastes sur le système cardiovasculaire et la formation des os [23].

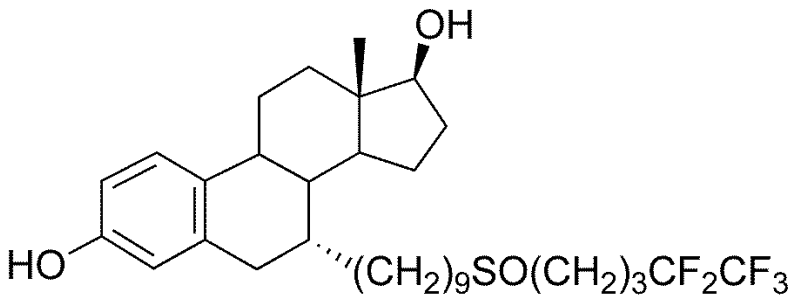


Figure 2. Structure du fulvestrant.

Les antiestrogènes purs sont efficaces pour bloquer le récepteur des estrogènes mais n'agissent pas spécifiquement selon le tissu. De nouveaux composés synthétisés ont démontré cette capacité de moduler sélectivement le récepteur des estrogènes α (SERM) selon le tissu. Un de ces composés est le tamoxifène (Fig. 3), un SERM qui agit à titre d'antagoniste dans le tissu mammaire mais qui a le rôle d'agoniste dans l'utérus et le tissu osseux. Malheureusement, son rôle en tant qu'agoniste dans l'utérus augmente le risque de développer un cancer de l'utérus chez les patientes traitées avec le tamoxifène pour le cancer du sein [24]. Un autre SERM est le raloxifène (Fig. 3), un composé utilisé dans le traitement de l'ostéoporose. Le raloxifène est agoniste dans le tissu osseux mais antagoniste dans le tissus mammaire et utérin, ce qui lui donne un avantage par rapport au tamoxifène. Malgré l'utilité thérapeutique du tamoxifène et du raloxifène dans le traitement du cancer du sein, les deux composés augmentent le risque de complications cardiovasculaires [25-26].

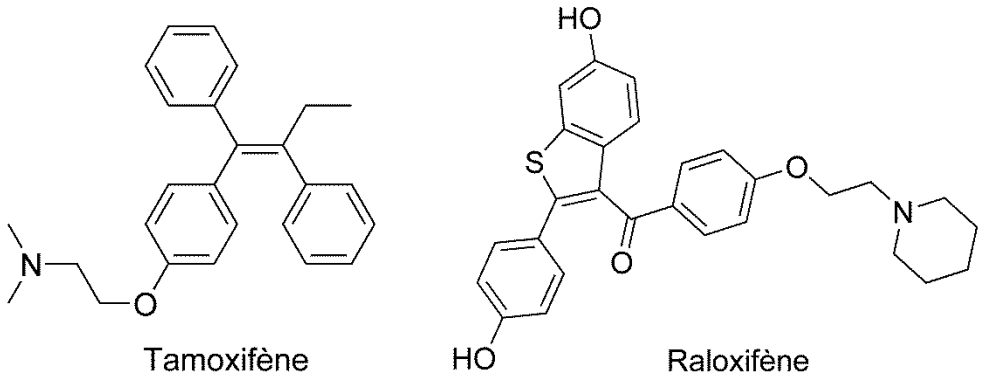


Figure 3. Structure du tamoxifène et du raloxifène.

Le développement de nouveaux SERMs est un domaine de recherche très actif pour trouver des composés qui diminueraient les risques de complication tout en gardant les caractéristiques essentielles d'un SERM, c'est-à-dire de jouer le rôle d'antagoniste dans les tissus mammaires et utérins et le rôle d'agoniste dans le tissu osseux.

4. Tissu osseux

4.1 Ostéogénèse

La formation du tissu osseux est un mécanisme complexe régulé par des facteurs de croissance, des cytokines, l'hormone parathyroïde (PTH) et les estrogènes ou les androgènes selon le sexe [27]. L'intégrité du tissu osseux est maintenue grâce à l'équilibre entre la formation des os par les ostéoblastes et la résorption des os par les ostéoclastes. Le rôle des estrogènes dans cet équilibre entre les ostéoblastes et les ostéoclastes sera détaillé à la section 6.3.

Initialement, lorsque la formation ou la modification du tissu osseux est nécessaire, les ostéoclastes sont recrutés au site de formation pour détruire une partie de la structure. Après résorption, les macrophages viennent digérer les débris cellulaires et les préostéoblastes sont recrutés au site de formation. Les préostéoblastes sont ensuite activés par différents facteurs, dont les hormones sexuelles, pour être davantage différentiés en ostéoblastes et initialiser la formation du nouveau tissu. Tout d'abord, les ostéoblastes synthétisent une matrice extracellulaire principalement constitué de collagène de type 1. Ensuite, cette matrice est calcifiée par les ostéoblastes dû à l'insertion de cristaux d'hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) dans l'échafaudage de protéines extracellulaires [28]. Cette calcification est en partie faite par l'alkaline phosphatase, une enzyme qui clive différents composés contenant du phosphate, comme le pyrophosphate ou le pyridoxal 5'-phosphate, pour libérer du phosphate inorganique essentiel à la formation d'hydroxyapatite [29]. La reconstruction du nouveau tissu se termine lorsque les ostéoblastes sont emprisonnés dans la matrice extracellulaire nouvellement synthétisée et calcifiée; les cellules sont alors appelées ostéocytes. Les ostéocytes communiquent entre eux par des jonctions

intercellulaires de leur membrane plasmique et régulent la formation du tissu osseux. Lorsque cela est nécessaire, les ostéocytes initialisent la reconstruction du tissu, à la suite d'un stress mécanique par exemple, et recrutent les ostéoclastes au site de formation.

4.2 Ostéoporose

L'ostéoporose est une maladie résultant du débalancement entre la formation et la résorption des os. La diminution de la densité osseuse est causée par une augmentation excessive de la résorption par les ostéoclastes et la formation inadéquate et insuffisante du tissu par les ostéoblastes. L'augmentation du taux de résorption entraine ainsi une fragilisation de la microarchitecture du tissu osseux et, conséquemment, le risque de fractures. Le mécanisme par lequel l'ostéoporose se développe reste à clarifier mais il semble évident que la diminution du niveau d'hormones sexuelles dans le sang, dû par exemple à la ménopause ou à l'oophorectomie chez la femme, entraine une diminution de la densité osseuse [27].

L'ostéoporose est une maladie qui affecte de nombreuses personnes. En effet, il est estimé qu'environ 55% de la population âgée de 50 ans et plus souffre d'ostéoporose ou a une faible densité osseuse (ostéopénie). Il est aussi estimé que jusqu'à 30% des femmes postménopausées souffrent d'ostéoporose [30]. Pour faciliter l'évaluation de la densité osseuse, l'organisation mondiale de la santé (OMS) propose une classification (Tableau 1) selon le T-score obtenue par la méthode d'absorption bi-photonique à rayons X (DXA en anglais) pour savoir à quel stade les patients se situent [31]. Le T-score représente la déviation standard calculée par rapport à une population de jeunes adultes.

Tableau 1. Classification de la densité osseuse par l'OMS

Stade	Normal	Ostéopénie	Ostéoporose	Ostéoporose sévère
T-Score	>-1	>-2.5 et ≤-1	≤-2.5	≤-2.5 et au moins une fracture liée à l'ostéoporose

Plusieurs alternatives de traitement sont disponibles afin de maintenir ou d'augmenter la densité osseuse et, par le fait-même, diminuer le risque de fractures [32] :

- La prise de suppléments de calcium et de vitamine D
- Les bisphosphonates (e.g. Alendronate)
- Les SERMs (e.g. Raloxifène)
- La thérapie hormonale substitutive (l'estradiol seul ou en combinaison avec un progestatif)
- L'hormone parathyroïde (PTH)
- La calcitonine
- Une combinaison des différents traitements disponibles (e.g. Alendronate + PTH)

5. Stéroïdogénèse

Les hormones stéroïdiennes jouent un rôle clé dans la régulation de l'homéostasie du corps humain et sont formées à partir d'un même précurseur, le cholestérol. Le cholestérol est un stéroïde qui contient quatre cycles fusionnés pour un total de 27 carbones (Fig. 4). Le cholestérol provient principalement de la consommation de produits d'origine animale (e.g. viandes et produits laitiers) car il est une composante essentielle et exclusive de la membrane cellulaire animale. Si la diète ne fournit pas tout le cholestérol nécessaire à l'organisme, il peut également être synthétisé *de novo* par la plupart des cellules [33]. En effet, les cellules possèdent toutes les enzymes nécessaires pour synthétiser le cholestérol à partir de l'acétyl-CoA en passant par la formation de différents intermédiaires métaboliques (e.g. mévalonate, farnésyl pyrophosphate et squalène).

La formation des cinq classes d'hormones stéroïdiennes, soit les glucocorticoïdes, les minéralocorticoïdes, les progestatifs, les androgènes et les estrogènes, est effectuée par la stéroïdogénèse (Fig. 5), qui est une voie métabolique impliquant plusieurs enzymes différentes. Ces enzymes sont exprimées spécifiquement dans certains tissus et permettent aux stéroïdes d'être utilisés directement dans le tissu où ils ont été formés (action

intracrine) ou d'être relargués dans la circulation pour agir sur d'autres tissus périphériques (action endocrine).

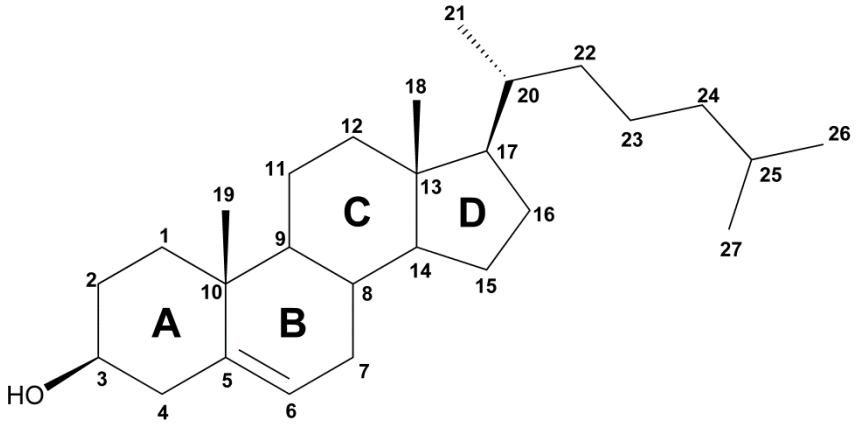


Figure 4. Structure et nomenclature du cholestérol

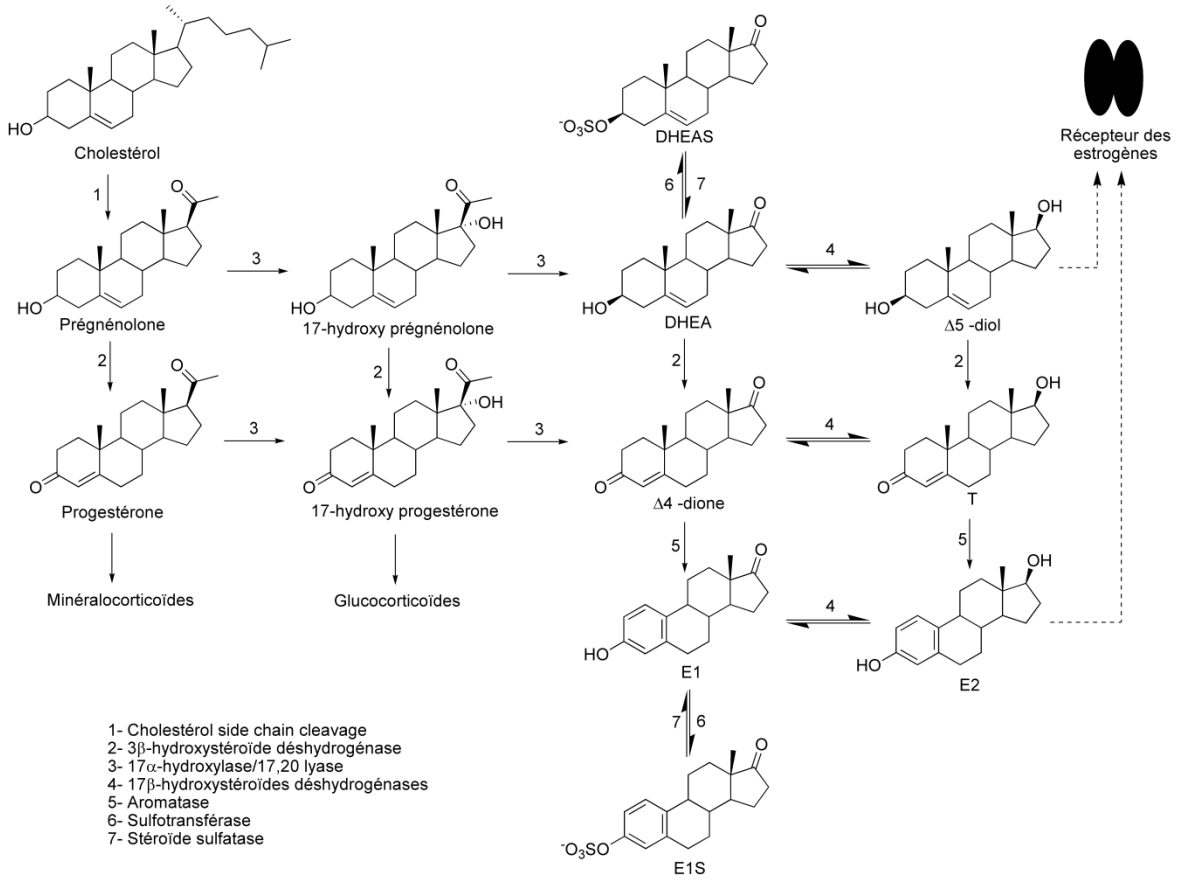


Figure 5. Schéma simplifié de la stéroïdogénèse

Les ovaires, lesquels possèdent toutes les enzymes nécessaires à la conversion du cholestérol en estrogènes actifs (E1 et E2), agissent à titre de tissu endocrinien. Les estrogènes actifs relargués dans la circulation sanguine par les ovaires ont un effet dans les différents tissus cibles périphériques (e.g. glandes mammaires, os, utérus et cerveau) par leur liaison aux récepteurs des estrogènes (α et β). Plusieurs tissus périphériques possèdent également les enzymes nécessaires à la conversion de précurseurs stéroïdiens présents en quantité importante dans le sang, soit le déhydroépiandrostérone (DHEA) et le déhydroépiandrostérone sulfate (DHEAS). Ces deux précurseurs sont produits par les glandes surrénales et sont responsables de la majorité des estrogènes synthétisés dans les tissus périphériques [34]. Avant la ménopause, 75% de la biosynthèse des estrogènes provient de la transformation des précurseurs surrénaliens dans les tissus périphériques. Après la ménopause, tous les estrogènes sont produits dans les tissus périphériques intracrines à partir du DHEA et du DHEAS en circulation.

Dans le cas d'un cancer du sein sensible aux estrogènes, il est donc crucial de bloquer la biosynthèse des stéroïdes estrogéniques, soit E1, E2 et le Δ5-diol, car la glande mammaire possède toutes les enzymes nécessaires à leur synthèse [35]. Tel que mentionné auparavant, certaines enzymes jouent un rôle important dans la formation des estrogènes actifs et sont donc des cibles de choix pour le développement d'inhibiteurs.

5.1 Aromatase

L'aromatase est une enzyme qui fait partie de la famille des cytochromes P450 et qui catalyse la conversion irréversible (aromatisation du cycle A des stéroïdes) de certains stéroïdes C-19 androgéniques en stéroïdes C-18 estrogéniques. Les 2 principaux substrats de l'enzyme sont l'androstènedione (4-dione) et la testostérone (T), lesquels sont convertis en E1 et E2, respectivement. L'aromatase est exprimée dans de nombreux tissus (e.g. gonades, placenta, os, adipocytes, glandes mammaires) et est localisée principalement dans le réticulum endoplasmique. L'aromatase forme un complexe enzymatique avec le cytochrome P450 réductase qui est une enzyme permettant le transfert des électrons du cofacteur nicotinamide adénine dinucléotide phosphate réduit (NADPH) essentiel à la

catalyse [36]. En effet, pour chaque mole de stéroïdes C-19 transformés, l'aromatase a besoin de 3 moles d'oxygène et 3 moles de NADPH. Cette réaction enzymatique est stabilisée par le complexe hème-fer situé dans le site catalytique, ce qui permet l'aromatisation du cycle A du substrat stéroïdien.

Catalysant une étape cruciale de la formation des estrogènes, l'aromatase fut étudiée extensivement pour déterminer sa structure tridimensionnelle et son mécanisme catalytique. Des études de relations structure-activité ont aussi permis de développer des inhibiteurs sélectifs de l'aromatase pouvant être utilisés dans le traitement des cancers du sein estrogénodépendants [37]. L'inhibition de l'aromatase s'est avérée être une bonne stratégie de traitement, car les deux tiers des carcinomes mammaires expriment l'aromatase et synthétisent localement des quantités non-négligeables d'estrogènes.

Deux catégories distinctes d'inhibiteurs de l'aromatase ont été développés : les composés de type 1, qui sont des inhibiteurs irréversibles généralement stéroïdiens, et les composés de type 2, qui sont des inhibiteurs réversibles majoritairement non-stéroïdiens. Dans la première catégorie de composés, on retrouve l'inhibiteur irréversible exemestane (Fig. 6) qui est présentement utilisé en clinique pour le traitement des cancers du sein estrogénodépendants chez les femmes postménopausées. Il a été démontré qu'une dose quotidienne de 25 mg d'exemestane permet d'inhiber 97.9% de l'activité aromatase et ainsi de diminuer significativement la concentration plasmatique des estrogènes [38]. Dans la deuxième catégorie de composés, on retrouve les inhibiteurs réversibles anastrozole et letrozole (Fig. 6) qui sont présentement utilisés en clinique. Ces deux inhibiteurs compétitionnent avec les substrats endogènes de l'enzyme et ont une structure similaire, soit par la présence d'un groupement triazole essentiel à la liaison avec le complexe hème-fer de l'aromatase. Une inhibition de l'activité de l'aromatase de 93% et de 99% fut d'ailleurs obtenue en utilisant l'anastrozole (1 mg/jour) et le letrozole (2.5 mg/jour), respectivement [39-40].

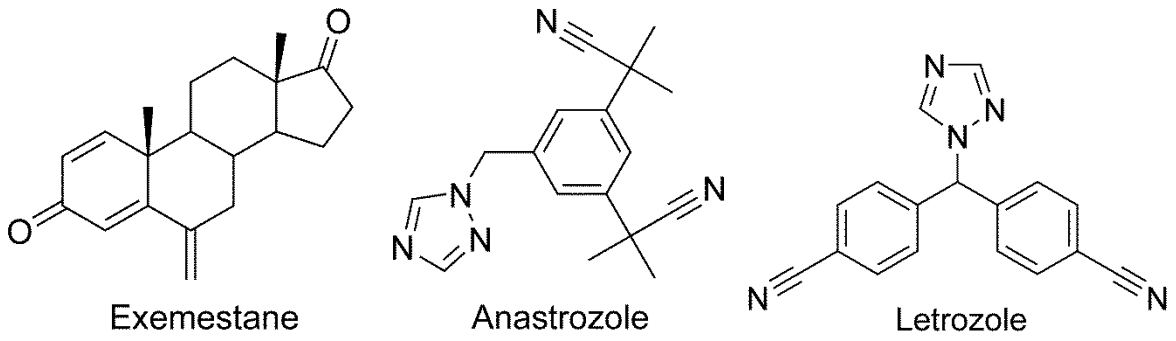


Figure 6. Structure d'inhibiteurs de l'aromatase.

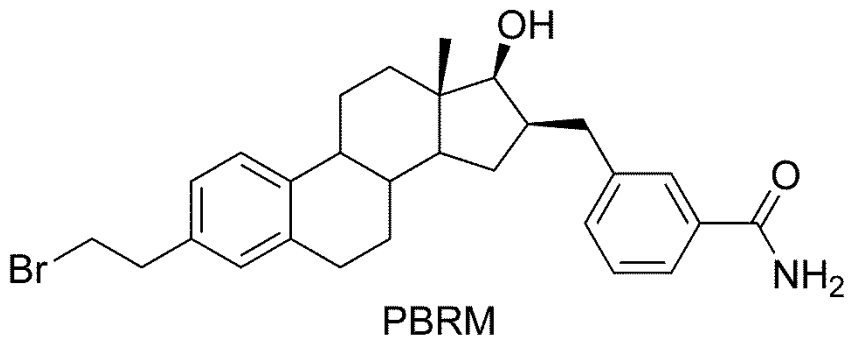
5.2 17β-hydroxystéroïdes déshydrogénases

Les 17β-hydroxystéroïdes déshydrogénases (17β-HSDs) sont des enzymes qui catalysent la réaction d'oxydation ou de réduction en position 17 des stéroïdes [41]. Ces enzymes, impliquées dans la dernière étape de biosynthèse des estrogènes et des androgènes, permettent la conversion des stéroïdes inactifs (e.g. E1 et Δ^4-dione) en hormones pleinement actives (e.g. E2 et T) sur les récepteurs nucléaires hormonaux. Les 17β-HSDs dépendent des cofacteurs NAD(P)H et $NAD(P)^+$ pour effectuer leur catalyse enzymatique de réduction ou d'oxydation, respectivement. Jusqu'à présent, 15 différents isoformes (types) de 17β-HSDs ont été identifiés chez les mammifères [42]. À l'exception de la 17β-HSD 5, qui fait partie de la famille des aldo-kéto réductases, les 17β-HSDs sont membres de la famille des déshydrogénases/réductases à courte chaîne (SDR). Les 17β-HSDs diffèrent par leur spécificité de substrats et leur expression tissulaire (e.g. ovaires, testicules, placenta, tissu mammaire). De plus, certains isoformes se retrouvent sous forme soluble dans le cytosol tandis que d'autres sont ancrés à la membrane du réticulum endoplasmique.

Dans le cas d'un cancer du sein estrogénodépendant, les 17β-HSDs impliqués dans la biosynthèse des estrogènes sont une cible thérapeutique importante. Les 17β-HSDs 1, 7 et 12 sont en mesure de faire la conversion d'E1 en E2 et sont toutes exprimées dans le tissu mammaire. Bien que les 17β-HSDs 7 et 12 soient en mesure de former E2, leur rôle dans le tissu mammaire reste flou car la majorité d'E2 synthétisé provient de la transformation d'E1 par la 17β-HSD 1 [43]. La 17β-HSD 1 peut également convertir le DHEA en Δ^5-diol,

un estrogène faiblement actif mais possédant tout de même une affinité non-négligeable pour le récepteur des estrogènes. La 17β-HSD 1 est donc une cible de choix pour le traitement des cancers du sein estrogénodépendants.

Durant les dernières décennies, de nombreux groupes de recherche ont travaillé sur les 17β-HSDs. Cette recherche extensive a permis de caractériser les différents isoformes [44], mais également de développer plusieurs inhibiteurs spécifiques à ces isoformes [45]. Malgré les nombreux inhibiteurs développés, il y a présentement aucun inhibiteur qui a été utilisé pour un essai clinique [46]. Cela s'explique en partie par la découverte de nouveaux isoformes dont les rôles physiologiques doivent être davantage précisés. Les composés développés doivent donc inhiber spécifiquement l'isoforme pour lequel ils ont été synthétisés tout en affectant aucunement l'activité des autres isoformes de la 17β-HSD. De plus, l'utilisation et l'efficacité des inhibiteurs de l'aromatase ont, initialement, fait ombrage au développement d'inhibiteurs des 17β-HSDs. Malgré cela, plusieurs inhibiteurs des 17β-HSDs semblent prometteurs. L'un d'entre eux est un inhibiteur de la 17β-HSD 1 récemment développé dans notre laboratoire de chimie médicinale [47]. Le composé dénommé PBRM (Fig. 7) inhibe très bien la transformation de E1 en E2 par l'enzyme (IC_{50} = 68 nM) et est dépourvu d'activité estrogénique indésirable sur des cellules cancéreuses du sein estrogénodépendantes (cellules MCF-7).



PBRM

Figure 7. Structure d'un inhibiteur de la 17β-HSD 1.

5.3 Stéroïde sulfatase

Les sulfatases, une famille d'enzymes dont la stéroïde sulfatase (STS) fait partie, ont la capacité d'hydrolyser les liens sulfate esters de nombreux substrats (e.g. stéroïdes et protéoglycanes). Jusqu'à présent, 17 protéines sulfatases humaines et leurs gènes correspondant ont été identifiés [48]. Les différentes sulfatases ont une homologie de séquence variant entre 20 et 60%, mais possèdent tout de même des structures tertiaires similaires [49]. Toutes les sulfatases possèdent deux éléments essentiels à l'hydrolyse des groupements sulfates, soit un acide aminé formylglycine, modifié post-traductionnellement à partir d'une cystéine, ainsi qu'un atome de calcium. La catalyse enzymatique des sulfatases semble fonctionner par un mécanisme de transestérification-élimination et implique ces deux éléments clés [49].

L'arylsulfatase C (STS) est une protéine transmembranaire de 583 acides aminés associée principalement au réticulum endoplasmique, mais également présente dans l'appareil de Golgi et la membrane plasmique. La majeure partie de la protéine se situe dans la lumière du réticulum endoplasmique à l'exception des deux hélices α hydrophobes qui sont incrustées dans la membrane (Fig. 8). L'entrée du site catalytique se trouve à l'interface entre la protéine et la membrane lipidique du réticulum ce qui facilite ainsi l'entrée des stéroïdes sulfatés provenant du cytoplasme.

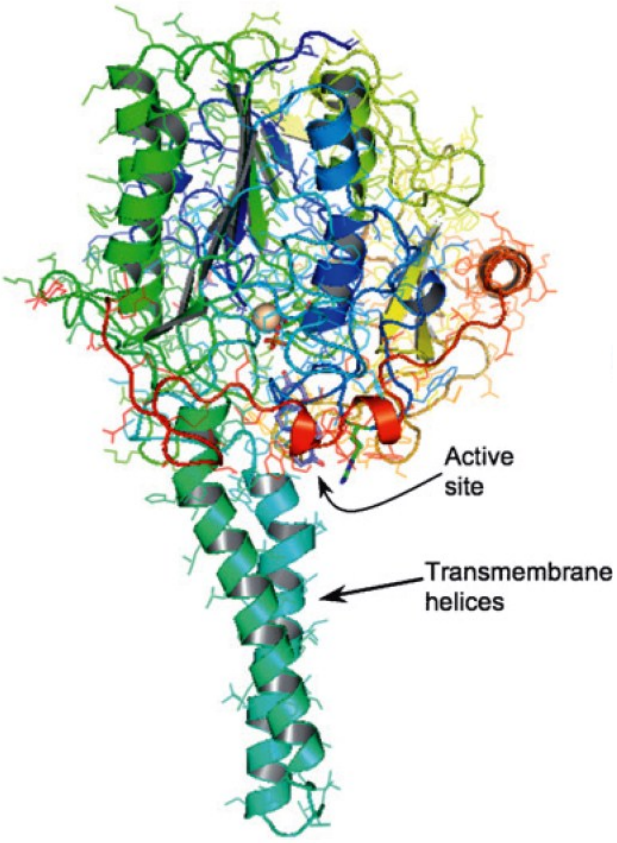


Figure 8. Structure tridimensionnelle de la stéroïde sulfatase [49].

La STS est la seule enzyme en mesure d'hydrolyser les stéroïdes sulfatés en hydroxystéroïdes non-conjugués, et ses principaux substrats sont le cholestérol sulfate, la prégnénolone sulfate, le déhydroépiandrostérone sulfate (DHEAS) et l'estrone sulfate (E1S) (Fig. 9) [50]. La présence d'un groupement sulfate sur les stéroïdes augmente leur polarité et, par le fait même, leur solubilité dans le sang. Les stéroïdes sulfatés, représentant la majorité des hormones stéroïdiennes en circulation, sont des précurseurs pouvant être convertis par la STS dans les tissus périphériques. En effet, E1S est le principal estrogène en circulation dans le sang et le DHEAS est le stéroïde le plus abondant [51].

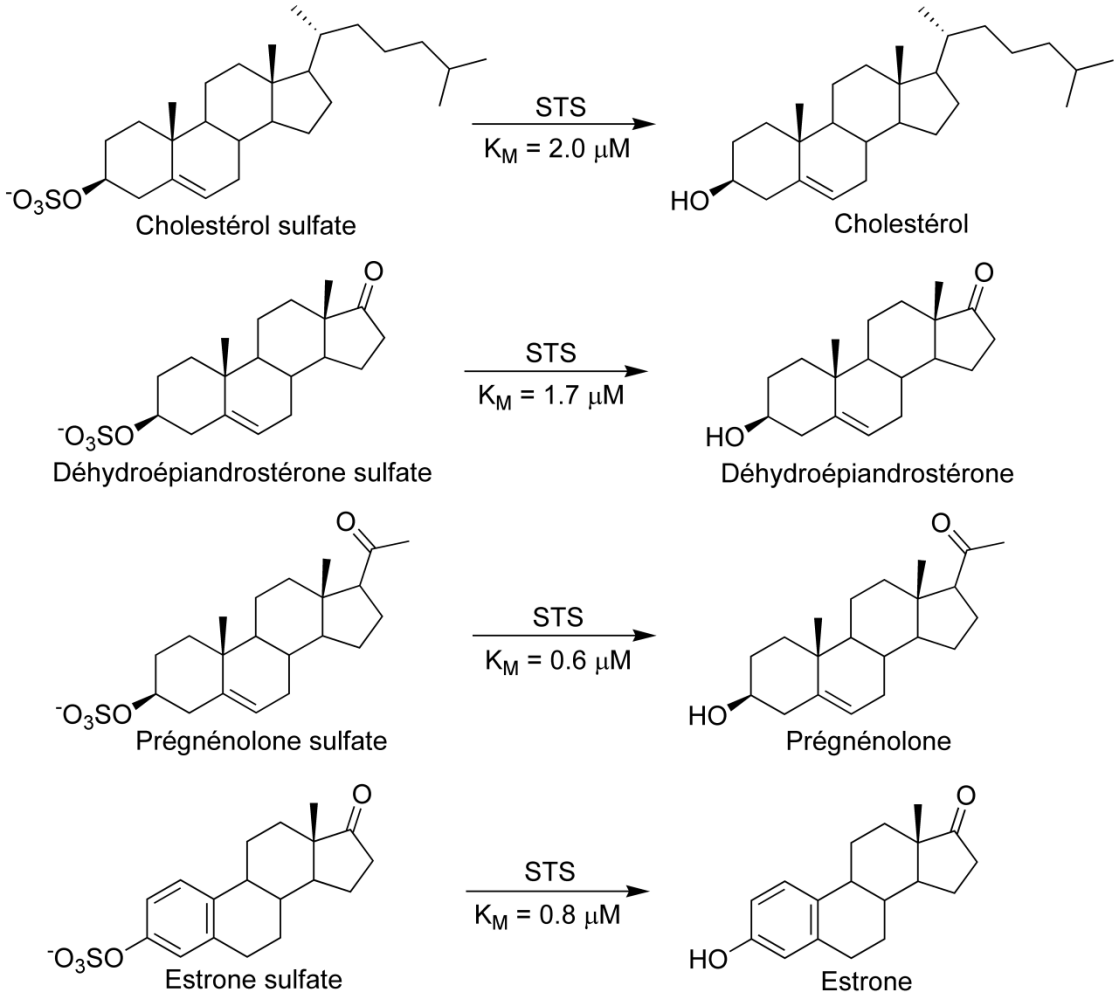


Figure 9. Principaux substrats de la stéroïde sulfatase (STS).

La stéroïde sulfatase a été détectée dans de nombreux tissus comme la glande mammaire, les ostéoblastes et le placenta et elle permettrait la synthèse *in situ* des hormones estrogéniques ou androgéniques en périphérie [52-53]. La synthèse intracrine d'estrogènes, à partir des précurseurs sulfatés, jouerait également un rôle majeur dans le développement des cancers du sein hormonodépendants. Effectivement, il a été démontré que l'activité enzymatique de la STS est plus élevée dans les tissus cancéreux du sein que dans les tissus mammaires normaux [54-55]. Il a aussi été démontré que l'activité STS dans les tumeurs cancéreuses du sein est plus élevée que l'activité de l'aromatase et que la formation *in situ* d'E1 et E2 provient principalement de la voie STS plutôt que de la voie de l'aromatase [56-58].

L'inhibition de la STS est une nouvelle approche thérapeutique prometteuse qui permettrait le blocage de la biosynthèse intracrine des estrogènes dans les tumeurs cancéreuses du sein estrogénodépendantes. Dans les dernières années, plusieurs équipes de recherche ont développé de très bons inhibiteurs [59]. Les inhibiteurs de la STS peuvent être séparés en plusieurs catégories selon leur structure stéroïdienne ou non-stéroïdienne et selon la présence ou non d'un groupement sulfamate ($-OSO_2NH_2$). Les inhibiteurs stéroïdiens ont généralement une bonne affinité pour la STS mais possèdent souvent une affinité pour le récepteur des estrogènes entrainant ainsi une estrogénicité indésirable. Les inhibiteurs non-stéroïdiens, quant à eux, ont habituellement une affinité moindre pour la STS et pour le récepteur des estrogènes.

Le groupement sulfamate s'est avéré être un très bon pharmacophore permettant l'inhibition irréversible de l'enzyme. Un des premiers inhibiteurs développés utilisant ce groupement est l'estrone sulfamate (EMATE) (Fig. 10). Ce composé inhibe bien l'enzyme (IC_{50} = 0.08 μM) mais relargue E1 après le clivage du groupement sulfamate par la STS [60]. Au cours des dernières années, les efforts ont été mis dans le développement d'inhibiteurs contenant un groupement sulfamate et possédant aucune activité estrogénique. Un de ces composés a été développé dans notre laboratoire de chimie médicinale et possède ces deux caractéristiques. Ce composé, le EM-1913 (Fig. 10), inhibe fortement la STS (IC_{50} = 0.024 nM) et ne possède pas d'activité estrogénique *in vitro* et *in vivo* [61]. Un autre composé très prometteur est le STX64 (Fig. 10), ou 667 coumate, un inhibiteur irréversible non-stéroïdien contenant également un groupement sulfamate. Le STX64 est actif oralement, possède une bonne biodisponibilité et est en mesure de réduire la taille des tumeurs mammaires chez des souris ovariectomisées en présence d'E1S [62]. Il est présentement le seul inhibiteur de la STS en étude clinique de phase 1 pour le traitement du cancer du sein chez les femmes postménopausées.

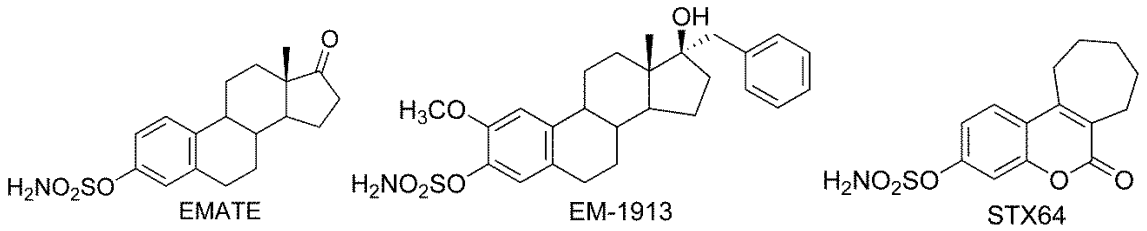


Figure 10. Structure d'inhibiteurs de la stéroïde sulfatase.

6. Récepteurs des estrogènes

6.1 Récepteurs des estrogènes α et β : structure et mécanisme

Les récepteurs des estrogènes sont des facteurs de transcription qui font partie de la grande famille des récepteurs nucléaires. Le premier récepteur des estrogènes a été cloné en 1986 et était alors considéré comme étant le seul récepteur en mesure de lier l'estradiol (E2) [63]. Mais en 1996, un second récepteur des estrogènes fut cloné et les 2 récepteurs furent alors nommés REα et REβ [64]. Le REα est une protéine de 66 kDa composée de 595 acides aminés tandis que REβ est une protéine de 59 kDa composée de 530 acides aminés. Les deux récepteurs possèdent une structure similaire composée de trois principaux domaines structuraux : le domaine N-terminal (NTD en anglais), le domaine de liaison à l'ADN (DBD en anglais) et le domaine de liaison du ligand (LBD en anglais) (Fig. 11) [65]. Ils possèdent également une homologie de séquence des acides aminés élevée, soit d'environ 95% dans le DBD et environ 55% dans le LBD.

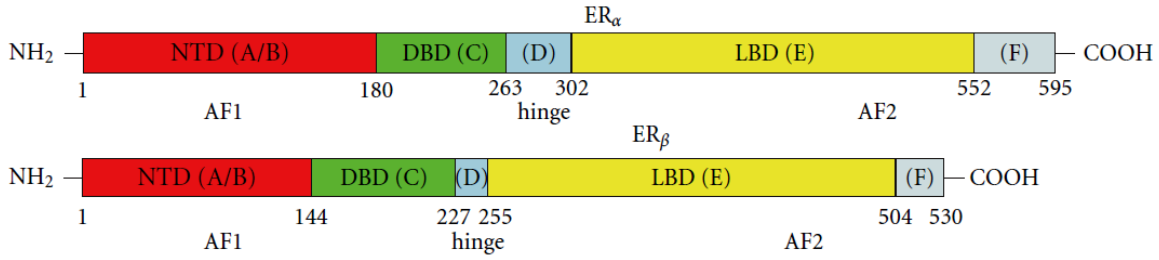


Figure 11. Domaines structuraux des récepteurs des estrogènes α et β [65].

Pour les 2 isomères du récepteur des estrogènes, le domaine de liaison au ligand est composé de 12 hélices α et de 1 feuillet β (Fig. 12) [66]. Bien que REα et REβ aient seulement 55% d'homologie de séquence en acides aminés dans ce domaine, la cavité de liaison du ligand diffère par seulement 2 acides aminés. Par contre, la grosseur de la cavité (REβ plus petite que REα) et les différents acides aminés en dehors de la cavité affectent l'affinité pour les ligands. À la suite de la liaison d'un ligand, par exemple E2, le domaine change de conformation et permet la dimérisation du récepteur, l'interaction avec différentes protéines corégulatrices et la translocation au noyau [67]. Effectivement, les récepteurs peuvent former des homodimères REα:REα ou REβ:REβ ou des hétérodimères REα:REβ pour se lier aux éléments de réponse des estrogènes (ERE) sur l'ADN. La liaison des protéines coactivatrices et corépressives joue également un rôle central dans la transcription des gènes. Lorsqu'un ligand se lie dans la cavité de liaison du domaine, le changement de conformation entraîne le déplacement de l'hélice α 12 qui forme, par la suite, avec les hélices α 3 et 5 une surface permettant la liaison des protéines corégulatrices [68]. Les protéines coactivatrices et corépressives contiennent une ou plusieurs séquences consensus riches en leucine LXXLL (X étant n'importe quel acide aminé) qui se lient à la fonction d'activation de la transcription-2 (AF-2 en anglais), soit la structure formée par les hélices 3, 5 et 12 [69].

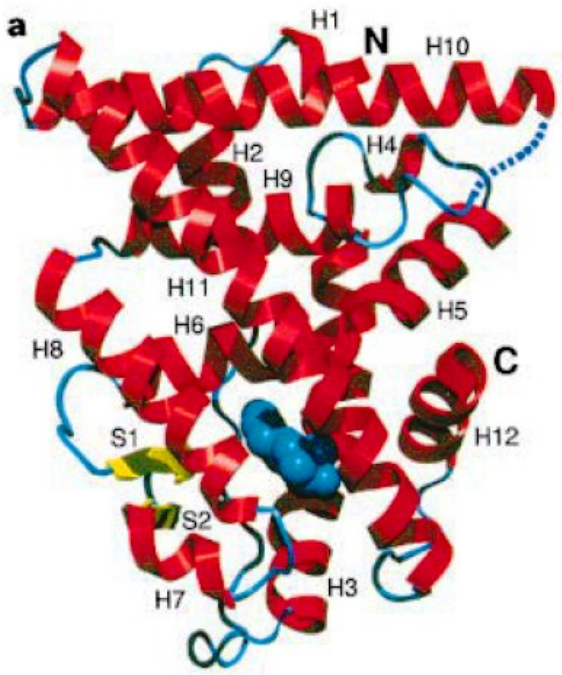


Figure 12. Structure du domaine de liaison au ligand du récepteur des estrogènes α [66].

La transcription de gènes est initialisée une fois que le dimère de récepteurs est entré dans le noyau. Le domaine de liaison à l'ADN se lie alors aux ERE situés à proximité des régions promotrices des gènes ou situés dans des régions amplificatrices distantes du site d'initiation de la transcription. Le domaine possède deux sous domaines en doigt de zinc, composé de 8 cystéines coordonnant 2 atomes de zinc, qui interagissent directement avec l'ADN [65]. Les deux doigts de zinc reconnaissent spécifiquement ERE, soit la séquence palindromique de 12 nucléotides 5'-AGGTCAnnnTGACCT-3', et initialisent la transcription. Les estrogènes ont donc la capacité d'augmenter ou diminuer l'expression des gènes associés à ERE via les récepteurs REα et REβ.

6.2 Rôles des récepteurs dans le tissu mammaire et osseux

Les estrogènes jouent un rôle important dans la régulation de nombreux tissus. En effet, les récepteurs des estrogènes α et β ont été détectés dans plusieurs tissus humains, dont le cerveau, l'utérus, les testicules, les muscles, le foie, la prostate, la glande mammaire et les os [70-71]. Même si le rôle des estrogènes dans certains tissus reste à élucider, la présence

des récepteurs nucléaires estrogéniques laisse supposer une fonction spécifique. Pour d'autres tissus, le rôle des estrogènes est plus évident.

Dans le tissu mammaire, les estrogènes assurent le développement des glandes mammaires et des canaux galactophores afin de produire et d'acheminer le lait pour les nouveau-nés. Les effets des estrogènes dans ce tissu seraient perpétrés par l'isomère α du récepteur des estrogènes et non β. En effet, il fut démontré que des souris déficientes (knock-out) pour REα ont un développement très rudimentaire de leurs glandes mammaires tandis que les souris déficientes pour REβ ont des glandes mammaires normales [72]. L'isomère α serait également responsable du développement des cellules cancéreuses du sein estrogénodépendantes. Des études démontrèrent qu'E2 active REα et régule l'expression de plusieurs gènes de prolifération cellulaire tels que c-myc et les cyclines D1, B1 et A dans les cellules cancéreuses du sein MCF-7 [73]. Par contre, lorsque REβ est coexprimé avec REα dans les cellules MCF-7, ces gènes de prolifération cellulaire ne sont pas activés. Cela suggère donc que REα serait responsable des effets prolifératifs des estrogènes dans les cellules cancéreuses du sein et que REβ aurait plutôt un rôle antiprolifératif en bloquant les effets attribuable à REα. Le blocage sélectif de REα, par des antiestrogènes pures ou des SERMs, est donc souhaitable afin d'empêcher la prolifération des cellules cancéreuses du sein sensibles aux estrogènes.

Dans le tissu osseux, les estrogènes ont le rôle de maintenir l'intégrité du tissu en contrôlant l'équilibre entre les ostéoblastes et les ostéoclastes. Des études ont démontré, par l'utilisation de composés sélectifs et de souris « knock-out », que REα est majoritairement responsable de la régulation de l'homéostasie dans les os [74-76]. Le rôle de REβ dans ce tissu reste toutefois à clarifier. Les estrogènes, via REα, régulent plusieurs gènes importants à la prolifération et à la différentiation des ostéoblastes et des ostéoclastes. Les estrogènes activent la différentiation des ostéoblastes en activant plusieurs gènes (e.g. alkaline phosphatase et collagène de type 1) importants à la synthèse et à la calcification de la matrice extracellulaire. Les ostéoclastes sont également régulés par les estrogènes, mais cette régulation serait principalement attribuable aux ostéoblastes [77]. En effet, les

ostéoblastes contrôlent la prolifération et la différentiation des ostéoclastes en synthétisant, en excrétant de façon paracrine et en exprimant certaines protéines à leur surface. Fas Ligand (FasL) est une protéine produite par les ostéoblastes qui se lie aux récepteurs Fas présents dans la membrane des ostéoclastes. Elle a le rôle d'enclencher l'apoptose des ostéoclastes afin de bien contrôler leur prolifération, et par le fait même, de maintenir l'homéostasie du tissu [78]. L'osteoprotégérine (OPG) et RANKL (Receptor-Activator of Nuclear-factor Kappa-B Ligand), quant à eux, contrôlent étroitement la différentiation des ostéoclastes [79]. RANKL est produit par les ostéoblastes sous deux formes distinctes, soit membranaire et soluble, et a pour rôle d'activer RANK (Receptor-Activator of Nuclear-factor Kappa-B), un récepteur à la surface des ostéoclastes qui active leur différentiation. L'OPG, qui est surexprimée en présence de E2 et sécrétée par les ostéoblastes, est un récepteur soluble qui se lie à RANKL, empêchant ainsi l'activation des ostéoclastes. Les SERMs, tout comme les estrogènes, sont également en mesure d'activer ces gènes essentiels au maintien de l'équilibre entre les ostéoblastes et les ostéoclastes.

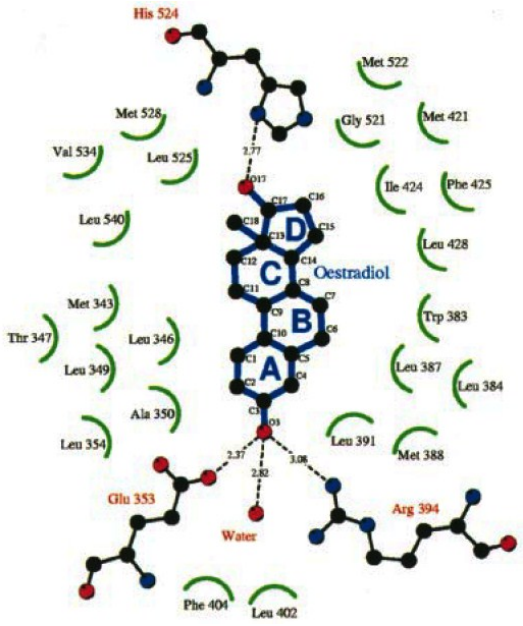
6.3 Mécanisme des SERMs

Les SERMs sont des composés qui ont été développés afin de moduler l'activité de REα dans les différents tissus. Le récepteur des estrogènes α (REα) étant responsable de l'activité estrogénique dans le tissu mammaire, le développement des SERMs pour le traitement des cancers du sein estrogénodépendants fut orienté vers cet isomère. Comme mentionné précédemment, un bon SERM doit être antagoniste dans le tissu mammaire et agoniste dans le tissu osseux. Pour réaliser leurs effets spécifiques dans ces tissus, les SERMs doivent tout d'abord posséder une bonne affinité pour REα. La cristallisation du récepteur permit de mettre en évidence certains acides aminés importants pour la liaison des ligands. En effet, lorsqu'on examine E2 dans la cavité de liaison, on remarque que le groupement hydroxyle en position C-3 fait des liaisons hydrogènes avec Glu353, Arg394 et une molécule d'eau tandis que l'hydroxyle en position C-17 fait une liaison hydrogène avec His524 (Fig. 13a) [66]. Le raloxifène, quant à lui, fait également des liaisons hydrogènes avec ces trois acides aminés (Fig. 13b), mais possède une longue chaine latérale qui joue un rôle majeur dans sa modulation du récepteur [66]. En effet, la chaine latérale sort de la cavité de liaison et vient interagir avec d'autres acides aminés. Ces interactions modifient le

changement de conformation habituellement observé avec E2 et bloquent partiellement le repliement de l'hélice α 12 du récepteur.

Ainsi, dans le cas des SERMs, le repliement partiel de l'hélice 12 influence directement la structure d'AF-2 et la liaison des protéines coactivatrices et corépressives [68]. De plus, l'expression différentielle des protéines corégulatrices dans les différents tissus influencent beaucoup l'activité des récepteurs des estrogènes. Les protéines coactivatrices et corépressives ne se lient pas tous de la même manière à AF-2 et peuvent être ou ne pas être affectées par liaison d'un SERM. Également, les SERMs se lient tous différemment au récepteur et peuvent différer quant à leur effet dans les tissus. Par exemple, dans l'utérus, le tamoxifène est agoniste tandis que le raloxifène est antagoniste. Donc, le développement des SERMs est un processus très complexe, car l'effet observé dans les tissus est dû à plusieurs aspects structuraux et mécanistiques.

a)



b)

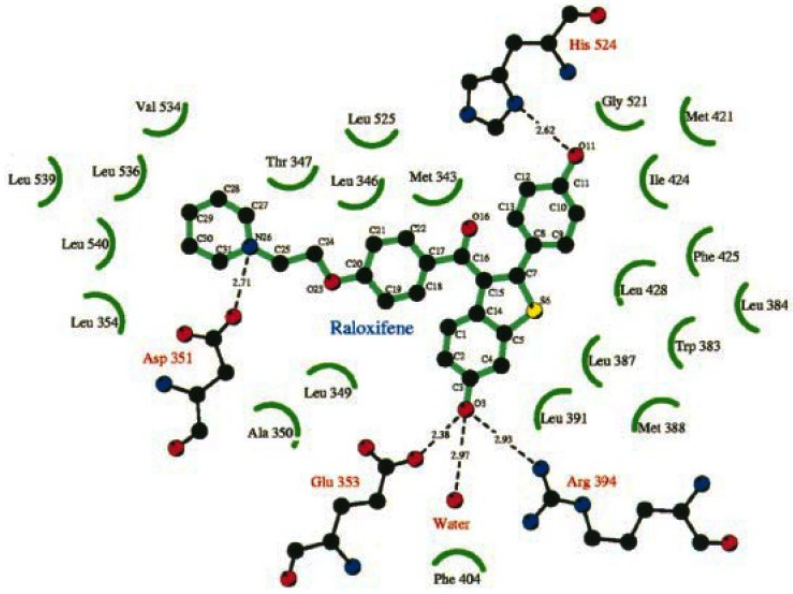


Figure 13. Liaisons hydrogènes entre le récepteur des estrogènes α et a) E2 et b) raloxifène [66].

7. Aperçu du projet de recherche

Ce projet de recherche a pour but d'évaluer la capacité d'inhibiteurs à double-action pour le traitement des cancers du sein estrogénodépendants. Les différents composés qui seront présentés dans ce mémoire ont été préalablement synthétisés dans notre laboratoire de chimie médicinale. Pour permettre la création rapide de librairies, tous les composés ont été synthétisés sur support solide en parallèle. Un noyau non-stéroïdien de type tétrahydroisoquinoline fut utilisé et permis l'ajout de divers groupements pour obtenir une grande diversité moléculaire (Fig. 14). Suite à l'utilisation d'une résine et d'un ancrage sulfamate, le clivage final des composés permet d'obtenir en condition acide des composés sulfamates ($R = SO_2NH_2$) ou, en condition nucléophile, des composés phénoliques ($R = H$). La diversité moléculaire des composés provient principalement des divers groupements X, R^1 et R^2 ainsi que du positionnement de l'amine tertiaire sur le noyau benzylique (Fig. 14).

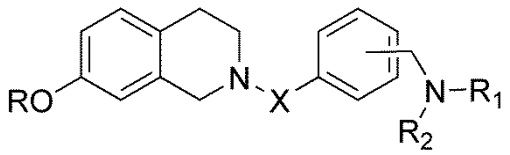


Figure 14. Structure de base des composés à double-action

Les composés ont été créés afin d'inhiber la STS et de bloquer sélectivement le récepteur des estrogènes de type alpha (REα) dans le tissu mammaire sans toutefois nuire au développement du tissu osseux. En ce qui a trait à la STS, seul les composés possédant un groupement sulfamate sont en mesure d'inhiber l'enzyme. Lorsqu'un composé sulfamate entre dans le site catalytique, l'enzyme clive le groupement sulfamate et relargue le composé phénolique correspondant (Fig. 15). Le groupement sulfamate reste lié de façon covalente à un des acides aminés du site actif entraînant ainsi l'inhibition irréversible de l'enzyme et éventuellement la dégradation par le protéasome. Afin d'évaluer le potentiel d'inhibition des composés sulfamates, des homogénats de cellules HEK-293 transfectées pour la STS furent utilisés.

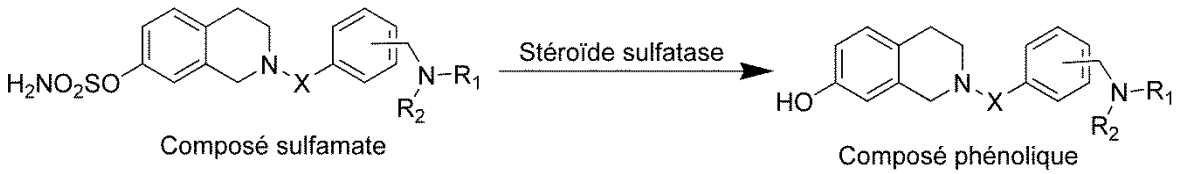


Figure 15. Conversion d'un composé sulfamate en composé phénolique par la stéroïde sulfatase (STS).

L'évaluation du potentiel SERM d'un composé sur les tissus recherchés nécessite plusieurs tests différents. Il faut mentionner que l'effet SERM, contrairement à l'inhibition de l'activité STS, peut être médié par les composés phénoliques et sulfamates. Premièrement, les composés ne doivent pas posséder une activité estrogénique qui stimulerait la prolifération des cellules cancéreuses du sein hormonodépendantes. Deuxièmement, ils doivent posséder un caractère antiestrogénique, c'est-à-dire d'être en mesure de bloquer la stimulation prolifératives des cellules cancéreuses du sein induite par E2. Pour évaluer ces

deux paramètres, des cellules cancéreuses du sein sensibles aux estrogènes (cellules T-47D et MCF-7) seront incubées avec les différents produits en absence ou en présence d'E2.

À l'opposé du tissu mammaire, il est nécessaire que les composés stimulent la prolifération et la différentiation des cellules osseuses responsables de la formation du tissu osseux, soit les ostéoblastes. Une lignée cellulaire d'ostéosarcome humain (cellules Saos-2), qui possède de nombreuses caractéristiques similaires aux ostéoblastes normaux, sera utilisée. La capacité de stimuler la prolifération et l'activité de l'alkaline phosphatase sera étudiée en incubant les cellules ostéoblastiques avec les divers composés. Finalement, l'affinité de certains composés pour REα sera évaluée pour s'assurer que les effets observés sont bel et bien médiés par l'activation ou le blocage du récepteur.

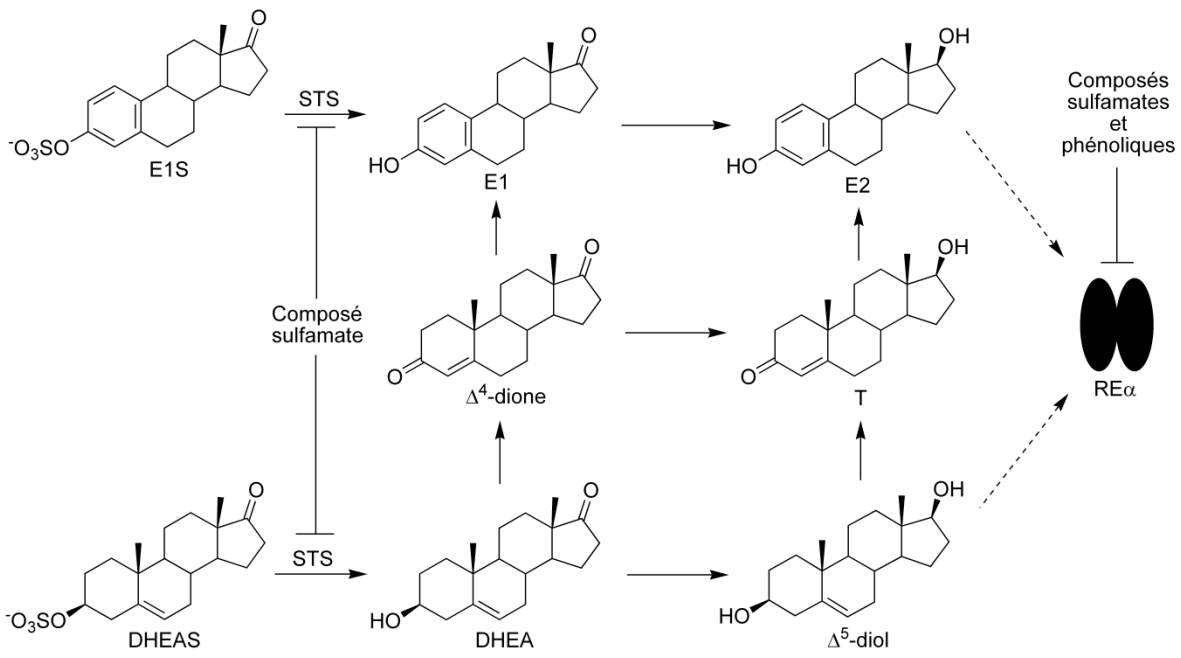


Figure 16. Site d'action des composés à double-action dans le tissu mammaire.

Afin de traiter un cancer du sein hormonodépendant, ce serait un composé sous sa forme sulfamate qui serait administré. Le composé sulfamate serait en mesure d'inhiber la STS et de bloquer l'activation de REα dans le tissu mammaire (Fig. 16). Le composé phénolique relargué suite à l'inhibition de la STS contribuerait également au blocage du récepteur.

Donc, l'inhibition de la STS empêcherait la tumeur de pouvoir utiliser le DHEAS et l'E1S présents en grande quantité dans le sang tandis que le blocage de REα empêcherait son activation par E1, E2 ou le Δ^5-diol qui pourrait entrer dans la tumeur sous forme non-sulfaté. Cette double-action permettrait à la fois de bloquer la synthèse *in situ* des estrogènes et de bloquer l'activation de REα pour ainsi empêcher complètement la stimulation de la prolifération des cellules cancéreuses du sein. L'aspect SERM de notre composé à double-action permettrait également l'activation de REα dans les ostéoblastes pour maintenir l'intégrité du tissu osseux.

Ce mémoire sera séparé en trois chapitres pour les deux générations de composés ayant été synthétisés et testés biologiquement. La 1^{ere} génération de composés (108 composés) sera détaillée dans les chapitres 1 et 2 tandis que la 2^{e} génération de composés (16 composés) sera détaillée dans le chapitre 3.

Chapitre 1

Résumé

Divers dérivés d'un noyau tétrahydroisoquinoline furent préparés comme inhibiteurs à double-action de la stéroïde sulfatase (STS). Les dérivés sulfamates et les dérivés phénoliques correspondants ont été synthétisés par chimie parallèle en phase solide. Les composés sulfamates furent testés pour leur capacité à inhiber l'activité STS tandis que les composés phénoliques furent testés pour leur capacité ou incapacité à induire la prolifération des cellules hormonodépendantes MCF-7 et des cellules Saos-2. Il fut intéressant de voir que plusieurs dérivés sulfamates montrèrent une bonne inhibition de la STS (80-90% d'inhibition à 0.1 μM). Également, quatre dérivés phénoliques n'ont pas présenté une activité estrogénique indésirable sur les cellules MCF-7 mais ont induit la prolifération et la différentiation des cellules Saos-2, suggérant ainsi un potentiel de modulation sélective du récepteur des estrogènes.

Manuscript soumis à *Medicinal Chemistry Communications*

Investigation of a tetrahydroisoquinoline scaffold as dual-action steroid sulfatase inhibitors generated by parallel solid-phase synthesis §

Étienne Ouellet, René Maltais, Charles Ouellet, Donald Poirier*

Laboratory of Medicinal Chemistry
CHU de Québec (CHUL) - Research Center and Laval University
2705 Laurier Boulevard, Québec (Québec) G1V 4G2, Canada

§ Electronic supplemental information (ESI) available: General information for the chemistry, characterization and LRMS data (Tables 1S-3S and chromatograms) of members of libraries 1-EO, 2-EO, 3-EO, 4-EO, 3'-EO, and 4'-EO. Table 4S: Chemical structures of phenol derivatives from libraries 3'-EO and 4'-EO and their effect on MCF-7 (ER^+) cell proliferation (%).

* Corresponding author:
Dr. Donald Poirier
Laboratory of Medicinal Chemistry
CHU de Québec (CHUL) - Research Center
2705 Laurier Boulevard, Québec (Québec) G1V 4G2, Canada
Phone: (418) 654-2296; Fax: (418) 654-2761
E-mail: donald.poirier@crchul.ulaval.ca

Abstract: Various derivatives of a tetrahydroisoquinoline core were designed as non-steroidal dual-action inhibitors of steroid sulfatase (STS). Sulfamate derivatives and corresponding phenol derivatives were both synthesized by parallel solid-phase chemistry. The sulfamate compounds were tested for their ability to inhibit STS activity, whereas the phenol compounds were tested for their ability or non-ability to induce the proliferation of estrogen-dependent MCF-7 and Saos-2 cells. Interestingly, many sulfamate derivatives showed good inhibitory activity toward the enzyme (80-90% of inhibition at 0.1 μM), whereas four phenol derivatives did not present unwanted estrogenic activity on MCF-7 cells but induced a proliferation of Saos-2 cells, thus suggesting a selective-estrogen receptor modulator potential.

Key words: steroid sulfatase, steroid, estrone sulfate, inhibitor, enzyme, breast cancer

Introduction

Steroid sulfatase (STS) is an enzyme responsible for the hydrolysis (desulfatation) of sulfated steroids to the free (unconjugated) hydroxylated version (Fig. 1A).[1] It is clear that STS plays a crucial role in the production of biologically active hydroxysteroids from inactive sulfated steroids. Hydroxysteroids such as estrone (E1) and estradiol (E2) are known to have a major impact on the development and growth of tumors in estrogen-dependent diseases such as endometriosis, uterine cancer and breast cancer.[2-3] Sulfated derivatives are unable to activate the estrogen receptor and it is impossible for steroidogenic enzymes other than STS to transform sulfated precursor. Also, most of the circulating estrogens are found to be of the sulfated version, considering that conjugated steroids are more hydrophilic, thus facilitating their transportation. It is highly probable that circulating sulfated steroids act as a reservoir for the formation of active steroids by the action of STS.[4] The main substrates known for STS are pregnenolone sulfate (PREGS), estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS) (Fig. 1A). The K_m values for these substrates are 0.6 μM, 0.8 μM and 1.7 μM, respectively.[5] DHEAS is the major sulfated steroid found in circulation and is a precursor of potent estrogens.[6] It is also important to note that STS is not a reversible enzyme and that sulfatation is done by the

sulfotransferase enzyme.[7] As pointed out previously, STS is a key enzyme in the production of biologically active steroids. Additionally, many *in vitro* studies suggest that STS would be the enzyme mainly responsible for the intratumoral production of estrone in estrogenic-dependent tumors.[8-9] Therefore, inhibition of this enzyme is a suitable approach for the treatment of estrogen-dependent diseases. Many STS inhibitors have been synthesized and have shown great potency in *in vitro* and *in vivo* studies,[10-15] and inhibitor STX64 has gone through pre-clinical and clinical studies.[16] These results support the relevance of developing potent inhibitors of this enzyme.

An approach recently investigated in our laboratory is based on dual-action inhibitors of STS. The goal is to develop a non-steroidal sulfamoylated inhibitor of the enzyme, having a selective estrogen receptor modulator (SERM) under its phenolic form. Indeed, it has already been shown that an aryl sulfamate is a good pharmacophore for the inactivation of STS (Fig. 1B).[17,18] Once the aryl sulfamate moiety (Ar-OSO_2NH_2) has inactivated the enzyme, or has been chemically hydrolyzed, the corresponding phenolic compound (Ar-OH) is released,[19-22] and may have further biological implications, especially as a SERM, a class of products acting as an estrogen receptor (ER) antagonist in the breast and the uterus, and acting as agonist in the bone and in the brain.[23] Dual-acting inhibitors of STS were previously reported by sulfamoylating the steroidal SERM SR-16137 [24-26] or by sulfamoylating the non-steroidal antiestrogen tamoxifen.[27] As the first part of a global approach targeting dual-action inhibitors, we now report the chemical synthesis and biological evaluation of non-steroidal sulfamates and their corresponding phenol derivatives.

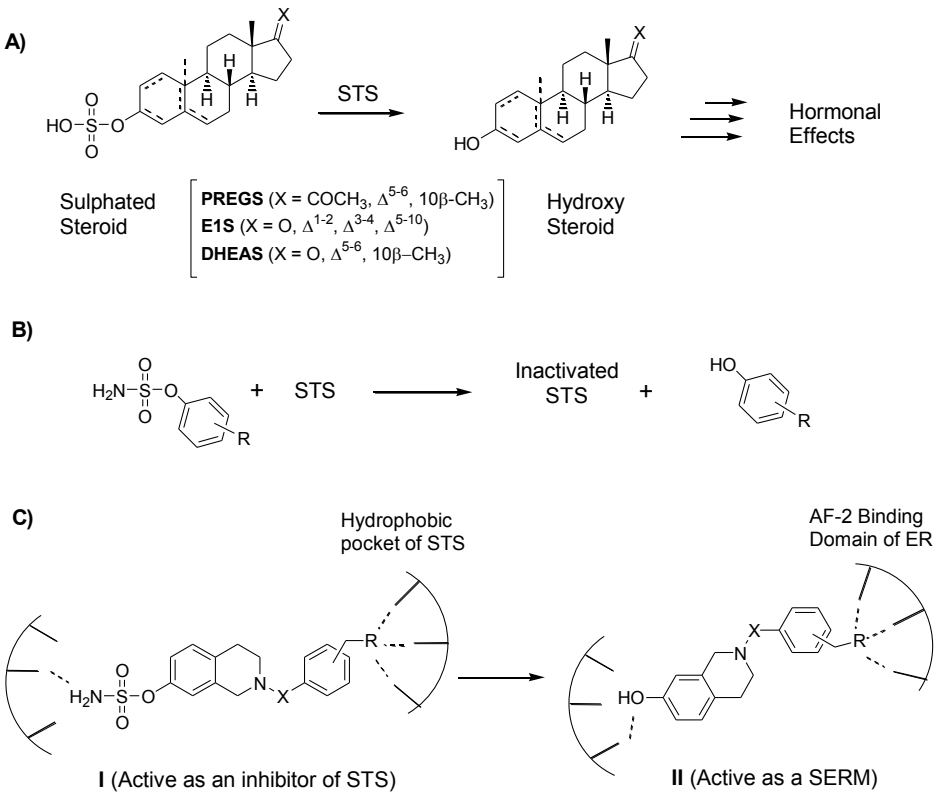


Fig. 1 A) Role and main substrates of steroid sulfatase (STS). B) Simplified mechanism of inactivation of steroid sulfatase (STS) by a sulfamate inhibitor (H_2NSO_2Ar). C) Potential dual role of designed compounds as STS inhibitors (**I**) and as SERMs (**II**). ER: estrogen receptor; SERM: selective estrogen receptor modulator.

Results and discussion

Chemistry

The primary objective of our ambitious project is to develop a synthetic route to access the sulfamate and hydroxy derivatives in order to see if our proposed inhibitors are potent (by testing the sulfamate derivatives) and if they present estrogenic activity (by testing the hydroxy derivatives). Solid-phase synthesis and combinatorial chemistry were perfectly suited for this project as we needed various levels of diversity from a common scaffold. The linker used for the solid-phase synthesis is the multidetachable sulfamate linker developed in our laboratory.[28-30] According to the conditions used for the cleavage of the resin, a sulfamate or a phenol derivative is obtained. Indeed, acidic conditions will give a

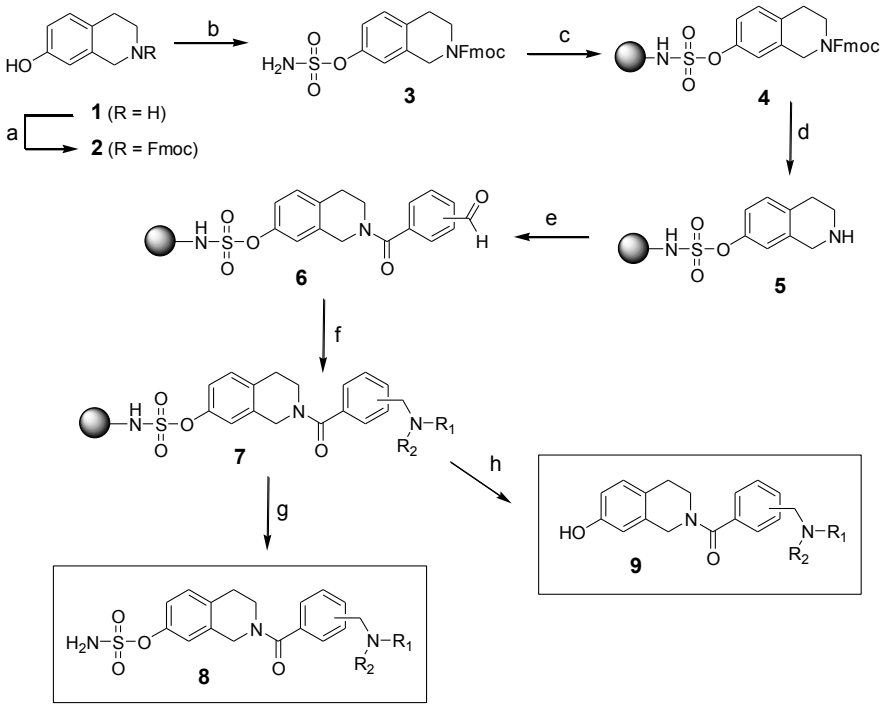
sulfamate derivative for STS inhibition while nucleophilic conditions will give a phenol derivative for SERM effect.

The starting unit and common scaffold for every compound is a non-steroidal 1,2,3,4-tetrahydroisoquinolin-7-ol core. This scaffold was chosen because of three main characteristics. First, we needed a phenol moiety to attach the sulfamate linker. Second, the amine could be used to install various lateral chains that will provide key interactions with well-defined hydrophobic STS pocket for potent inhibition.[31,32] Third, and most important, it is also believed that by pivoting the molecule longitudinally, the lateral chain will be able to interact with binding domain AF-2 and the co-activators of the ER, thus causing the SERM effect (Fig. 1C).[33] Interestingly, in a different therapeutic approach, a similar tetrahydroisoquinoline core has been recently reported as a microtubule disruptor showing the potential of this kind of molecular platform.[34]

*Amide derivatives **8** and **9***

The general synthetic methodology for the preparation of all library members is outlined in Scheme 1. The starting 1,2,3,4-tetrahydroisoquinolin-7-ol (**1**) was selectively protected as the *N*-Fmoc derivative **2**, which after a sulfamoylation of the phenol yielded the sulfamate **3**. This sulfamate derivative was then reacted with the trityl chloride resin to give the solid-phase bounded compound **4**. Deprotection of the *N*-Fmoc provided resin **5** with a free NH, which can be diversified with various spacers or different side chains. Since the first spacer considered was a benzamide, resin **6** was obtained by acylation of resin **5** with carboxybenzaldehyde. Another level of diversity was then added by performing a reductive amination of resin **6** with various secondary amines that yielded resin **7**. Finally, both the sulfamate derivatives **8** and the phenol derivatives **9** were obtained by cleavage from the solid support using acidic and nucleophilic conditions, respectively. We thus generated four libraries (1-EO, 2-EO, 3-EO and 4-EO; 54 sulfamate derivatives) as potential STS inhibitors (Tables 1 and 2) and two libraries (3'-EO and 4'-EO; 34 phenol derivatives) as potential SERM (Figures 4 and 5). For libraries 1, 3 and 3', the secondary amine is in *para*

position relative to the benzamide group, whereas for libraries 2, 4 and 4', the secondary amine is in *meta* position.

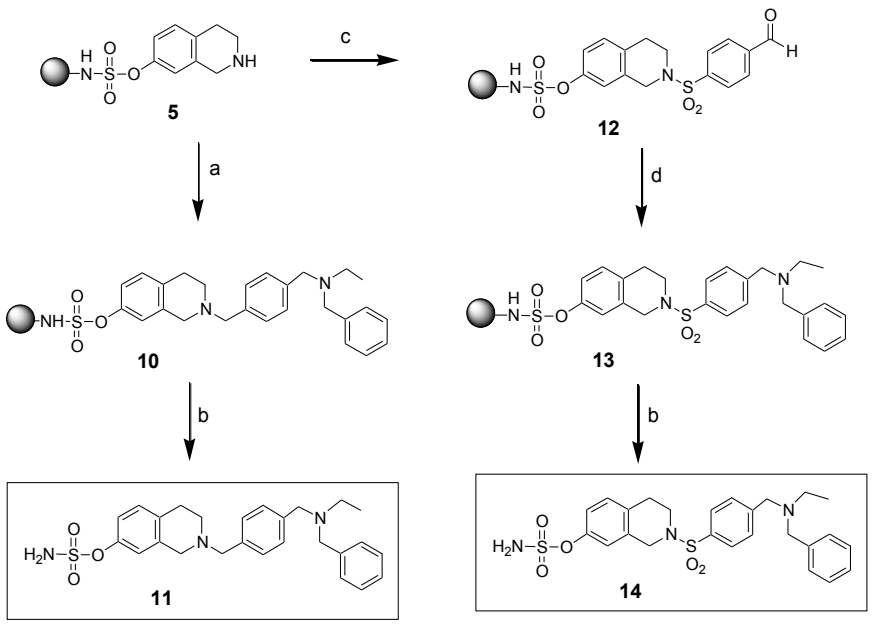


Scheme 1 Synthetic route to obtaining sulfamate derivatives **8** (Libraries 1-4) and phenol derivatives **9** (Libraries 3' and 4'). Reagents and conditions: (a) Fmoc-*O*-succinimide, $NaHCO_3$, H_2O; (b) NH_2SO_2Cl, 2,6-di-*tert*-butyl-4-methylpyridine, DCM; (c) trityl chloride resin, DIPEA, DMA/DCM; (d) 20% piperidine in DMF; (e) carboxybenzaldehyde, DIPEA, HOBt, PyBOP, DMF; (f) secondary amine (R_1R_2NH), $NaBH(OAc)_3$, 10% AcOH in NMP; (g) 30% HFIP in DCM; (h) 30% DEA in THF, 60°C.

*Amine and sulfonamide derivatives **11** and **14***

In order to determine the relative importance of chemical functionality on inhibitory activity (amide, amine, sulfonamide) at the junction point between the tetrahydroisoquinoline and the substituted benzylamine moiety, we synthesized the amine and sulfonamide analogs of the best amide derivative (**1-EO-9**) of library 1 (Scheme 2). The amine **11** was obtained by first performing a reductive amination on resin **5** using the N-ethylbenzylamine-benzaldehyde building block to give the corresponding amine

intermediate **10**, which was then submitted to an acidic cleavage to release the corresponding sulfamate **11**. On the other side, the sulfonamide **14** was obtained by having the amine resin **5** react with the 4-sulfonylchloride benzaldehyde to give the aldehyde intermediate resin **12**, which was then submitted to a reductive amination with N-ethylbenzylamine to provide resin **13**. The sulfonamide **14** was finally cleaved from the solid support in mild acidic conditions.



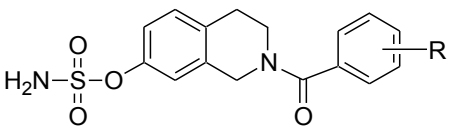
Scheme 2 Synthetic route to obtaining sulfamates **11** (amine) and **14** (sulfonamide). Reagents and conditions: (a) i) 4-{[benzyl(ethyl)amino]methyl}benzaldehyde, AcOH, NMP rt, 45 min; ii) $NaBH(OAc)_3$, NMP, 18 h, rt; (b) 30% HFIP in DCM, rt; (c) 4-sulfonylchloride-benzaldehyde, triethylamine, DCM, rt; (d) i) N-benzylethanamine, AcOH, NMP, rt, 45 min; ii) $NaBH(OAc)_3$, NMP, rt, 18 h.

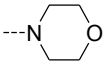
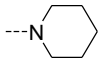
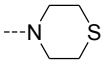
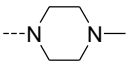
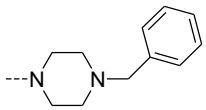
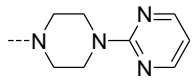
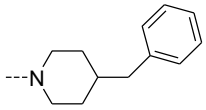
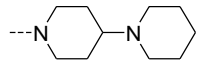
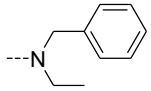
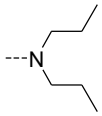
STS inhibition studies

The enzymatic assay was performed using homogenate of STS transfected HEK-293 cells as the source of enzyme activity. The transformation of [^{3}H]-E1S into [^{3}H]-E1 was measured using scintillation counting of labeled E1S and E1 in the aqueous and organic phases, respectively. Newly synthesized sulfamate compounds were tested at two

concentrations (0.1 μM and 1 μM). Libraries **1-EO** and **2-EO** were synthesized using the *p*- and *m*-benzamide spacers as 1st level of molecular diversity and the same wide variety of secondary amines as 2nd level of diversity (Table 1), which were chosen in a somewhat random fashion while including a different pattern, format and heteroatom. At first glance to the inhibition results, it can be seen that library 1 (*para*) seems to present more inhibitory potency toward the enzyme compared to library 2 (*meta*). Compounds **1-EO-07**, **1-EO-09** and **1-EO-10** represent a good starting point for optimization, as they show more than 36% of inhibition when tested at a concentration of 0.1 μM. These compounds have relatively hydrophobic substituents compared to the rest of the library members. In fact, logP values for **1-EO-07**, **1-EO-09** and **1-EO-10** are 3.87, 3.08 and 3.07, respectively. The average logP value for the rest of the library, excluding these three compounds, is 1.93. Library 2 members with the secondary amine side-chain in *meta* position showed overall less potency of inhibition compared to library 2 members in *para* position. As an example, compound **2-EO-09** gave 17% of STS inhibition at 0.1 μM while the *para* equivalent (**1-EO-09**) displayed 67% of inhibition at the same concentration. However, we decided to keep both orientations for the next libraries in order to see if optimized secondary amines in *meta* position would yield better results. It is important to keep in mind that the objective is not only to develop a potent STS inhibitor (sulfamate derivatives), but also to obtain a compound that can act as a SERM in its phenolic form. The usual interaction between a SERM, generally a phenol derivative, and the ER depends on the hydrogen bond.[35] Knowing that we have to find a compromise between STS inhibition and SERM activity, we focused on developing potent STS inhibitors that bear side chains that are able to form hydrogen bonds.

Table 1 Structure of the sulfamate compounds of libraries **1-EO** and **2-EO** and their inhibitory activity (%) for the transformation of [^{3}H]-estrone sulfate into [^{3}H]-estrone by STS (HEK-293 transfected cells)



R	ID	*Para compound* STS inhibition (%) [a]		ID	*Meta compound* STS inhibition (%) [a]	
		0.1 µM	1 µM		0.1 µM	1 µM
	**1-EO-01**	10.9 ± 3.5	50.6 ± 1.2	**2-EO-01**	0.0 ± 4.7	20.9 ± 4.4
	**1-EO-02**	11.5 ± 2.4	38.3 ± 3.6	**2-EO-02**	1.0 ± 5.1	11.9 ± 2.6
	**1-EO-03**	32.3 ± 1.0	80.2 ± 0.9	**2-EO-03**	2.3 ± 0.1	39.6 ± 2.8
	**1-EO-04**	2.9 ± 0.5	23.3 ± 1.3	**2-EO-04**	0.0 ± 12.2	12.6 ± 2.3
	**1-EO-05**	20.8 ± 3.7	75.8 ± 1.6	**2-EO-05**	0.0 ± 3.1	23.7 ± 0.6
	**1-EO-06**	14.7 ± 3.6	41.9 ± 0.8	**2-EO-06**	1.6 ± 1.6	24.4 ± 0.4
	**1-EO-07**	41.4 ± 0.8	81.3 ± 0.5	**2-EO-07**	1.4 ± 0.6	41.9 ± 2.7
	**1-EO-08**	8.5 ± 1.2	53.7 ± 4.7	**2-EO-08**	0.0 ± 2.4	8.0 ± 1.1
	**1-EO-09**	66.9 ± 1.5	83.6 ± 0.2	**2-EO-09**	17.3 ± 2.5	69.0 ± 0.0
	**1-EO-10**	36.6 ± 1.3	84.7 ± 0.2	**2-EO-10**	8.3 ± 0.0	54.8 ± 1.2

[a] Compounds were tested at two concentrations of 0.1 µM and 1 µM. Results are expressed as mean ± SEM of one experiment performed in triplicate.

Before the synthesis of subsequent libraries, we were concerned about the influence of the chemical functionality acting at the junction point between the tetrahydroisoquinoline moiety and the benzylamine portion on STS inhibition. We thus synthesized the amine and sulfonamide analogs (sulfamate derivatives **11** and **14**) of the best inhibitor of library 1 (**1-EO-9**) to see if the amide represented the best functionality in order to maximize the interaction of the two key parts of the molecule inside the STS enzyme. As a result, the inhibition levels of amine analog **11** (1% at 0.1 µM and 17% at 1 µM) and sulfonamide analog **14** (10% at 0.1 µM and 31% at 1 µM) were clearly lower than the inhibition of the amide derivative **1-EO-9** (67% at 0.1 µM and 84% at 1 µM) (Fig. 2). We thus conserved

the amide functionality at the junction point for elaboration of subsequent libraries **3-EO** and **4-EO**.

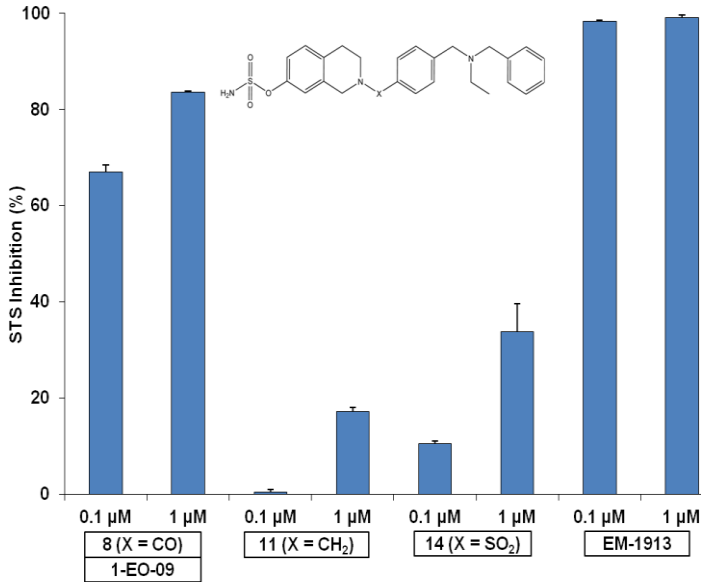
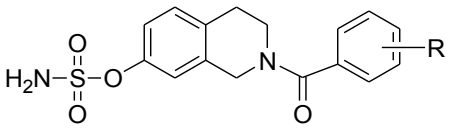


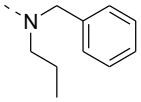
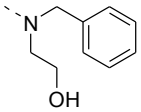
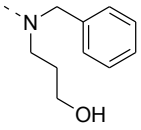
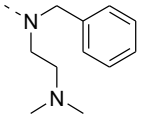
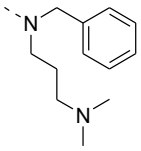
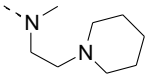
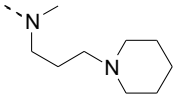
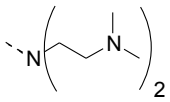
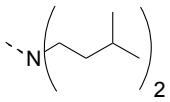
Fig. 2 The effect of amide (X = CO), amine (X = CH_2) and sulfonamide (X = SO_2) on STS inhibition. EM-1913 is a potent inhibitor of STS used as reference compound.[36]

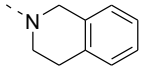
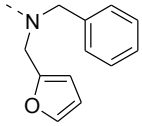
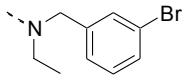
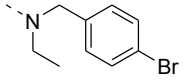
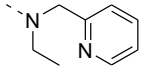
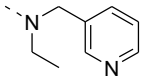
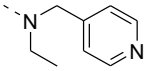
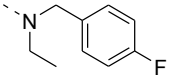
Based on hit compounds previously highlighted from libraries 1 and 2, we selected new amines to yield libraries **3-EO** and **4-EO** (Table 2). It can be seen that the inhibitory potency was increased for both orientations, but the *para* position (library 3) still gave the best results. As expected, the most hydrophobic substituents produced the best inhibitory results. For instance, compound **3-EO-11** bearing a *N*-furyl-*N*-benzyl side chain gave 91% of STS inhibition at 0.1 µM. Hydrophobic compounds **3-EO-12** and **3-EO-13** bearing a *N*-ethyl-*N*-bromobenzyl side chain gave good results as well with 80% and 78% of inhibition at 0.1 µM. Also, it is important to note that good inhibition has also been achieved with more polar side chains. Interestingly, compounds **3-EO-14**, **3-EO-15** and **3-EO-16** showed inhibition of 36-47% at 0.1 µM and 85-88% at 1 µM. These three sulfamate derivatives possess a *N*-pyridine-*N*-ethyl side chain, with a difference in the position of the nitrogen in the pyridine ring. Such side chains with a heteroatom capable of forming hydrogen bonds are promising to obtain a SERM effect. Some inhibitors of library 4 gave good inhibition only at higher concentrations. In fact, only three compounds (**4-EO-09**, **4-EO-11** and **4-**

EO-13) gave more than 75% of inhibition at 1 µM. With these results in hand, it is clear that with the benzamide spacer, the best orientation is in *para* position.

Table 2 Structure of the sulfamate compounds of libraries **3-EO** and **4-EO** and their inhibition (%) of the transformation of [^{3}H]-estrone sulfate into [^{3}H]-estrone by STS (HEK-293 transfected cells)



R	ID	*Para compound* STS inhibition (%) [a]		ID	*Meta compound* STS inhibition (%) [a]	
		0.1 µM	**1 µM**		**0.1 µM**	**1 µM**
	**3-EO-01**	64.8 ± 3.8	94.3 ± 0.3	**4-EO-01**	23.1 ± 7.5	67.4 ± 10.9
	**3-EO-02**	27.9 ± 2.6	78.0 ± 1.1	**4-EO-02**	26.7 ± 7.2	32.4 ± 6.3
	**3-EO-03**	5.7 ± 4.4	64.3 ± 1.6	**4-EO-03**	25.8 ± 3.9	37.3 ± 7.0
	**3-EO-04**	13.9 ± 4.8	57.8 ± 3.4	**4-EO-04**	0.0 ± 0.0	28.6 ± 2.2
	**3-EO-05**	16.2 ± 4.6	61.6 ± 4.9	**4-EO-05**	23.8 ± 4.1	26.0 ± 8.5
	**3-EO-06**	0.3 ± 1.7	26.8 ± 13.5	**4-EO-06**	24.5 ± 7.4	30.8 ± 6.4
	**3-EO-07**	17.4 ± 12.3	40.7 ± 8.6	**4-EO-07**	24.1 ± 0.4	30.6 ± 6.9
	**3-EO-08**	3.8 ± 9.6	15.9 ± 21.5	**4-EO-08**	0.0 ± 0.0	17.1 ± 7.6
	**3-EO-09**	47.0 ± 13.0	90.8 ± 1.7	**4-EO-09**	32.3 ± 2.9	75.9 ± 4.2

	**3-EO-10**	56.0 ± 7.2	83.0 ± 11.9	**4-EO-10**	24.1 ± 5.6	50.2 ± 1.9
	**3-EO-11**	90.8 ± 2.6	98.2 ± 0.4	**4-EO-11**	27.5 ± 0.9	90.5 ± 1.4
	**3-EO-12**	80.2 ± 8.0	97.2 ± 0.6	**4-EO-12**	20.9 ± 7.1	24.9 ± 0.6
	**3-EO-13**	78.2 ± 1.7	95.9 ± 0.6	**4-EO-13**	24.2 ± 4.9	84.5 ± 3.7
	**3-EO-14**	42.5 ± 3.5	87.7 ± 0.7	**4-EO-14**	6.5 ± 6.9	19.9 ± 12.6
	**3-EO-15**	35.7 ± 4.3	85.2 ± 1.7	**4-EO-15**	14.3 ± 4.7	33.5 ± 5.8
	**3-EO-16**	47.0 ± 1.8	86.6 ± 1.8	**4-EO-16**	33.3 ± 0.0	52.8 ± 1.3
	**3-EO-17**	69.1 ± 1.9	91.2 ± 2.7	**4-EO-17**	10.9 ± 7.4	66.5 ± 4.5

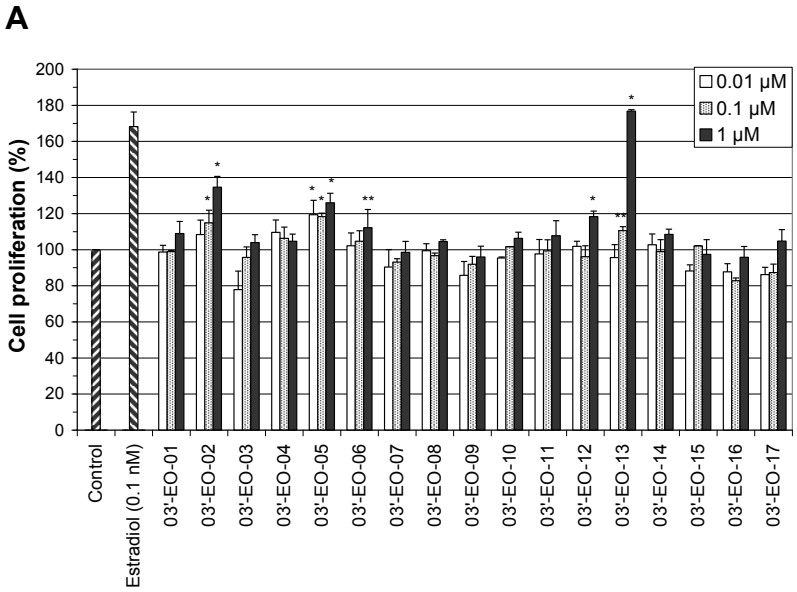
[a] Compounds were tested at two concentrations of 0.1 µM and 1 µM. Results are expressed as mean ± SEM of one experiment performed in triplicate.

Proliferative (estrogenic) activity (MCF-7 (ER^+) cells)

The next step was to determine the presence or absence of estrogenic activity. To do so, cell proliferative assays were carried out on MCF-7 cells. This breast cancer cell line is known to express the estrogen receptor (ER).[37] This means that molecules possessing estrogenic activity, such as the potent estrogen E2 used as a reference compound, will activate the ER, thus inducing cell growth over the control fixed at 100%. Phenols of the corresponding sulfamate library **3-EO** and **4-EO** are referred to library **3'-EO** and library **4'-EO**, respectively. Proliferative activities of all members of these two libraries of phenol derivatives have been tested to investigate their estrogenic activity at three concentrations of 0.01 µM, 0.1 µM and 1 µM (Fig. 3).

At first sight, library **3'-EO**, with the side chain in *para* position, has only a few phenol derivatives that display an estrogenic activity (Fig. 3A). Thus, only phenol derivatives **3'-EO-02**, **3'-EO-05**, **3'-EO-12** and **3'-EO-13** induce the proliferation of ER^+ cell line MCF-7

at a concentration of 1 µM (135, 126, 118 and 177%, respectively). As a point of comparison, the potent estrogen E2 induced a proliferation of 176% at the lower concentration of 0.1 nM. Although these proliferative effects are low in comparison to the effect of a potent estrogen, we need compounds without estrogenic potency. The other phenol derivatives of library **3'-EO** are more interesting. For example, the phenol derivative **3'-EO-11**, corresponding to the most potent sulfamate derivative **3-EO-11**, did not stimulate the ER-cell growth at the concentrations tested. In contrast to library **3'-EO**, library **4'-EO**, with the side chain in *meta* position, seemed to contain more estrogenic compounds (Fig. 3B). Most of the compounds stimulated the growth of the estrogen-dependent MCF-7 cells at 1 µM. Thus, not only were the sulfamate derivatives from library **4-EO** less potent than those from library **3-EO**, but most of the phenol derivatives from library **4'-EO** were estrogenic. With the results of estrogenic assays for phenol derivatives and considering the results of STS inhibition for the sulfamate derivatives, we can confirm that phenol derivatives **3-EO-9** to **11** and **3-EO-14** to **17** are interesting for further investigation concerning the SERM effect.



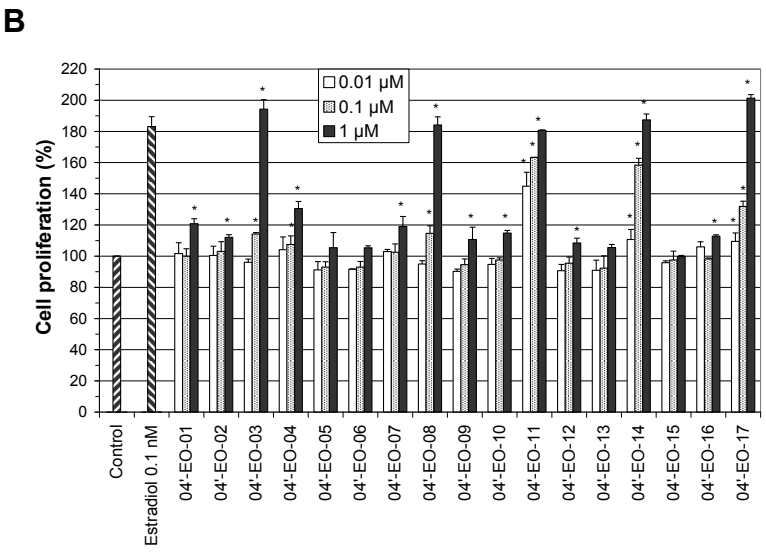


Fig. 3 Effect of estradiol and the phenol derivatives of library **3'-EO** (**A**) and **4'-EO** (**B**) on the growth of estrogen-starved MCF-7 (ER^+) cells after seven days of treatment. Control is fixed at 100% of cell proliferation. The potent natural estrogen estradiol was used as a reference compound. Results are expressed as mean ± SEM of one experiment in triplicate. ** = $P<0.05$ vs. control; * = $P<0.01$ vs. control.

Proliferative and alkaline phosphatase activities (Saos-2 (ER^+) cells)

Estrogens are known to have a beneficial effect on bone. Indeed, osteoblast cells express the ER and modulate the formation of bone tissue.[38] Osteoblast cells are thus a good *in vitro* model for the evaluation of SERM compounds because their proliferation is partly mediated by estrogenic molecules such as E2. The phenol derivatives **3'-EO-09** to **3'-EO-11** and **3'-EO-14** to **3'-EO-17** were tested on the osteoblast-like cell line Saos-2 to further evaluate their SERM capacity (Fig. 4A). The reference compounds raloxifen (SERM) and E2 (estrogen), at a concentration of 1 µM, stimulated the proliferation of Saos-2 by 123% and 144%, respectively. All of the phenolic compounds tested were also able to significantly stimulate Saos-2 cell proliferation, but the proliferative effects are more important for **3'-EO-14** to **3'-EO-17** than for **3'-EO-09** to **3'-EO-11**. Compound **3'-EO-14** produced the best stimulation of Saos-2 cell proliferation (140%). This compound

surpasses the stimulation induced by raloxifen and reaches a similar level of cell proliferation as E2.

We also investigated the effect of selected phenol derivatives on alkaline phosphatase (ALP) activity in Saos-2 cells (Fig. 4B). ALP activity is a more sensitive test than cell proliferation and allows us to determine if a compound can activate osteoblast maturation and mineralization. As reference compounds, tested at a concentration of 1 nM, E2 and the SERM tamoxifen increased ALP activity to 148% and 143%, respectively, when compared to control (no treatment) fixed at 100%. At the same concentration, the phenol derivatives increased the ALP activity 133 to 152%, and the best results were obtained with **3'-EO-14** (147%) and **3'-EO-17** (152%).

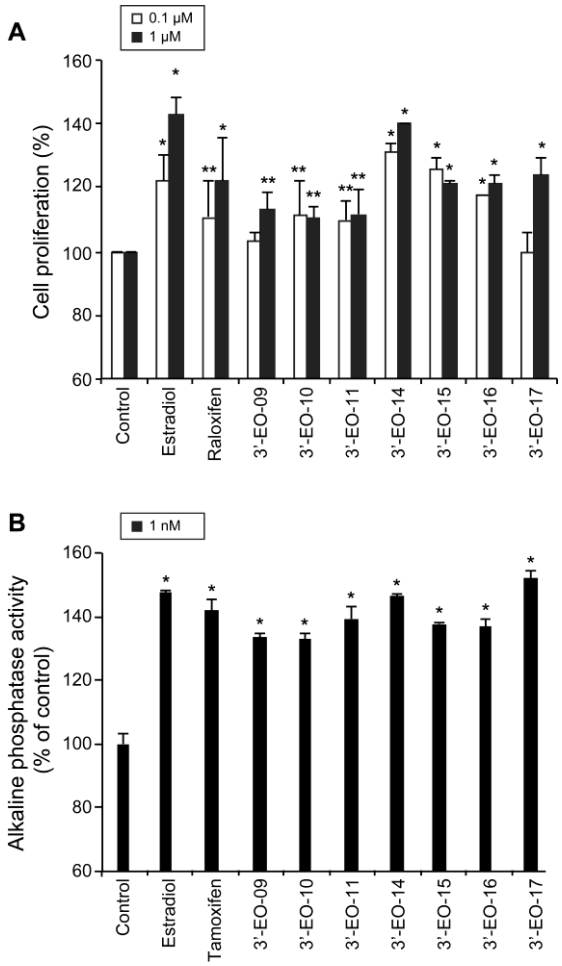


Fig. 4 Effect of estradiol, raloxifen and phenol derivatives **3'-EO-9** to **11** and **3'-EO-14** to **17** on the growth (**A**) and alkaline phosphatase activity (**B**) of estrogen-starved Saos-2 cells (ER^+) after seven days of treatment. Control is fixed at 100% of cell proliferation. The potent natural estrogen estradiol and SERM raloxifen were used as a reference compounds. Results are expressed as mean ± SEM of one experiment in triplicate. ** = $P<0.05$ vs. control; * = $P<0.01$ vs. control.

Conclusion

Four libraries of diversified 1,2,3,4-tetrahydroisoquinoline sulfamate derivatives (54 compounds) and their corresponding phenol derivatives (54 compounds) were synthesized using parallel solid-phase synthesis. The sulfamate derivatives were tested for their ability to inhibit STS in homogenized HEK-293 transfected cells, while the corresponding phenol derivatives were tested to investigate their estrogenicity on an estrogen-dependent cell lines, MCF-7 and Saos-2. Sulfamate compounds bearing the amino side chain in *para* position gave better inhibition of STS and most of the corresponding phenolic compounds showed no unwanted residual estrogenicity. The most potent STS inhibitor is the sulfamate derivative **3-EO-11** (98% of inhibition at 1 μM and 91% of inhibition at 0.1 μM) and the corresponding phenol derivative **3'-EO-11** showed no estrogenic activity. Although less potent as STS inhibitors, other compounds such as the pyridine derivatives **3-EO-14**, **3-EO-15** and **3-EO-16** showed very good inhibitory results. Interestingly, their corresponding phenol derivatives **3'-EO-14**, **3'-EO-15** and **3'-EO-16** showed no proliferative (estrogenic) activity on estrogen-sensitive MCF-7 cells, induced a proliferation of bone Saos-2 cells and increased the alkaline phosphatase activity in Saos-2 cells, which are three characteristics of a SERM.

Our screening study converged toward a series of sulfamate derivatives with potent STS inhibitory activity, and their corresponding phenolic derivatives have the characteristics of a SERM, but additional experiments will be necessary to fully confirm the dual-acting potency of these non-steroidal compounds. Specifically, *in vivo* experiments evaluating the femur weight or bone density of ovariectomized rats will be necessary to determine the

efficiency of a treatment using a dual-acting STS inhibitor (sulfamate derivatives) compared to a treatment using the corresponding phenol derivative to generate a SERM effect. In fact, divergent hypotheses exist regarding the potential of using a dual-acting STS inhibitor. In one hypothesis, the quantity of phenol derivative released by STS would be too weak to produce a SERM effect at a clinically efficacious level, especially because the enzyme would be irreversibly inhibited by the sulfamate inhibitor. In another hypothesis, however, the sulfamate group could be chemically hydrolyzed, thus providing the phenol in sufficient quantity.[19] It is also possible that both enzymatic and chemical hydrolysis of the sulfamate group are involved in the synthesis of the active phenolic compound.

Nevertheless, using a dual-acting STS inhibitor would be an attractive strategy to reduce the level of estrogen generated from sulphated steroids, especially for the treatment of estrogen-dependent diseases. In addition, the quantity of phenolic SERM generated by enzymatic or/and chemical hydrolysis of the inhibitor could be beneficial to at least partly reduce osteoporosis or other side-effects associated with an estrogen-deprivation treatment, but this last point remains to be confirmed *in vivo*. In summary, the chemical solid-phase methodology we presented herein could be advantageously used to optimize the dual-acting potency of this new family of non-steroidal compounds (STS inhibitors with a SERM effect) by generating additional and more diversified libraries of analog compounds for SAR studies. However, an important initial step will be to confirm the capacity of such non-steroidal STS inhibitors to release enough phenol derivatives to produce a SERM effect.

Experimental

Chemistry

***N*-Fmoc protection of 1,2,3,4-tetrahydroisoquinolin-7-ol (synthesis of 2):** To a stirred solution of 1,2,3,4-tetrahydroisoquinolin-7-ol hydrobromide (5.0 g) in 500 mL of THF/H_2O (1:1) and 65 mL of a solution of $NaHCO_3$ (1 N) was added Fmoc-*O*-succinimide (7.69 g). The solution was vigorously stirred for 2 h at room temperature under argon atmosphere.

Water was then added and the mixture was extracted with EtOAc. The organic phase was washed with water and with brine, and dried over Na_2SO_4 and evaporated to dryness. Purification by flash chromatography with hexanes/EtOAc (1:1) yielded 7.36 g (91%) of phenol derivative **2**.

(9*H*-Fluoren-9-yl)methyl-7-hydroxy-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (2). White solid; IR (film) υ: 3321 (OH), 1674 (C=O, carbamate); ^{1}H NMR (acetone-d_6) δ: 2.68 (m, 2H), 3.61 (t, J = 6.0 Hz, 2H), 4.32 (t, J = 6.6 Hz, 1H), 4.45 (d, J = 6.6 Hz, 2H), 4.49 (s, 2H), 6.62 (s, 1H), 6.68 (d, J = 6.4 Hz, 1H), 6.98 (d, J = 8.2 Hz, 1H), 7.33 (br s, 2H), 7.40 (m, 2H), 7.68 (br s, 2H), 7.86 (br s, 2H), 8.23 (br s, 1H); ^{13}C NMR (acetone-d_6) δ: 42.6, 46.4, 48.1, 67.7, 113.2, 114.7, 120.7, 125.9, 126.0, 127.9, 128.4, 130.4, 142.1, 145.1, 155.7, 156.5. APCI-MS (+): 372 m/z $[M + H]^+$

Sulfamoylation of *N*-Fmoc protected 1,2,3,4-tetrahydroisoquinolin-7-ol (synthesis of 3): The stirred solution of *N*-Fmoc protected 1,2,3,4-tetrahydroisoquinolin-7-ol (5.0 g) (**2**) in 100 mL of dry dimethylacetamide (DMA) under argon was cooled to 0°C, followed by addition of sulfamoyl chloride (4.6 g).[39] After 1 h of stirring at room temperature, the reaction was cooled back to 0°C followed by another addition of sulfamoyl chloride (4.6 g). The mixture was then allowed to react for 3 h at room temperature. The reaction was quenched with water, and the crude product was extracted with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and evaporated to dryness. Purification by trituration with diethyl ether yielded 5.16 g (85%) of sulfamate **3**.

(9*H*-Fluoren-9-yl)methyl-7-(sulfamoyloxy)-3,4-dihydroisoquinoline-2(1*H*)-carboxylate (3). White solid; IR (film) υ: 3213 (NH_2), 1682 (C=O, carbamate), 1381 and 1180 (S=O, sulfamate); ^{1}H NMR ($CDCl_3$) δ: 2.78 (br s, 2H), 3.65 (br s, 2H), 4.26 (t, J = 6.4 Hz, 1H), 4.49 (m, 3H), 4.61 (s, 1H), 5.47 (br s, 2H), 7.07 (d, J = 19.7 Hz, 1H), 7.14 (s, 2H), 7.31 (d, J = 6.7 Hz, 2H), 7.39 (d, J = 6.1 Hz, 2H), 7.57 (br s, 2H), 7.75 (d, J = 12.9 Hz); ^{13}C NMR (acetone-d_6) δ: 42.7, 46.2, 48.2, 67.9, 120.7, 120.8, 121.3, 125.9, 128.0, 128.5, 130.8, 142.2, 145.1, 155.7; APCI-MS (+): 451 m/z $[M + H]^+$.

Coupling of NFmoc protected 7-sulfamate-1,2,3,4-tetrahydro-isoquinoline with trityl resin (synthesis of 4): Trityl chloride resin (1.75 mmol/g theoretical loading) (10 g) was swollen under argon in 50 mL of dry CH_2Cl_2. After 5 min of stirring, sulfamate **3** (9.49 g) was added as a solution in 50 mL of dry DMA/CH_2Cl_2 (1:1) followed by the addition of DIPEA (18.3 mL), and the mixture was shaken for 24 h at room temperature. The resin was filtered and washed successively with CH_2Cl_2 (3x), MeOH (3x), CH_2Cl_2 (3x) and MeOH (3x), then dried overnight under vacuum to afford 14.05 g of resin **4**. The coupling (loading) yield calculated by the means of the mass increase was 56%. The filtrate was collected and evaporated to dryness to isolate 3.74 g of unreacted sulfamate **3**. The loading yield calculated by the recovered amount of compound **3** was 60%.

Procedure for the NFmoc deprotection of resins 4 (synthesis of 5): The *N*-Fmoc protected resins **4** (13.85 g) were reacted for 2 h with 260 mL of a solution of piperidine in DMF (20%) for the cleavage of the Fmoc protecting group. The resins were filtered and washed with CH_2Cl_2 (3x), with MeOH (3x), and again with CH_2Cl_2 (3x), then dried overnight under vacuum to give 11.00 g of resin **5**.

Acylation of the resins 5 with carboxybenzaldehyde (synthesis of 6): Resins **5** (2.4 g) were swollen under argon in 25 mL of dry DMF. After 5 min of shaking, the resins were filtered and 3-carboxybenzaldehyde (or 4-carboxybenzaldehyde) (2.16 g), N-hydroxybenzotriazol (HOBt) (1.95 g) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (7.95 g) were added as a solution in 25 mL of dry DMF followed by addition of a solution of DIPEA (5.02 mL) in 24 mL of dry DMF. The resins were shaken for 3 h at room temperature, then filtered and washed with DMF (3x) and with CH_2Cl_2 (4x). The resins were dried overnight under vacuum to give 2.61 g of resin **6**. The coupling (loading) yield calculated by the means of the mass increase was 81%.

Reductive amination of the resins 6 with secondary amines (synthesis of 7): Resins **6** were weighted, and then divided in 10 or 17 wells to perform reductive amination (70 mg of resin **6**/well for libraries 1 and 2) (125 mg of resin **6**/well for libraries 3 and 4). Each well was swollen under argon in 2 mL of *N*-methyl-2-pyrrolidone (NMP). After 2 min of

stirring, the resins were filtered and secondary amine (0.5 mmol) was added as a solution in 1 mL of NMP followed by the addition of 1 mL of a solution of acetic acid in NMP (30%). The resins were then stirred for 10 min followed by the addition of $NaBH(OAc)_3$ (0.7 mmol) as a solution in 1 mL of NMP. The resins were stirred for 18 h at room temperature, then filtered and washed successively with DMF, H_2O, EtOH, DMF, H_2O, EtOH and CH_2Cl_2. The resins were dried overnight under vacuum to afford resin **7**.

Acid cleavage of the resins 7 (synthesis of 8; Libraries 1-4): Resins **7** were allowed to react for 1 h with 3 mL of a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in CH_2Cl_2 (30%), then filtered and washed with CH_2Cl_2. The organic layer was collected in pre-weighed tubes and the solvent was evaporated under reduced pressure. Residual solvents were removed by co-evaporation with toluene and CH_2Cl_2, and then dried under vacuum pump to generate sulfamate compounds **8**. Range and average quantities obtained for each library: **1-EO** = 6.0 - 25.0 mg, average = 13.8 mg; **2-EO** = 11.0 - 26.0 mg, average = 17.7 mg; **3-EO** = 18.0 - 33.6 mg, average = 27.1 mg; **4-EO** = 18.6 - 40.9, average = 32.4 mg. All library members were obtained as one major compound (by TLC analysis) having the right mass (by LRMS analyses). Three compounds from both libraries **1-EO** and **2-EO** and four compounds from both libraries **3-EO** and **4-EO** were randomly chosen for IR, ^1H NMR and LRMS characterizations.

Nucleophilic cleavage of the resins 7 (synthesis of 9): Resins **7** were allowed to react for 24 h at 60°C with 3 mL of a solution of DEA in THF (30%), then filtered and washed with DMF. The organic layer was collected in pre-weighed tubes and the solvent was evaporated under reduced pressure. Residual solvents were removed by co-evaporation with toluene and CH_2Cl_2, and then dried under vacuum pump to generate phenol derivatives **9**. Range and average quantities obtained for each library: **3'-EO** = 15.6 - 32.6 mg, average = 28.1 mg; **4'-EO** = 28.2 - 35.9, average = 31.9 mg. All library members were obtained as one major compound (by TLC analyses) that possesses the right mass (by LRMS analyses). Four compounds from each library (**3'-EO** and **4'-EO**) were randomly chosen for IR, ^1H NMR and LRMS characterizations.

Reductive amination of resin 5 with 4-{[benzyl(ethyl)amino]methyl} benzaldehyde and cleavage from the resin (synthesis of 11): The resin **5** (70 mg, 0.06 mmol) was first swollen in 2 mL of NMP and stirred for 2 min. The resin was filtered and 4-{[benzyl(ethyl)amino]methyl} benzaldehyde (43 mg, 0.17 mmol) was added as a solution in 0.5 mL of NMP followed by the addition of 0.5 mL of a solution of acetic acid in NMP (30%). The resin was then stirred for 45 min under argon atmosphere followed by the addition of $NaBH(OAc)_3$ (0.7 M) in 0.5 mL of NMP. The resins were stirred for 18 h at room temperature under argon atmosphere, then filtered and washed successively with DMF, H_2O, EtOH, DMF, H_2O, EtOH and CH_2Cl_2. The resin was dried overnight under vacuum to afford resin **10**. This resin was allowed to react for 1 h with 3 mL of a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in CH_2Cl_2 (30%), then filtered and washed with CH_2Cl_2. The organic layer was evaporated under reduced pressure. Residual solvents were removed by co-evaporation with toluene and CH_2Cl_2, and then dried under vacuum. The crude compound was purified by flash chromatography using DCM/MeOH (9:1) to give compound **11** as yellow amorpheous solid; IR (KBr) υ: 3394 (NH_2), 1373 and 1180 (S=O, sulfamate); ^{1}H NMR (400 MHz, methanol-d_4) δ: 1.11 (t, J = 7.1 Hz, 3H), 2.53 (q, J = 7.1 Hz, 2H), 2.79 (t, J = 6.1 Hz, 2H), 2.91 (t, J = 5.9 Hz, 2H), 3.60 (s, 4H), 3.66 (s, 2H), 3.71 (s, 2H), 6.99 (d, J = 2.2 Hz, 1H), 7.09 (dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, 1H), 7.17 (d, J = 8.4 Hz, 1H), 7.24 (t, J = 7.2 Hz, 1H), 7.31 (t, J = 7.2 Hz, 2H), 7.37 (s, 5H); APCI-MS (+): 466.4 m/z $[M + H]^+$.

Sulfonylation of resin 5 (synthesis of 12): Resin **5** (100 mg, 0.08 mmol) was first swollen in 2 mL of DCM and stirred for 2 min. The resin was filtered and 2 mL of a solution of 4-sulfonylchloride benzaldehyde in DCM (0.3 M) was added. The suspension was stirred for 12 h under argon atmosphere at room temperature. The resin was then filtered and washed thoroughly with DCM and dried under vacuum.

Reductive amination of resin 12 with 4-ethylaminobenzyl and cleavage from the resin (synthesis of 14): The resin **12** (70 mg, 0.06 mmol) was first swollen in 2 mL of NMP and stirred for 2 min. The resin was filtered and N-benzylethanamine (23 mg, 0.17 mmol) was added as a solution in 0.5 mL of NMP followed by the addition of 0.5 mL of solution of

acetic acid in NMP (30%). The resin was then stirred for 45 min under argon atmosphere followed by the addition of a solution of $NaBH(OAc)_3$ (0.7 M) in 0.5 mL of NMP. The resin was stirred for 18 h at room temperature under argon atmosphere, then filtered and washed successively with DMF, H_2O, EtOH, DMF, H_2O, EtOH and CH_2Cl_2. The resin was dried overnight under vacuum to afford resin **13**. This resin was allowed to react for 1 h with 3 mL of a solution of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in CH_2Cl_2 (30%), then filtered and washed with CH_2Cl_2. The organic layer was evaporated under reduced pressure. Residual solvents were removed by co-evaporation with toluene and CH_2Cl_2, and then dried under vacuum. The crude compound was purified by flash chromatography using EtOAc/Hexanes (7:3) to give compound **14** as light yellow solid; IR (KBr) υ: 3356 and 3271 (NH_2), 1381 and 1180 (S=O, sulfamate and sulfonamide); ^{1}H NMR (400 MHz, acetone-d_6) δ: 1.07 (t, *J* = 7.1 Hz, 3H), 2.50 (q, J = 7.1 Hz, 2H), 2.92 (t, J = 5.8 Hz, 2H), 3.35 (t, J = 6.0 Hz, 2H), 3.59 (s, 2H), 3.67 (s, 2H), 4.24 (s, 2H), 7.07-7.22 (m, 4H), 7.30 (d, J = 7.7 Hz, 2H), 7.38 (d, J = 7.5 Hz, 2H), 7.65 (d, J = 8.2 Hz, 2H), 7.81 (d, J = 8.4 Hz, 2H); APCI-MS (+): 516.3 m/z $[M + H]^+$.

Biological assays

Inhibition of STS activity: This enzymatic assay was carried out according to a procedure previously described for the transformation of substrate [^{3}H]-E1S (100 μM) into [^{3}H]-E1 by homogenated HEK-293 cells over-expressing the enzyme activity.[30,31]

Proliferative (estrogenic) activity on MCF-7 (ER^+) cells: This cell assay was carried out according to a procedure previously described to determine the estrogenic activity of enzyme inhibitors on the growth of estrogen-sensitive MCF-7 cells.[40] All chemicals tested were first dissolved in DMSO and subsequent dilutions were done in the proper cell culture media. The final concentration of DMSO in the culture medium was 0.1% or less. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium (MTS) was used as an indirect colorimetric measurement of cell proliferation according to the manufacturer's instructions (Promega, Madison, WI). At the end of the treatments, 20 μL of MTS solution was added to each well (100 μL) and the MCF-7 cells were incubated

4 h at 37°C. The absorbance at 490 nm was then measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of cell proliferation.

Proliferative activity on Saos-2 (ER^+) cells: The osteoblast-like Saos-2 cells were maintained in culture flasks (175 cm^2 growth area, BD Falcon) at 37°C in a 5% CO_2 humidified atmosphere and grown in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Penicillin/streptomycin mix, L-Glutamine, normal and charcoal-stripped FBS were purchased from Wisent, Inc. (St-Bruno, QC, Canada), phenol-red free McCoy's 5A was purchased from PromoCell (Heidelberg, Germany). Chemicals tested were first dissolved in DMSO and subsequent dilutions were done in the cell culture medium. The final concentration of DMSO in the culture medium was 0.1% or less. For the proliferation assay, the 5% FBS in the culture medium was replaced with 10% charcoal-stripped FBS. The cells were seeded in 96-well plates at a density of 10 000 cells/well and allowed to attach 24 h. After 24 h, the phenolic compounds diluted in culture medium were added to the wells and replaced every 2 days for 7 days of treatment. As reported above, MTS was used as an indirect colorimetric measurement of cell proliferation according to the manufacturer's instructions, except the plate were incubated 2 h instead of 4 h. Results were reported as cell proliferation in % (mean ± SD of one experiment) where the control of cell proliferation was fixed at 0%.

Alkaline phosphatase assay (Saos-2 cells): Saos-2 cells were used similarly as reported in the cell proliferation assay. The cells were seeded at a density of 2 000 cells/well and treated for three days with each compound to be tested. The alkaline phosphatase (ALP) activity was measured using the Sensolyte® pNPP Alkaline Phosphatase Assay Kit *Colorimetric* (AnaSpec, Freemont, Ca) following the manufacturer's protocol. Briefly, after three days of treatment, the cells were washed twice with washing buffer (provided with the kit) and lysed with 0.2% Triton X-100. The cell lysates were centrifuged and the supernatants were used to determine ALP activity. The supernatants were deposed in a 96-well plate and incubated for 30 minutes with a *p*-nitrophenyl phosphate solution (provided

with the kit). The absorbance at 405 nm was measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of alkaline phosphatase activity.

Acknowledgments

We wish to thank the Fondation du cancer du sein du Québec (FCSQ) for operating grants and Mrs. Micheline Harvey for careful reading of the manuscript.

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Chapitre 2

Résumé

Les modulateurs sélectifs du récepteur des estrogènes (SERMs) sont présentement utilisés comme thérapie hormonale pour le cancer du sein. Une nouvelle approche de traitement hormonal consiste à inhiber la stéroïde sulfatase (STS), l'enzyme convertissant les précurseurs stéroïdiens sulfatés en hormones actives. Ici nous rapportons le potentiel d'une molécule à double-action, c'est-à-dire un inhibiteur de la STS ayant une activité SERM. L'inhibiteur de la STS 6-EO-14, lequel contient un groupement sulfamate, relarguerait l'analogue phénolique 8-EO-14 après l'inhibition irréversible de la STS. La molécule sulfamoylé non-stéroïdienne a été testé sur des cellules HEK-293 surexprimant la STS et a démontré une bonne inhibition de l'enzyme (IC_{50} = 0.3 μM). Pour valider le potentiel SERM, le composé phénolique fut testé sur les cellules ostéoblastiques Saos-2 et l'activité d'un marqueur spécifique de différentiation, l'alkaline phosphatase (ALP), fut évalué. Le composé 8-EO-14 a stimulé la prolifération des cellules Saos-2 (21% à 1 μM) et a induit l'activité ALP (31% à 0.1 nM) via le récepteur des estrogènes alpha (REα) d'une façon similaire à celle du SERM raloxifène. De plus, l'affinité des deux composés pour REα fut testée et les résultats montrèrent qu'ils possèdent une faible affinité avec des valeurs d'IC_{50} de 3.1 μM pour 6-EO-14 et de 24.6 μM pour 8-EO-14. Les deux composés furent également testés sur deux lignées cellulaires cancéreuses du sein hormonodépendantes (MCF-7 et T-47D) pour évaluer leur potentiel estrogénique et antiestrogénique. Malheureusement, les composés ont montré une activité estrogénique sur les cellules T-47D et n'ont pas été en mesure de bloquer la stimulation induite par l'estradiol dans les deux lignées cellulaires.

Manuscrit à soumettre

In vitro evaluation of a steroid sulfatase inhibitor with a potential selective estrogen receptor modulator (SERM) capacity

Charles Ouellet, Étienne Ouellet, Donald Poirier*

Laboratory of Medicinal Chemistry
CHU de Québec (CHUL) – Research Center and Laval University
2705 Laurier Boulevard, Québec (Québec), G1V 4G2, Canada

*Corresponding author:
Dr. Donald Poirier
Laboratory of Medicinal Chemistry
CHU de Québec (CHUL) - Research Center
2705 Laurier Boulevard, Québec (Québec), G1V 4G2, Canada
Phone : (418) 654-2296; Fax : (418) 654-2761
E-mail : donald.poirier@crchul.ulaval.ca

Abstract:

Selective estrogen receptor modulators (SERMs) are currently in use as hormonal therapy for breast cancer. A new approach of hormonal treatment is the inhibition of steroid sulfatase (STS) which converts inactive sulfated steroid precursors into active hormones. Here we investigated the potential of a dual action STS inhibitor with a selective estrogen receptor modulator capacity. The STS inhibitor 6-EO-14, which contains a sulfamate moiety, releases the phenol analogue 8-EO-14 after the irreversible inhibition of STS. The sulfamoylated non-steroidal molecule was tested on STS-transfected HEK-293 cells and showed a good inhibition of the enzyme (IC_{50} = 0.3 μM). To validate its SERM potential, we tested the phenolic compound 8-EO-14 on osteoblast-like Saos-2 cells to investigate the effect on cell proliferation and a specific differentiation marker activity, alkaline phosphatase (ALP). The compound stimulated Saos-2 proliferation (21% at 1 μM) and induced ALP activity (31% at 0.1 nM) via the estrogen receptor alpha (ERα) similarly as the SERM raloxifene. Furthermore, the affinity of both molecules for the human ERα was tested and they showed weak affinities with IC_{50} values of 3.1 μM and 24.6 μM for 6-EO-14 and 8-EO-14, respectively. The estrogenicity and antiestrogenicity of both compounds were also tested on two estrogen-dependent breast cancer cell lines, MCF-7 and T-47D. Unfortunately, they stimulated the growth of T-47D cells and were unable to block the estradiol-stimulated growth of both cell lines suggesting an estrogenic activity.

1. Introduction

Among women, breast cancer is the most diagnosed cancer and the second in term of deaths after lung cancer (Canadian Cancer Society, American Cancer Society). The majority of breast cancers are estrogen-dependent, with approximately 55% in premenopausal women and 75% in post-menopausal women (Jonat et al. 2006). Hormonal therapy has proved to be an effective way of blocking estrogens from stimulating breast cancer cells. Currently, hormonal treatments include SERMs, such as tamoxifen, and aromatase inhibitors which respectively block estrogen receptor (ER) and cytochrome P450 aromatase enzyme involved in estradiol (E2) formation. Even though they are interesting therapeutic agents, they have some negative side effects. Tamoxifen therapy induces some

adverse effects like hot flushes or vaginal bleeding and increases the risk of thromboembolic diseases (Duggan et al. 2003) and endometrial cancer (Bergman et al. 2000). Aromatase inhibitors block the conversion of 4-androstene-3,17-dione (Δ^4-dione) and testosterone (T) into estrone (E1) and E2, respectively, and thus limit the availability of estrogens in the organism. Although this decrease of estrogens level in blood is a good way of blocking the stimulation of breast cancer cells, it can also lead to osteoporosis in bone tissue because of the decrease in stimulation of osteoblasts (Riggs et al. 2002). Thus, focusing on the development of new drugs for hormonal treatments is very important toward a better survival and recovery of the disease without adverse side effects.

SERMs play an important role in hormonal therapy and extensive research is done to develop new SERMs with fewer side effects (Obiorah et al. 2011). Other SERMs like raloxifene and lasofoxifene can block breast cancer progression while being also useful as a treatment for osteoporosis (Obiorah et al. 2011). Osteoporosis is a misbalance between bone resorption by osteoclasts and bone formation by osteoblasts. Estrogens stimulate osteoblast differentiation and inhibit osteoclast proliferation and differentiation therefore resulting in an increased bone formation. To be effectively used in hormonal therapy, SERMs must act as ER-agonist in bone while acting as ER-antagonist in breast and uterus.

Inhibition of STS, the enzyme that catalyses the conversion of sulfated steroids into unconjugated (hydroxylated) hormones (Ghosh D. 2007), is a new approach for the treatment of estrogen-dependent breast cancer. In blood, estrone sulfate is the major circulating estrogen and dehydroepiandrosterone sulfate (DHEAS) is the most abundant steroid (Pasqualini et al. 1989). It was also shown that STS activity in breast cancer tumors is much higher than aromatase activity (Chetrite et al. 2000, Pasqualini et al. 1996) and that in situ formation of E1 and E2 is mainly done via the STS pathway rather than the aromatase pathway (Santner et al. 1984). Therefore blocking this key enzyme seems essential to prevent sulfated precursors from entering estrogen-dependant tumors. Consequently, a molecule being able to inhibit STS and act as a SERM would be beneficial as a treatment for breast cancer.

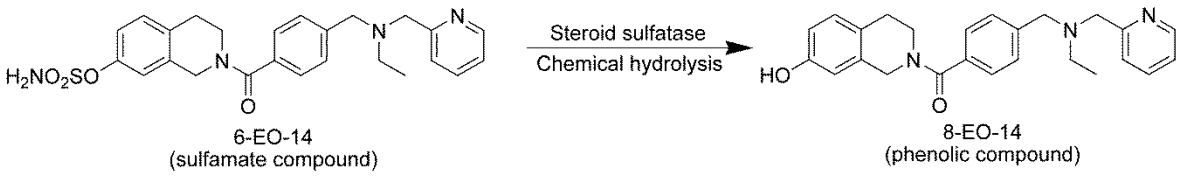


Fig. 1 Conversion of 6-EO-14 into 8-EO-14 by steroid sulfatase (STS) or by chemical hydrolysis. This enzyme cleaves 6-EO-14 and releases the phenolic analogue 8-EO-14. The sulfamate moiety is cleaved and remains irreversibly linked to the active site of the enzyme, thus inactivating the STS.

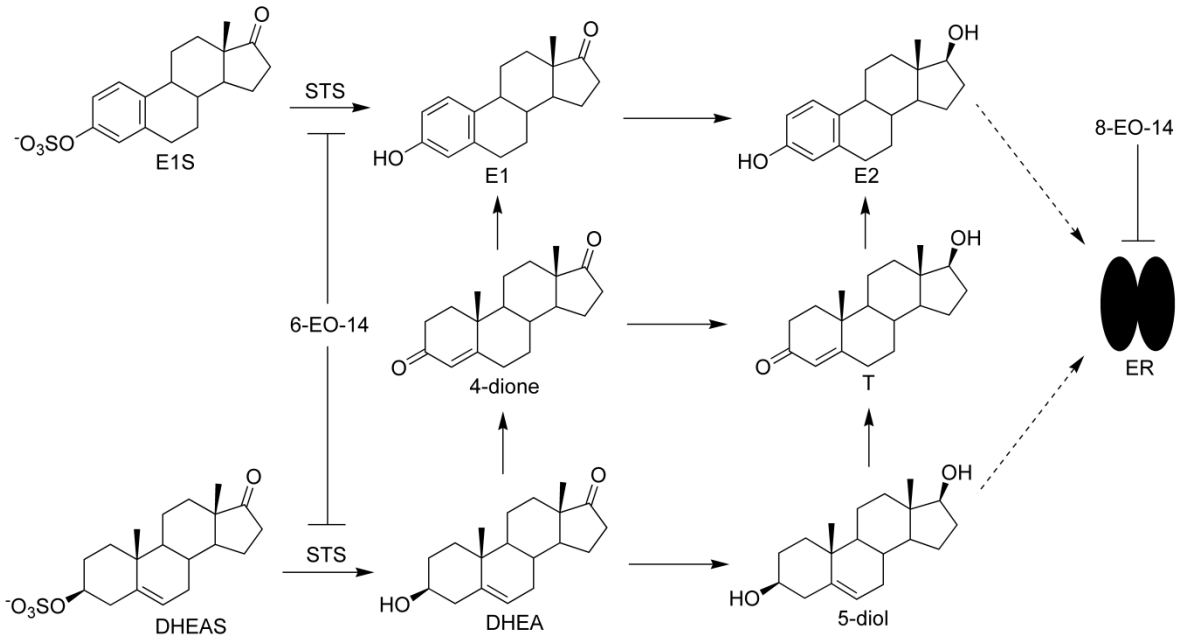


Fig. 2 Transformation of sulfated steroid E1S and DHEAS into active hormones and site of action of our dual-action inhibitor 6-EO-14. The sulfamoylated compound 6-EO-14 inhibits the conversion of the inactive sulfated precursors E1S and DHEAS into E1 and DHEA, respectively by releasing 8-EO-14. The phenolic compound 8-EO-14 is potentially blocking the estrogen receptor from activation by E2 and Δ^5-diol in breast tissue.

In our laboratory, we recently started a new research program aimed to develop STS inhibitors having a dual-action. We reported that some molecules synthesized are good inhibitors of STS (Ouellet E. et al.). The sulfamate compound 6-EO-14 as well as the corresponding phenolic compound 8-EO-14 were selected for further biological evaluation.

The compound 6-EO-14 is a sulfamate-containing non-steroidal STS inhibitor that releases the phenol compound 8-EO-14 after chemical hydrolysis or cleavage by STS (Fig. 1). 6-EO-14 inhibits STS and releases 8-EO-14 which potentially blocks the estrogen receptor selectively depending on the tissues (Fig. 2). In this study, we tested the sulfamate 6-EO-14 and the phenolic analogue 8-EO-14 on cell proliferation of T-47D, MCF-7 and Saos-2 cell lines as well as STS inhibition in transfected HEK-293 cells. To further evaluate the effect on the osteoblast-like Saos-2 cells, we determined alkaline phosphatase activity following incubation with the new synthesized compounds and different inhibitors. Finally, we determined their relative binding affinity (RBA) on the human estrogen receptor alpha (ERα).

2. Materials and methods

2.1. Chemicals and Reagents

17β-estradiol, estrone, tamoxifen, Tris, EDTA, EGTA, glycerol, KCl, dithiothreitol and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI). Radiolabeled [2,4,6,7-^{3}H] estradiol (89.2 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (Saint Louis, MO) and radiolabeled [6,7-^{3}H] estrone sulfate (54.3 Ci/mmol) was purchased from Perkin Elmer (Woodbridge, ON, Canada). Raloxifene hydrochloride was bought from Cayman Chemical (Ann Harbor, MI). The two selective estrogen receptor antagonists, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H pyrazole dihydrochloride (MPP) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), were purchased from Tocris Biosciences (Minneapolis, MN). Bio-Gel HTP hydroxyapatite was purchased from Bio-Rad (Mississauga, ON, Canada). Penicillin/streptomycin mix, L-glutamine, non-essential amino acids, sodium pyruvate, normal and charcoal-stripped Fetal Bovine Serum (FBS) and geneticin (G418 sulfate) were purchased from Wisent, Inc. (St-Bruno, QC, Canada). All cell culture medias were purchased from Life Technologies (Grand Island,

NY) except for phenol-red free McCoy's 5A medium that was purchased from PromoCell (Heidelberg, Germany). STS inhibitor EM-1913 (Ciobanu et al. 2003) and pure antiestrogen ICI-164,384 (EM-100) (Wakeling et al. 1988) were synthesized in our Laboratory of Medicinal Chemistry.

For the purpose of *in vitro* assays, all chemicals tested (inhibitors and reference compounds) were first dissolved in DMSO and subsequent dilutions were done in the proper buffer or cell culture media. The final concentration of DMSO in the culture medium was 0.1% or less.

2.2. Cell Culture

The ER^+ breast cancer cell lines (T-47D and MCF-7) and the osteoblast-like Saos-2 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The HEK-293 cell line overexpressing STS was obtained from Dr. Van Luu-The (CHUQ-CHUL Research Center). All cell lines were maintained in culture flasks (175 cm^2 growth area, BD Falcon) at 37°C in a 5% CO_2 humidified atmosphere. The MCF-7 and T-47D cell lines were grown in phenol red free Dulbecco's Modified Eagle Medium containing Ham's mixture F-12 (DMEM/F12) and in phenol red free RPMI 1640 medium, respectively. They were both supplemented with FBS (5% and 10% respectively), penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 17β-estradiol (1 nM). The Saos-2 cells were grown in McCoy's 5A medium supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The HEK-293 cells transfected with STS were maintained in Minimum Essential Medium supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and geneticin (G418 sulfate) (700 μg/ml).

2.3. Steroid sulfatase inhibition assay

An enzymatic assay previously described (Ciobanu et al. 2002) was used for the inhibition of the transformation of estrone sulfate (E1S) to estrone (E1) by STS. Briefly, the transfected HEK-293 cells were homogenized by repeated (5 times) cycles of freezing (-80°C) and thawing on ice (4°C). The homogenates were then incubated for 2 h at 37°C (shaking water bath) with or without inhibitors (0.01 μM – 1 μM) in presence of [^{3}H]-E1S (9 nM), adjusted to 1 μM with E1S, in a Tris-acetate buffer (pH 7.4) containing 5 mM EDTA and 10% glycerol. After the incubation, 1 ml of xylene was added to each tube and the solutions were then centrifuged at 3000 rpm for 20 minutes to separate the organic ([^{3}H]-E1) and aqueous ([^{3}H]-E1S) phases. Once 500 μl of each phase was added to 10 ml of Biodegradable Counting Scintillant (Amersham Biosciences), the radioactivity of the samples was recorded using a Wallac 1411 Liquid Scintillation Counter. The percentage of inhibition was determined by comparison with the control (Buffer + homogenate + [^{3}H]-E1S) which was set to 0% of inhibition. IC_{50} value for 6-EO-14 was obtained using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

2.4. Cell proliferation

CellTitter 96® Aqueous One Solution Cell Proliferation Assay was used as an indirect colorimetric measurement of cell proliferation according to the manufacturer's instructions. Briefly, 20 μl of MTS solution was added to each well (100 μl) of the plates and incubated at 37°C for 2 h (Saos-2) or 4 h (T-47D and MCF-7). The absorbance at 490 nm was then measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of cell proliferation.

2.4.1. T-47D and MCF-7 cells

T-47D and MCF-7 cells were suspended in their respective media (RPMI and DMEM/F12) supplemented with insulin (50 ng/ml), instead of 17β-estradiol, and 5% charcoal-stripped FBS to deprive the media of estrogens. The cells were plated in 96-well plates at a density

of 3 000 cells/well and allowed to attach for 48 h. After this pre-incubation, the inhibitors (6-EO-14 or 8-EO-14) and the reference compounds diluted in fresh culture media were added to the wells and replaced every 2 days for 7 days of treatment.

2.4.2. Saos-2 cells

Saos-2 cells were suspended in phenol-red free McCoy's 5A medium supplemented with 10% charcoal-stripped FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The cells were seeded in 96-well plates at a density of 10 000 cells/well and allowed to attach 24 h. After 24 h, the inhibitors (6-EO-14 or 8-EO-14) and the reference compounds diluted in fresh culture media were added to the wells and replaced every 2 days for 7 days of treatment.

2.5. Alkaline phosphatase assay

Saos-2 cells were treated similarly as reported in the cell proliferation assay. The cells were seeded at a density of 2 000 cells/well and were treated with the inhibitors (6-EO-14 or 8-EO-14) and the reference compounds for 3 days. The alkaline phosphatase (ALP) activity was measured using Sensolyte® pNPP Alkaline Phosphatase Assay Kit *Colorimetric* (AnaSpec, Fremont, CA) following the manufacturer's protocol. Briefly, after the 3 days of treatment, the cells were washed twice with washing buffer (provided with the kit) and lysed with 0.2% Triton X-100. The cell lysates were centrifuged and the supernatants were used to determine the ALP activity. The supernatants were deposed in a 96-well plate and incubated 30 minutes with a *p*-nitrophenyl phosphate solution (provided with the kit). The absorbance at 405 nm was measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of alkaline phosphatase activity.

2.6. ER alpha binding assay

A competitive binding assay using a purified full-length recombinant human ERα (Life Technologies, Grand Island, NY) was done as previously described (Arcaro et al. 1998, Davis et al. 2008). Briefly, each reaction consisted of 1.2 nM rhERα and 2.5 nM [^{3}H]-estradiol in assay buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 mg/ml BSA, pH 7.5) with different concentrations of the compounds or cold estradiol in a total reaction volume of 100 µl. Non-specific binding was determined by incubation with an excess of unlabeled estradiol (1 µM). After an overnight incubation at 4°C, 100 µl of cold 50% hydroxyapatite slurry was added to bind the receptor/ligand complex. After 15 minutes, 1 ml of wash buffer (40 mM Tris, 1 mM EDTA, 1 mM EGTA, 100 mM KCl, pH 7.4) was added and the tubes were centrifuged at 4500 rpm for 5 minutes at 4°C. The washing step was repeated twice. The radioactivity of the pellet was extracted by incubation with 1 ml of ethanol for 1 h at room temperature. The suspension was then put into 10 ml of Biodegradable Counting Scintillant and the radioactivity counted with a Wallac 1411 Liquid Scintillation Counter. IC_{50} values were obtained using GraphPad Prism 5 and RBA values were obtained by using the following equation: (IC_{50} of estradiol / IC_{50} of compound) x 100.

3. Results and discussion

3.1 Effect of 6-EO-14 on steroid sulfatase activity

The sulfamoylated compound 6-EO-14 was one of the best STS inhibitors from a previously reported screening study (Ouellet E. et al.) with 43% and 88% of inhibition at 0.1 µM and 1 µM, respectively. To further characterize its inhibition, we tested more concentrations to obtain an IC_{50} value. 6-EO-14 clearly demonstrated a dose-dependent inhibition of steroid sulfatase with an IC_{50} of 0.3 µM ± 0.05 µM (Fig. 3). The phenolic analogue 8-EO-14 was also tested but showed no significant inhibition in the range of concentrations tested. As well known, phenolic inhibitors of STS are less potent than their sulfamoylated analogues (Ciobanu et al. 2002). Therefore, steroid sulfatase inhibition is

limited to 6-EO-14 and released compound 8-EO-14 can potentially act as SERM on estrogen receptor.

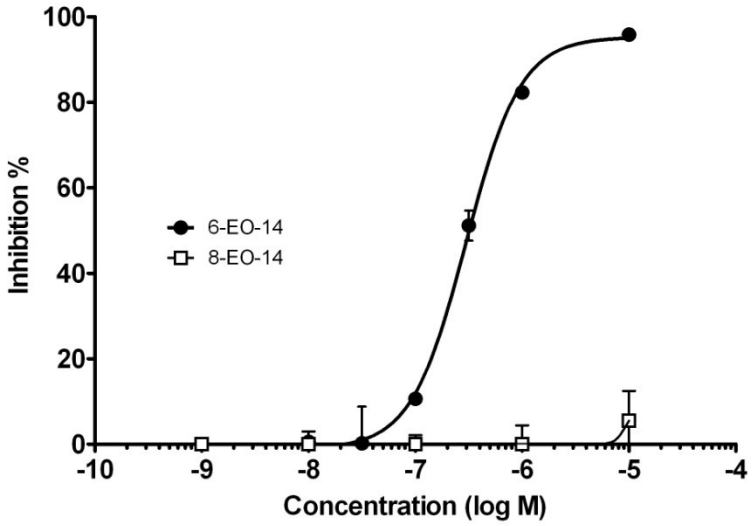


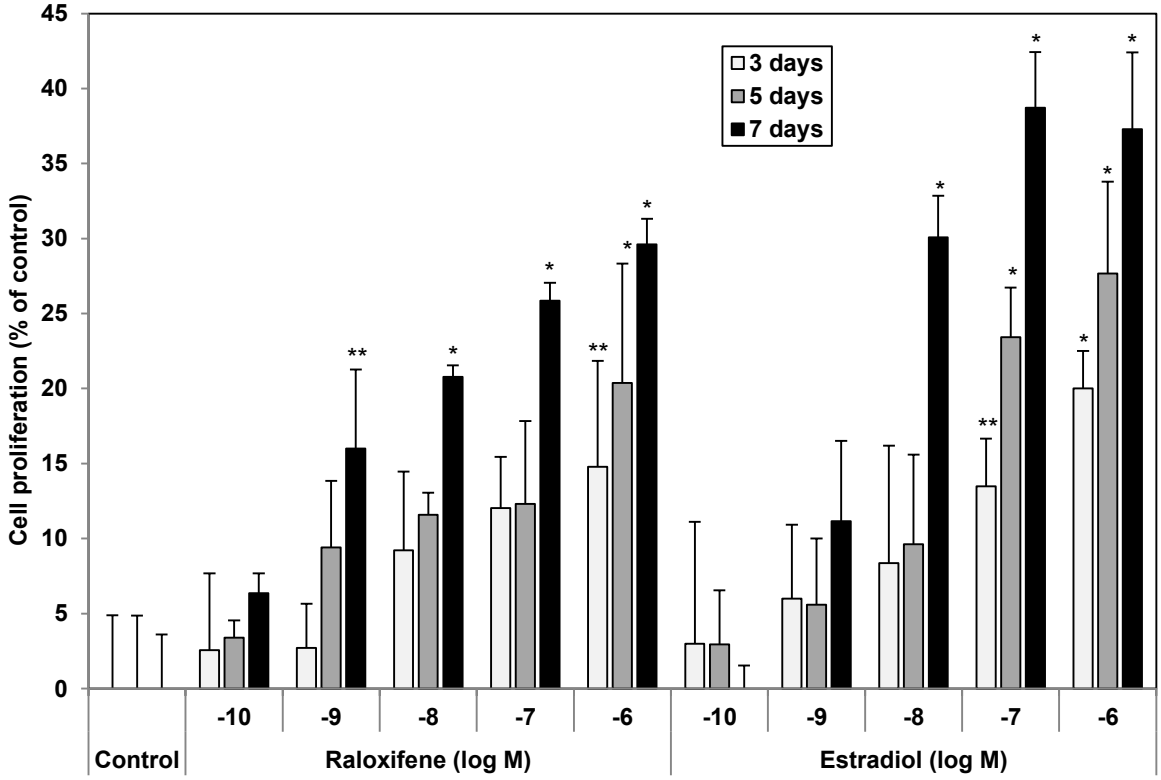
Fig. 3 Effect of 6-EO-14 and 8-EO-14 on steroid sulfatase activity in homogenated HEK-293 cells. An IC_{50} value of 0.3 μM ± 0.05 μM was obtained for 6-EO-14 while no significant inhibition of STS was obtained by 8-EO-14. Results are expressed as % inhibition of [^{3}H]-E1S conversion in control. Each point represents the mean ± SD of triplicate in one experiment.

3.2 Effect on Saos-2 cell proliferation

One important characteristic of SERMs is their ability to maintain the integrity of bone tissue. As such, we tested our 2 molecules on Saos-2 cells to see if they can stimulate their proliferation. First, we needed to optimize the conditions of the protocol to determine what concentrations would be tested and how long the treatments would last. Therefore we tested E2 and raloxifene at different concentrations (0.1 nM - 1 μM) on the cells for 3, 5 and 7 days of treatment (Fig. 4a). After observing an apparent dose-dependent stimulation, we decided that the best conditions would be an incubation of 7 days with 6-EO-14 and 8-EO-14 at concentrations of 0.1 μM and 1 μM (Fig. 4b). 8-EO-14 induced a stimulation of

proliferation similar to the stimulation by E2 and raloxifene, but 6-EO-14 showed no significant stimulation comparatively to control. Even though 8-EO-14 induced a significant growth of Saos-2 cells compared to control, the stimulation is still weak (21% and 16% at 1 μM and 0.1 μM, respectively).

a



b

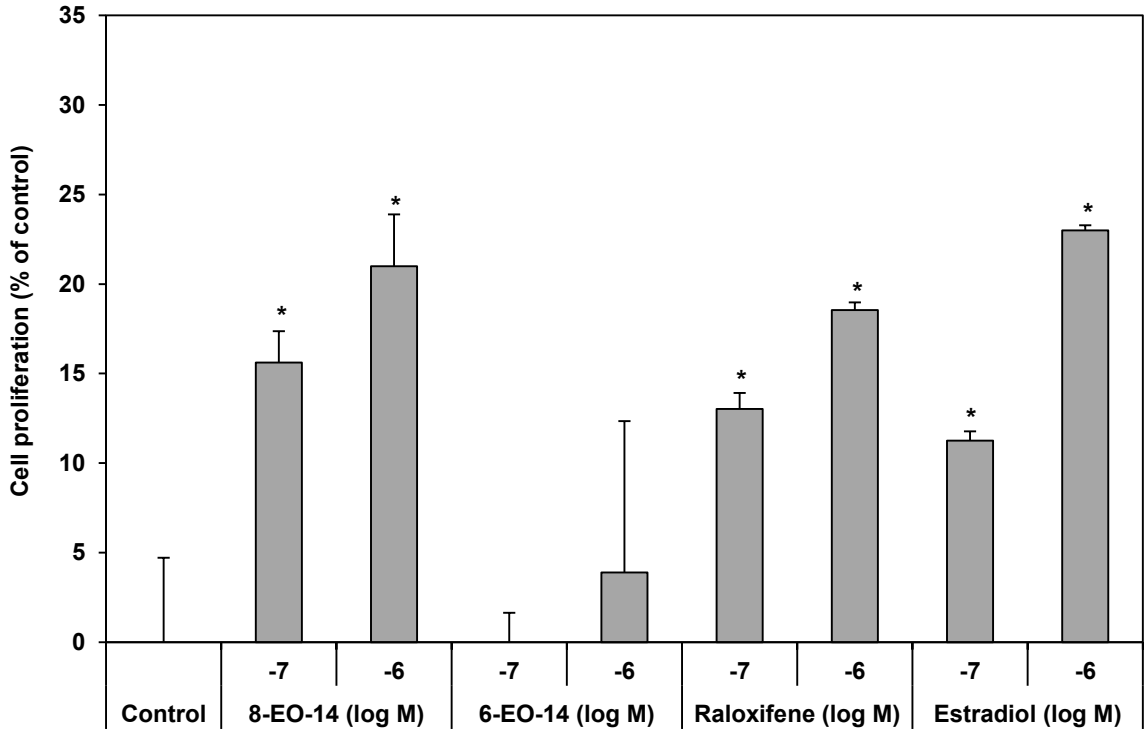


Fig. 4 (a) Effect of 17β-estradiol and raloxifene on Saos-2 cell proliferation after 3, 5 and 7 days of treatment. **(b)** Effect of 8-EO-14 and 6-EO-14 on Saos-2 cell proliferation after 7 days of treatment. The proliferation of control cells is set to 0%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. control, ** $p \leq 0.05$ vs. control.

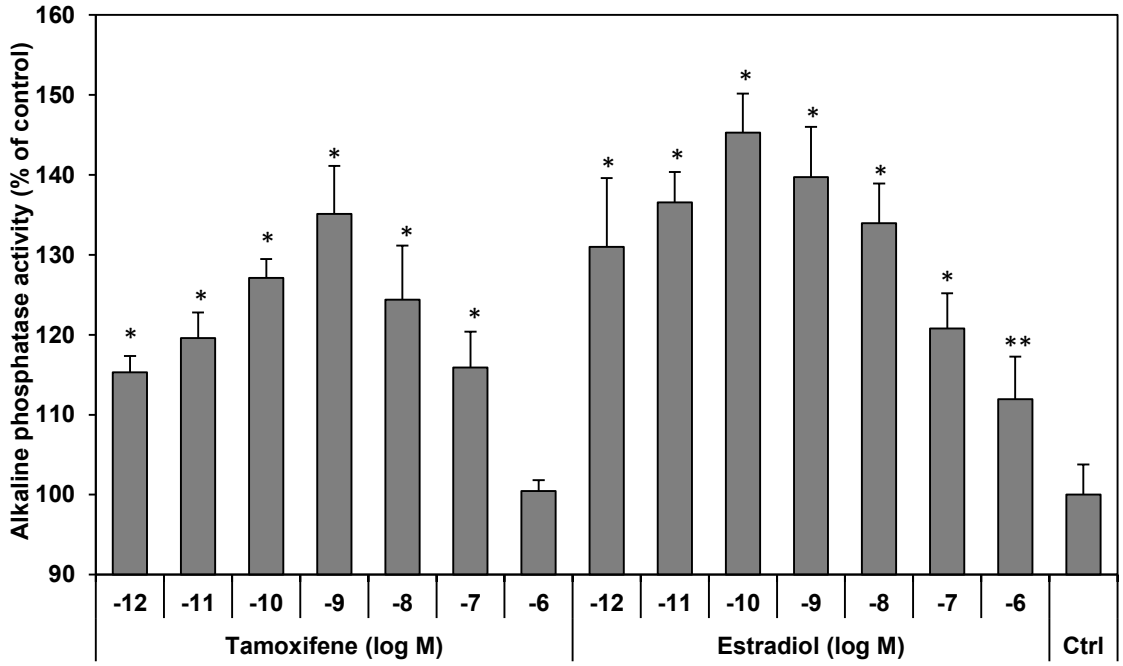
3.3 Effect on alkaline phosphatase (ALP) activity of Saos-2 cells

To partly validate its SERM capacity on bone tissue, we decided that cell proliferation was not enough and that a more sensitive test was required. We investigated the effect of 6-EO-14 and 8-EO-14 on ALP activity in Saos-2 cells to determine if they could activate osteoblast maturation and mineralization. After minor adjustments to the conditions of the assay on Saos-2 cells (data not shown), we determined the concentration of products required to stimulate significantly ALP activity. We tested 17β-estradiol (E2) and the SERM tamoxifen on Saos-2 cells and observed an unexpected bell pattern for the concentrations tested (Fig. 5a), instead of the dose-dependent effect that we anticipated. As previously reported (Qu et al. 1998, Qu et al. 1999), E2 and tamoxifen increase ALP

activity with a maximal effect at 0.1 nM and 1 nM respectively. We also did a preliminary test for 6-EO-14 and 8-EO-14 and we observed the same bell pattern with a maximal effect at 0.1 nM (data not shown). As a result, we tested all molecules at a concentration of 0.1 nM for the subsequent experiments.

For a better understanding of the mechanism of 6-EO-14 and 8-EO-14, we incubated Saos-2 cells with both molecules and in presence of different estrogen receptor (ER) antagonists. We used the pure antiestrogen ICI-164,384 to see if the increase in ALP activity induced by 6-EO-14 and 8-EO-14 was really acting via the ER and if the stimulation could be fully blocked. We also wanted to know by which estrogen receptor subtype they acted so we used MPP, which is an ERα selective antagonist (Sun et al. 2002) and PHTPP, which is an ERβ selective antagonist (Compton et al. 2004). We observed that the increase of ALP activity induced by 6-EO-14 and 8-EO-14 is exclusively done via ERα (Fig. 5b). The incubation in presence of MPP fully blocked the induced stimulation while the presence of PHTPP had no effect. In presence of both antiestrogens, the induced stimulation was fully blocked, but that was caused solely by MPP presence. ICI-164,384 also fully blocked the induced stimulation by 6-EO-14 and 8-EO-14. The sulfamate 6-EO-14 can activate the estrogen receptor in Saos-2 because of the presence of STS in osteoblasts (Fujikawa et al. 1997) which converts it into the phenol 8-EO-14. Such results indicate that our molecule can activate osteoblast maturation and have the capacity of being a SERM. When we observe E2 and raloxifene induced stimulations (Fig. 5b), we clearly see that the stimulation is done via both ER subtypes. In these cases, the selective estrogen receptor subtype antagonists either blocked partly or completely the induced stimulation while ICI-164,384 fully blocked the increase in ALP activity compared to control.

a


Alkaline phosphatase activity (% of control)
160
150
140
130
120
110
100
90
-12
-11
-10
-9
-8
-7
-6
Tamoxifene (log M)
Estradiol (log M)
Ctrl

b

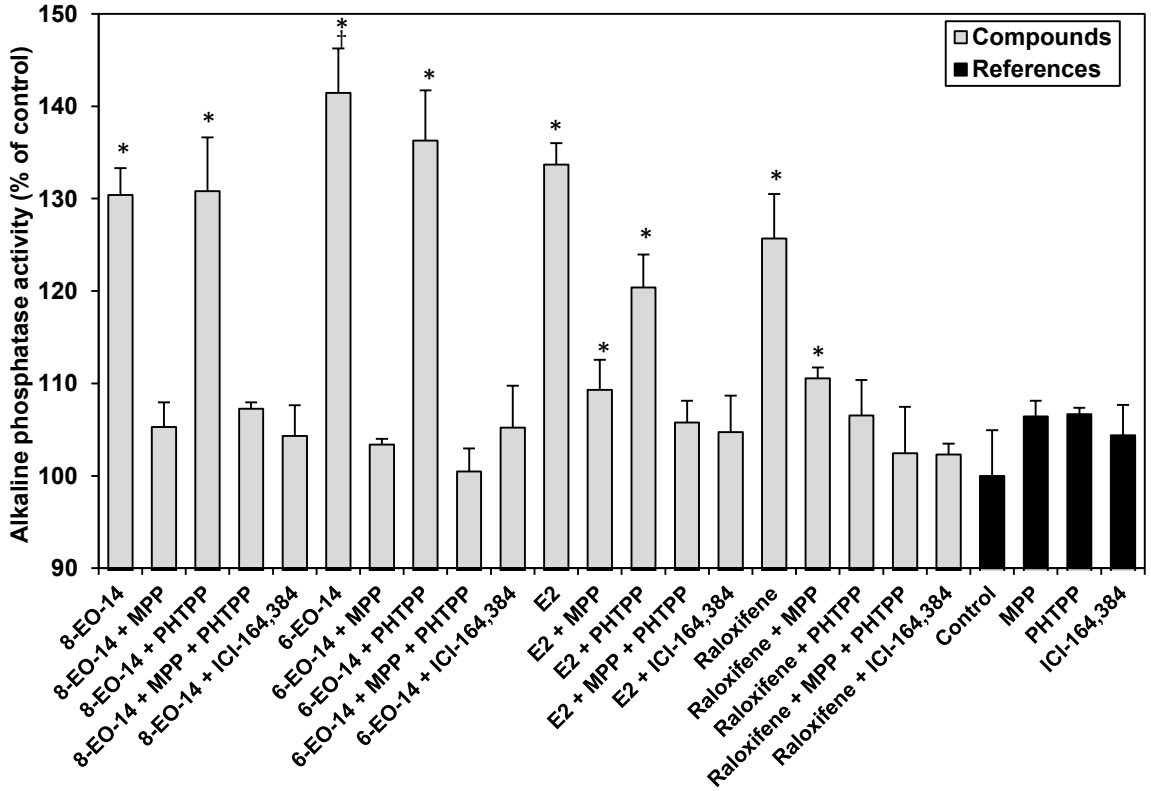

Alkaline phosphatase activity (% of control)
150
140
130
120
110
100
90
Compounds
References
8-EO-14
8-EO-14 + MPP
8-EO-14 + PHTPP
8-EO-14 + MPP + PHTPP
8-EO-14 + ICI-164,384
6-EO-14
6-EO-14 + MPP
6-EO-14 + PHTPP
6-EO-14 + MPP + PHTPP
6-EO-14 + ICI-164,384
E2
E2 + MPP
E2 + PHTPP
E2 + MPP + PHTPP
E2 + ICI-164,384
Raloxifene
Raloxifene + MPP
Raloxifene + PHTPP
Raloxifene + MPP + PHTPP
Raloxifene + ICI-164,384
Control
MPP
PHTPP
ICI-164,384

Fig. 5 (a) Effect of estradiol (E2) and tamoxifen on alkaline phosphatase (ALP) activity in Saos-2 cells **(b)** Effect of 6-EO-14 and 8-EO-14 on ALP activity. The cells were incubated for 3 days with 0.1 nM of 8-EO-14, 6-EO-14, 17β-estradiol or raloxifene with or without the different inhibitors (MPP, PHTPP, ICI-164,384) at a concentration of 1 μM. The ALP activity of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. control, ** $p \leq 0.05$ vs. control, † $p \leq 0.05$ vs. 8-EO-14.

3.4 Affinity of 6-EO-14 and 8-EO-14 for human ERα

As a result of the increase of alkaline phosphatase activity in Saos-2 induced by 6-EO-14 and 8-EO-14, we wanted to find out their affinity for the human estrogen receptor alpha (hERα). We also determined the affinity of estrone (E1) and estrone sulfamate (EMATE) as a comparative for our two molecules because E1 can be considered as the phenolic analogue of STS inhibitor EMATE. We had anticipated obtaining results as previously reported by Nussbaumer et al. (Nussbaumer et al. 2003) where they show that the sulfamate EMATE has a lower affinity than its phenolic analogue E1 for ERα. Similarly, we obtained IC_{50} values of 0.3 μM and 7 nM for EMATE and E1, respectively (Table 1). Surprisingly, we found that 6-EO-14 (IC_{50} of 3.1 μM) has a better affinity than 8-EO-14 (IC_{50} of 24.6 μM) for hERα (Fig. 6 and Table 1). This result was unexpected because we anticipated that the sulfamate moiety on 6-EO-14 would have decreased its affinity for ERα similarly to EMATE towards E1.

To explain the better affinity of 6-EO-14 than 8-EO-14 towards ERα, we calculated and compared the distance between atoms (Fig. 7) suspected to make hydrogen bonds with key amino acids Glu353, Arg394 and His524 in the ligand binding domain (LBD) of ER (Kumar et al. 2011). For 8-EO-14 and 6-EO-14, the distance between the oxygen of the carbonyl and either the hydrogen of the hydroxyl group or the hydrogen of the amine in the sulfamate group were calculated. We also calculated the distance between the oxygen of the carbonyl in position 17 and the hydrogen of the hydroxyl group in position 3 of E1. We chose estrone over E2 because the carbonyl group in position 17 of E1 could be compared

to the carbonyl groups in 8-EO-14 and 6-EO-14. The distances calculated were 7-8 Å, 10 Å and 11 Å for 8-EO-14, 6-EO-14 and E1, respectively. These results show that 6-EO-14 could make hydrogen bonds with amino acids Glu353 and Arg394 when bound to the LBD of ER because the hydrogens of the amine in the sulfamate group are in the same area of the hydrogen of the hydroxyl group in position 3 of E1. Comparatively, the hydrogen of the hydroxyl group of 8-EO-14 is a bit too far from those two amino acids and cannot make hydrogen bonds, thus resulting in a lower affinity. The better affinity of 6-EO-14 for ERα could also be noticed in the significantly higher stimulation of ALP activity in Saos-2 cells than 8-EO-14 (Fig. 5b). It is also surprising that even with low affinities for ERα, both molecules were able to stimulate ALP activity in Saos-2 similarly to E2.

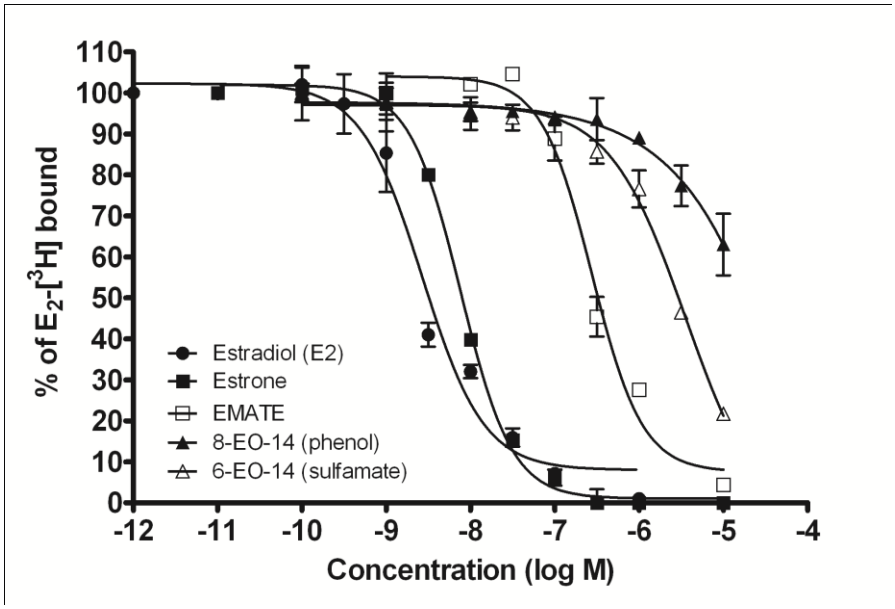


Fig. 6 Competition of the binding of [^{3}H]-E2 to human ERα by 6-EO-14, 8-EO-14 and reference compounds. Results are expressed as percent of [^{3}H]-E2 bound in comparison to control (buffer + hERα + [^{3}H]-E2) and each data points represent the mean ± SD of triplicate measurements.

Table 1 IC_{50} values and relative binding affinities (RBA) of 6-EO-14, 8-EO-14 and reference compounds for hERα

Compound	hERα affinity (IC_{50} ± SD)	RBA_{α} (%)
17β-estradiol (E2)	2.718×10^{-9} M ± 9.83×10^{-10} M	100
Estrone (E1)	7.677×10^{-9} M ± 7.57×10^{-10} M	35
EMATE	2.949×10^{-7} M ± 8.44×10^{-8} M	0.92
8-EO-14[a]	2.460×10^{-5} M ± 1.59×10^{-5} M	0.01
6-EO-14[a]	3.076×10^{-6} M ± 7.72×10^{-7}M	0.09

[a] IC_{50} values were determined by adding a fictional point at 10^{-2} M to help the software calculate better approximate values.

3.5 Effect on breast cancer cell lines

To be a SERM, our 2 molecules must not stimulate the estrogen-sensitive breast cancer cell proliferation and must have the capacity to block the E2-induced stimulation of estrogen-dependent cells. At first, we tested only 8-EO-14 on MCF-7 cells because we thought that only the phenolic compound 8-EO-14 could bind to ER (Fig. 7). The results obtained show that 8-EO-14, at the 3 concentrations tested, is not estrogenic (no cell proliferation). Unfortunately, this compound is unable to counter the stimulation induced by E2 so it is not an antiestrogen.

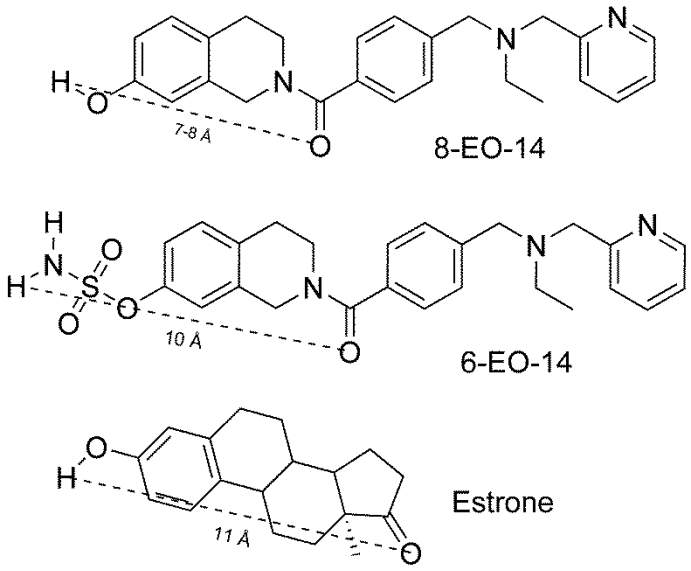


Fig. 7 Comparison between atom distances of 8-EO-14, 6-EO-14 and estrone (E1). For our two compounds, the distances between the oxygen of the carbonyl and either the hydrogen of the hydroxyl group or the hydrogen of the amine in the sulfamate group were calculated. In comparison, the distance between the oxygen of the carbonyl in position 17 and the hydrogen of the hydroxyl group in position 3 of E1 was calculated. We can see that the sulfamate group of 6-EO-14 could possibly make hydrogen bonds with the same amino acids of the ligand binding domain of ER similarly to the hydroxyl group of E1. The distances were calculated using Chem3D Ultra 8.0.

Following the results showing that 6-EO-14 has a better affinity for ERα than 8-EO-14, we decided to test both compounds on another breast cancer cell line. This time we observed that 6-EO-14 and 8-EO-14 clearly stimulated T-47D cell proliferation in a dose-dependent manner (Fig. 8a). We also noticed that in order to induce about 180% cell proliferation, the cells had to be incubated with different concentrations of 8-EO-14, 6-EO-14 and E2 (1 μM, 0.1 μM and 0.1 nM respectively). These results support the IC_{50} values obtained for ERα where the affinity of 6-EO-14 and 8-EO-14 is respectively about 1 000 and 10 000 times less than E2. We also incubated T-47D cells with both compounds in presence of 0.1 nM estradiol to reevaluate their potential to block the estradiol-induced stimulation. The results obtained clearly confirm that 6-EO-14 and 8-EO-14 are unable to prevent E2 binding to ER in a way raloxifene does (Fig. 8b).

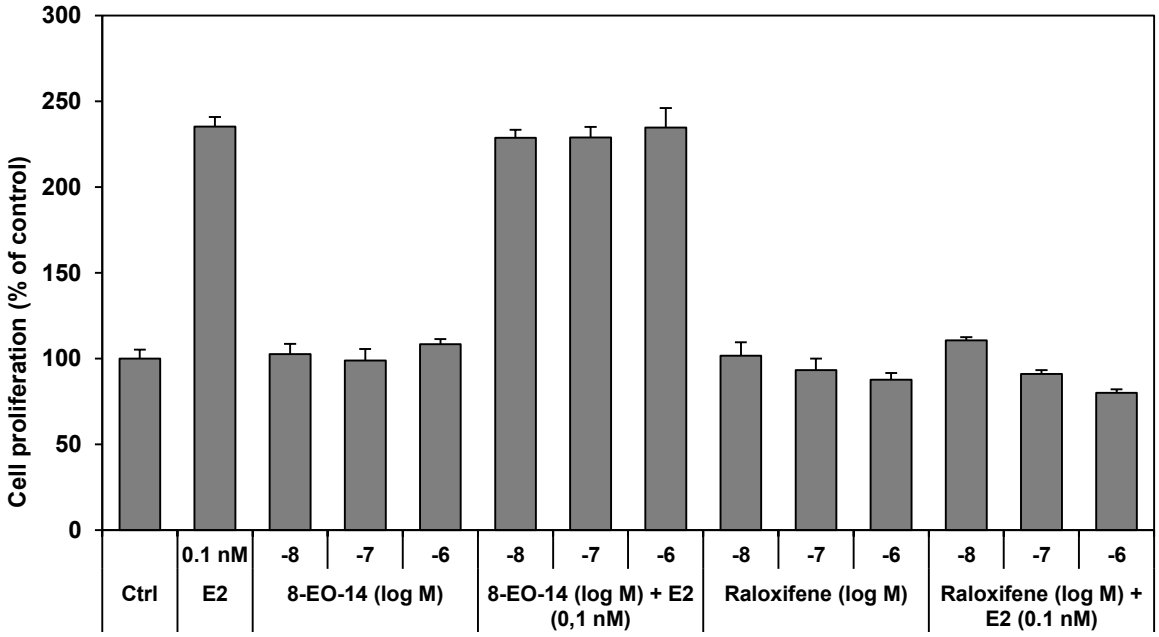
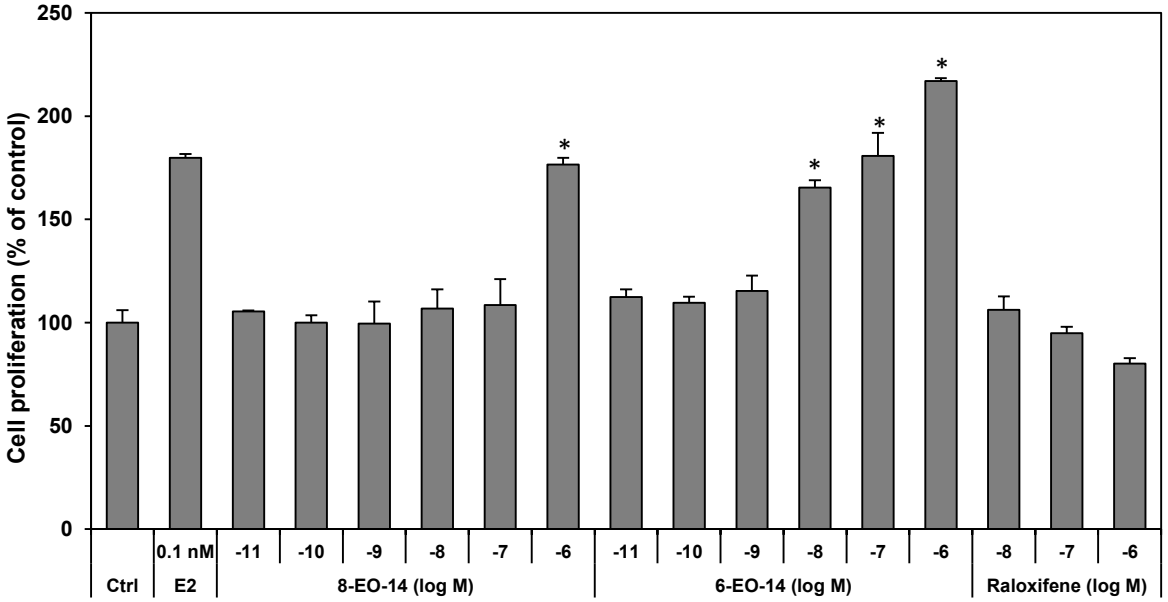


Fig. 8 Effect of 8-EO-14 on MCF-7 cell proliferation after 7 days of treatment. The cells were incubated with or without 0.1 nM of estradiol to evaluate 8-EO-14 estrogenicity and its potential to counter estradiol stimulation. The proliferation of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements.

a



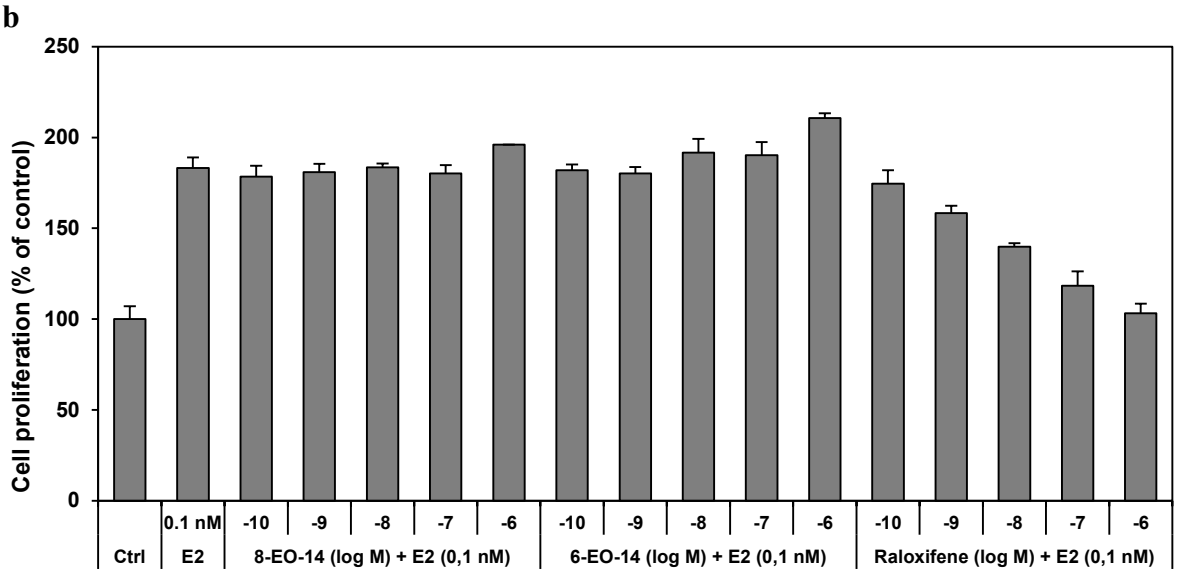


Fig. 9 Effect of 6-EO-14 and 8-EO-14 on T-47D cell proliferation after 7 days of treatment **(a)** The estrogenicity of 6-EO-14 and 8-EO-14 was evaluated by incubating the cells with different concentrations of both compounds. **(b)** The antiestrogenicity of 6-EO-14 and 8-EO-14 was investigated by incubating the cells with different concentrations of both compounds and 0.1 nM of estradiol (E2). The proliferation of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. control

4. Conclusion

We described the *in vitro* assays used to evaluate our dual-action steroid sulfatase inhibitor with a selective estrogen receptor modulator (SERM) capacity. The non-steroidal sulfamoylated molecule 6-EO-14 effectively inhibits STS (IC_{50} = 0.3 μM) and releases the phenolic analogue 8-EO-14. We showed that 8-EO-14, but not 6-EO-14, stimulated cell proliferation of the osteoblast-like Saos-2 cells similarly to potent estrogen E2 and SERM raloxifene. Both compounds stimulated alkaline phosphatase activity in Saos-2 cells by exclusively binding to ERα. Due to its better affinity for the receptor, the ALP activity induced by the sulfamate 6-EO-14 was slightly higher than the phenol 8-EO-14. In fact, the affinity of both molecules for the human ERα was tested and showed that 6-EO-14 had a better affinity than 8-EO-14. Furthermore, 6-EO-14 and 8-EO-14 were tested on the estrogen-dependent ER^+ breast cancer cell lines MCF-7 and T-47D. The results obtained

demonstrated that both compounds are estrogenic on T-47D cells and are unable to block the E2-induced stimulation of both cell lines. We showed that our sulfamate compound can inhibit STS but seems to be purely estrogenic on breast and bone tissues. These results unfortunately show that 6-EO-14 cannot work as a dual-action compound inhibiting STS and acting as a SERM. Clearly, our lead compound 6-EO-14 releases 8-EO-14, but this phenolic compound did not possess the characteristics needed for the partial folding of helix 12 of ER after binding to the LBD, the key conformational change induced by SERM compounds (Dahlman-Wright et al. 2006, Brzozowski et al. 1997). However, we described *in vitro* assays that can be effectively used in further structure-activity relationship studies to evaluate the potential of improved second generation of 6-EO-14 analogues as dual-action inhibitors. 6-EO-14 is also a potent STS inhibitor (IC_{50} = 0.3 μM) that could be used as a hormonal treatment for ER^+ breast cancers.

Acknowledgments

We thank the Fondation du Cancer du Sein du Québec for granting this study. We also thank Jenny Roy and Diana Ayan for the help with the *in vitro* assays.

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Chapitre 3

Résumé

Présentement, le traitement hormonal du cancer du sein inclut l'utilisation d'inhibiteurs de l'aromatase et des modulateurs sélectifs du récepteur des estrogènes (SERMs). La stéroïde sulfatase, l'enzyme convertissant les précurseurs stéroïdiens sulfatés inactifs en hormones actives, est une cible thérapeutique prometteuse pour le traitement des cancers du sein hormonodépendants. Nous rapportons ici la synthèse chimique et l'évaluation biologique *in vitro* d'inhibiteurs de la STS à double-action possédant une activité modulatrice sélective du récepteur des estrogènes. Une librairie de composés phénoliques et quelques analogues sulfamates correspondants furent synthétisés à l'aide de la chimie en parallèle sur support solide. Pour le criblage initial de la librairie, les composés phénoliques furent testés sur les cellules cancéreuses du sein sensibles aux estrogènes T-47D. Trois composés dépourvus d'estrogénicité et de cytotoxicité sont ressortis de ce criblage. Leurs trois sulfamates analogues furent alors synthétisés et testés sur des cellules HEK-293 surexprimant la STS et démontrèrent une excellente inhibition de l'enzyme (IC_{50} de 3.9, 8.9 et 16.6 nM). Ces composés sélectionnés (3 phénols et 3 sulfamates) furent testés sur des cellules T-47D et montrèrent aucune activité estrogénique en plus de bloquer la stimulation induite par l'estradiol, montrant ainsi des propriétés antiestrogéniques. Pour évaluer leur potentiel SERM, les composés furent testés sur des cellules ostéoblastiques Saos-2 et stimulèrent significativement leur prolifération à 1 μM. Les composés ont aussi stimulé significativement l'activité de l'alkaline phosphatase, un marqueur de différentiation dans les cellules Saos-2. Ces composés prometteurs seront utilisés dans de futurs tests *in vivo* sur des rongeurs pour confirmer leur double-action.

Manuscrit à soumettre au *Journal of Medicinal Chemistry*

Development of sulfamate tetrahydroisoquinoline-N-substituted derivatives as potent dual-action compounds for the treatment of estrogen-dependant breast cancer

Charles Ouellet, René Maltais, Étienne Ouellet, Donald Poirier*

Laboratory of Medicinal Chemistry, CHU de Québec (CHUL) – Research Center and Laval University, 2705 Laurier Boulevard, Québec (Québec), G1V 4G2, Canada

*Corresponding author:

Dr. Donald Poirier
Laboratory of Medicinal Chemistry
CHU de Québec (CHUL) - Research Center,
2705 Laurier Boulevard, Québec (Québec), G1V 4G2, Canada
Phone : (418) 654-2296; Fax : (418) 654-2761
E-mail : donald.poirier@crchul.ulaval.ca

Abstract

Steroid sulfatase (STS), the enzyme which converts inactive sulfated steroid precursors into active hormones, is a promising therapeutic target for the treatment of estrogen sensitive breast cancer. We report herein the synthesis and *in vitro* study of dual-action STS inhibitors with selective estrogen receptor modulator (SERM) effects. A library of phenolic compounds and some of their corresponding sulfamate analogues were synthesized by parallel solid-phase chemistry. For the initial screening of the library, the phenolic forms of the compounds were tested on estrogen-sensitive breast cancer cells T-47D. Three phenolic compounds devoid of estrogenic activity and toxicity emerged from this screening. Their sulfamate analogues were then synthesized and tested on STS transfected HEK-293 cells and found to be excellent inhibitor of the enzyme (IC_{50} of 3.9, 8.9 and 16.6 nM). These selected compounds (3 phenols and 3 sulfamates) were tested on T-47D cells and showed no estrogenic activity as well as being able to counter the estradiol-induced proliferation, thus showing antiestrogenic properties. To evaluate their SERM potential, the compounds were further tested on osteoblast-like Saos-2 cells and were found to stimulate their proliferation significantly at 1 μM. The compounds also stimulated significantly the activity of alkaline phosphatase (ALP), a differentiation marker. These promising compounds could be used in future *in vivo* assays on rodents to confirm their dual-action.

Introduction

Hormonal therapy is currently used for the treatment of estrogen-sensitive breast cancer. As the majority of breast cancers are initially estrogen-dependent, with approximately 55% in premenopausal women and 75% in post-menopausal women, this therapy efficiently blocks the stimulating effect of estrogens in breast cancer cells.[1] Selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, are compounds presently used to treat breast cancer.[2] In breast tissues, SERMs effectively blocks the activation of estrogen receptor alpha (ERα) by endogenous ligands and prevent the transcription of genes mediated by estrogen response elements (ERE).[3] This class of compounds possesses the particularity of having tissue specific effects on ERα, resulting in antagonist activity in

breast and uterus tissues and agonist activity in bone. Although tamoxifen and raloxifene possess the desired SERM activity, they also increase the risk of venous thromboembolism.[4-5] As a result, the development of new SERMs is still active and needed to obtain SERMs with fewer side effects.[6]

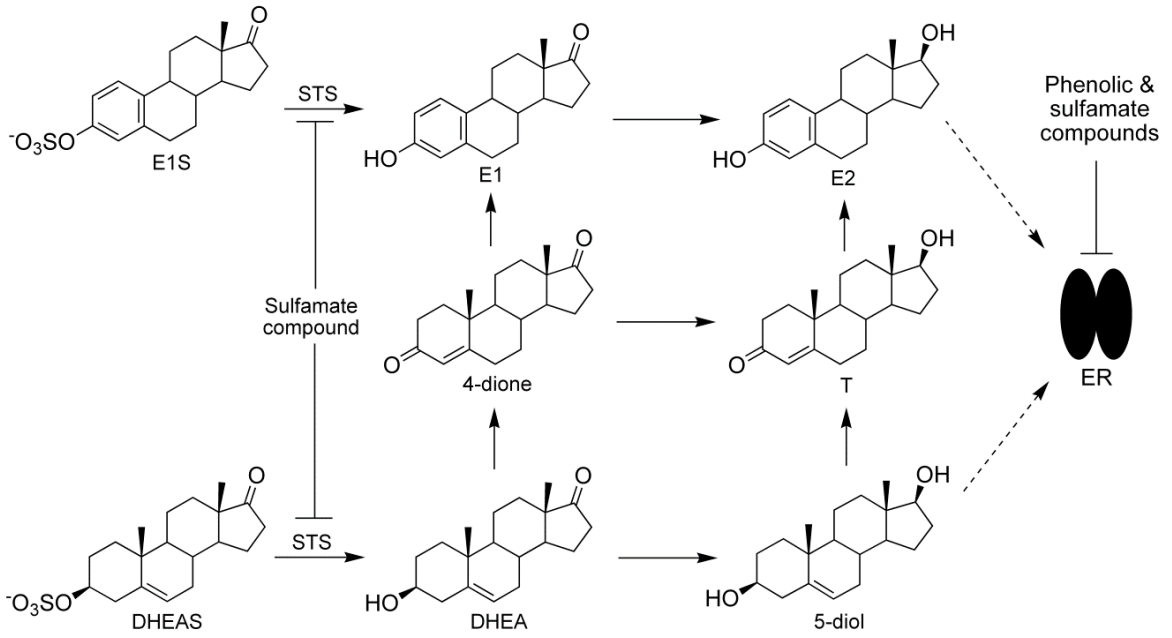


Fig. 1 Transformation by steroid sulfatase (STS) of sulfated steroid E1S and DHEAS into active hormones and site of action of dual-action compounds (sulfamates and phenols). The sulfamate compound inhibits the conversion of the inactive sulfated precursors E1S and DHEAS into E1 and DHEA, respectively, by releasing phenolic analogue. Both sulfamate and phenolic compounds are potentially blocking the estrogen receptor (ER) from activation by E2 and 5-diol in breast tissue.

Inhibition of steroid sulfatase (STS) is a therapeutic approach for the treatment of estrogen-dependent breast cancer and different kinds of inhibitors were developed during the past years.[7-9] STS is an enzyme that converts inactive sulfated steroids, mainly pregnenolone sulfate (PREGS), estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHEAS), into unconjugated hormones (Fig. 1).[10] E1S and DHEAS are particularly abundant in circulation and act as a reservoir of steroid precursors.[11] It was also shown that STS activity in breast

cancer tumors is much higher than aromatase activity and that in situ formation of E1 and E2 is mainly done via the STS pathway rather than the aromatase pathway.[12-14] Therefore blocking STS could prevent estrogen-sensitive carcinomas from transforming sulfated steroids into potent estrogens, mainly estrone (E1), estradiol (E2) and 5-androstenediol (5-diol).

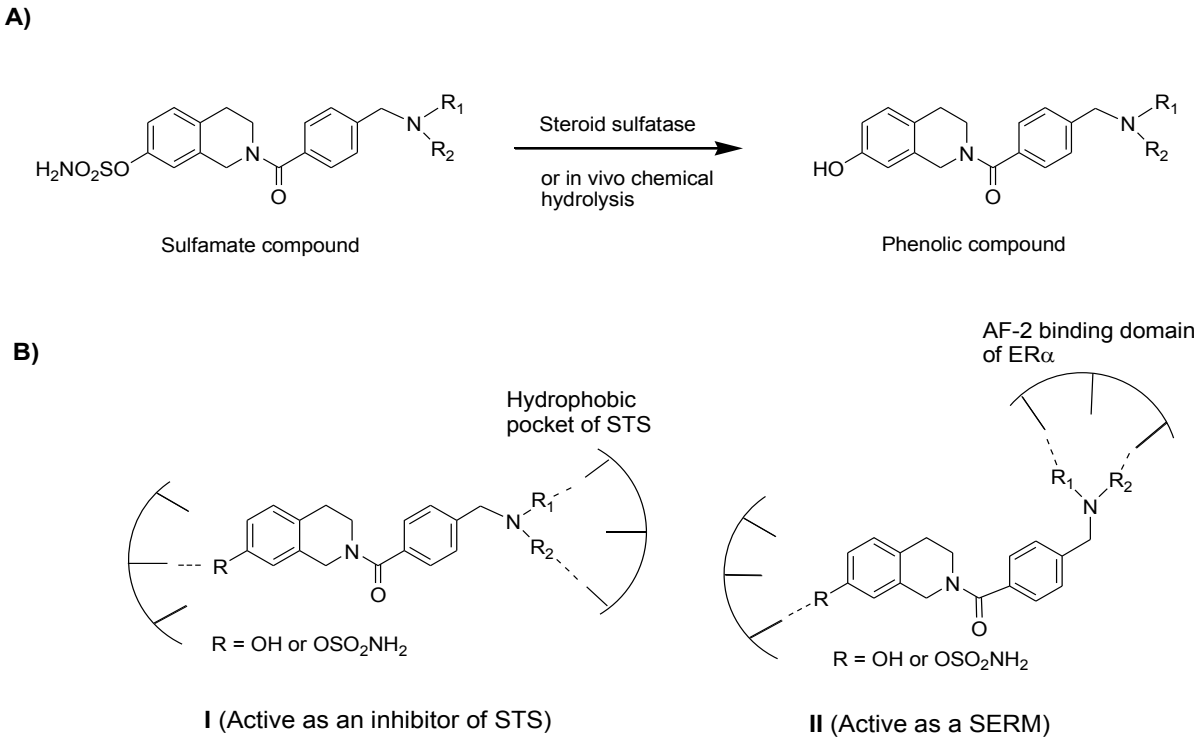


Fig. 2 A. Conversion of sulfamate compounds into phenolic compounds by steroid sulfatase (STS) or by chemical hydrolysis. **B.** The sulfamate binds to the active site of the enzyme, thus inactivating the STS. The sulfamate by itself or via released phenol could act as SERM compound on ERα.

The dual blocking of ERα and STS to reach a maximum estrogen blockade for the treatment of (ER^+) breast cancer represent an interesting therapeutic approach. However, the maximum estrogen blockade obtained by such treatment should induce an estrogen depletion condition that could provoke undesirable side effect like osteoporosis.[15] An

approach recently investigated in our laboratory resides on dual-action inhibitors of STS. The goal is to develop a non-steroidal sulfamoylated inhibitor of the enzyme having a selective estrogen receptor modulator (SERM) activity to attenuate the potential problem related with estrogen depletion induced by STS inhibitor. We recently reported the development of such dual-action compounds.[16] Many first-generation compounds showed a good inhibition of STS but did not possess the SERM capacity we were looking for. The compounds were found purely estrogenic on breast cancer and osteoblast-like cells.[17] Here we report the synthesis of second generation dual-action compounds designed to inhibit STS and act as a SERM (Fig. 1). The non-steroidal compounds were synthesized by parallel solid-phase chemistry using a multidetachable sulfamate linker.[18-20] The sulfamate-containing STS inhibitors release the phenolic analogues after the irreversible cleavage by STS or chemical hydrolysis (Fig. 2). For the initial screening, the library of phenolic compounds was tested on estrogen-sensitive breast cancer T-47D cells. Three phenolic compounds showed good initial results and were selected with corresponding sulfamate compounds for further testing. The six selected compounds were tested in HEK-293 transfected cells as STS inhibitors, on T-47D cells to evaluate their non-estrogenic and antiestrogenic properties and on osteoblast-like Saos-2 cells to evaluate their capacity to stimulate cell proliferation and alkaline phosphatase activity.

Results and discussion

Chemistry

Selection and chemical synthesis of secondary amines as building blocks

The choice of the secondary amines building blocks (compounds **1a-b**, **2a-b**, **3c**, **4c**, **5-11**) used to prepare the phenol library (compounds **19-31**) was guided by their potential capacity to interact either with STS enzyme (hydrophobic substituent) or with the estrogen receptor (H-bond acceptor groups). In the case of STS, it is well known that hydrophobic chains are well tolerated considering the presence of a large hydrophobic pocket in the

active site of the enzyme.[21] We thus selected secondary amines that bear hydrophobic substituent like 4-bromophenyl, furan and thiophene groups. Interestingly, the 4-bromophenyl and furan groups were previously found to be as the most potent substituents from a series of tetrahydroisoquinoline derivatives synthesized as STS inhibitors in a precedent SAR study.[16]

In parallel to hydrophobic chains for STS inhibition, we were interested by amines that bear hydrogen bond acceptor group, like pyridine, imidazole, morpholine or piperidine, to favor interaction with the estrogen receptor key amino acid. Indeed, it is well known that key amino acids involved in the stabilisation of the H12 helix of estrogen receptor like Asp351 could be targeted to induce SERM activity.[22] In that purpose, we selected amines of different sizes, shape and hydrogen bond acceptor capacity. Particularly, we synthesized the phenoxypropyl-piperidine chains which have been frequently reported as an important pharmacophore in several SERM compounds.[23]

Chemical synthesis of secondary amines as building blocks (compounds **1a-b**, **2a-b**, **3c**, **4c**, **5-11**)

The secondary amines **1a-b**, **2a-b**, **3c**, **4c** and **5-11** were synthesized by reacting the aldehydes **1-4** and appropriate amines **a-c** under classic conditions of reductive amination using molecular sieves in ethanol followed by the reduction of the intermediate imine with sodium borohydride (Scheme 1).[24] The aldehydes **1** and **2** were beforehand synthesized by reacting 2- or 4-hydroxybenzaldehyde and 1-(3-chloropropyl)piperidine with sodium carbonate and sodium iodide in refluxing acetone.

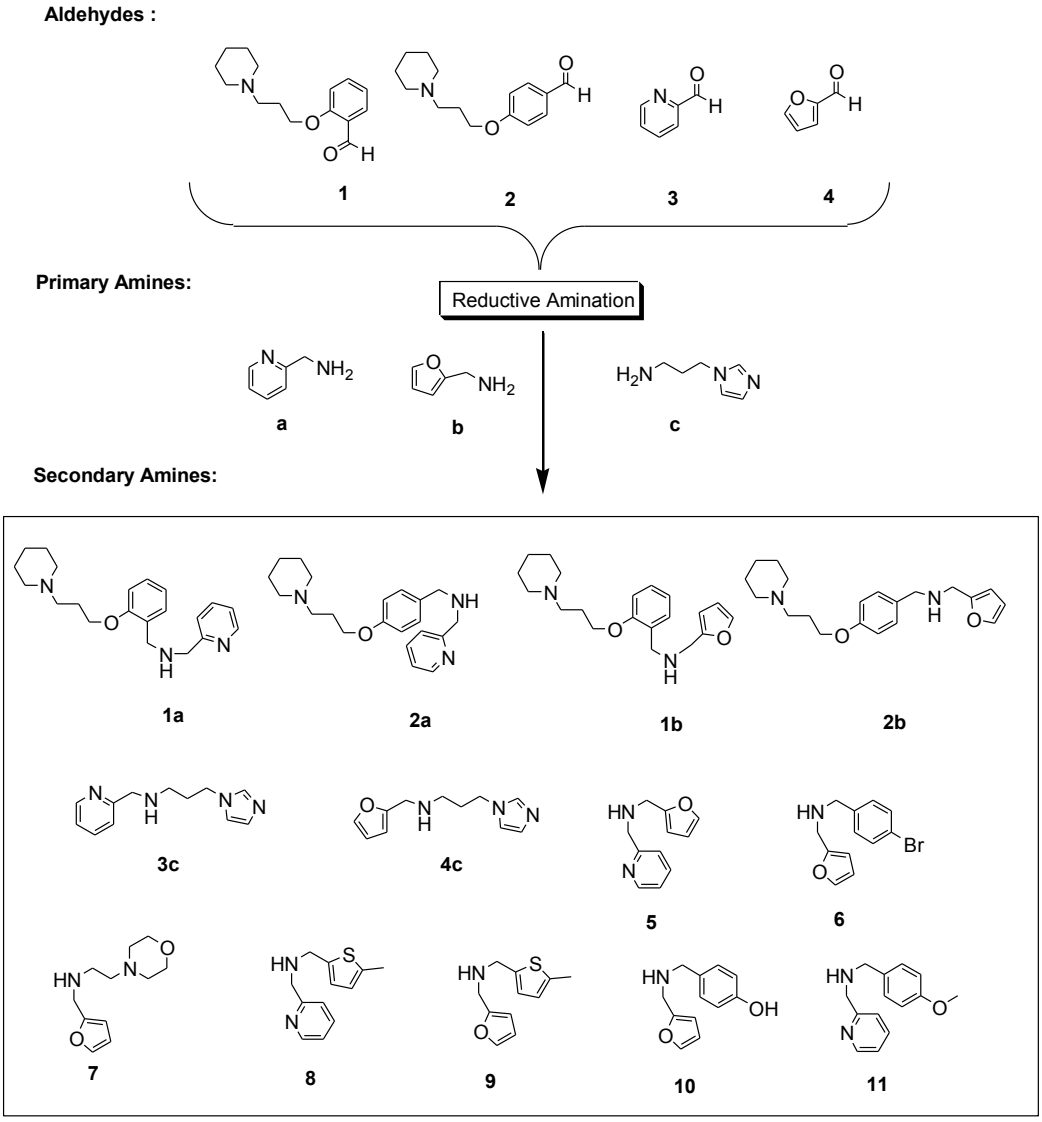
*Chemical synthesis of phenol library (compounds **19-31**)*

The general synthetic methodology for the preparation of all library members is outlined in Scheme 2. The starting 1,2,3,4-tetrahydroisoquinolin-7-ol (**12**) was selectively protected as the *N*-Fmoc derivative **13**, which after a sulfamoylation of the phenol yielded the sulfamate **14**. This sulfamate derivative was then reacted with the trityl chloride resin to give the

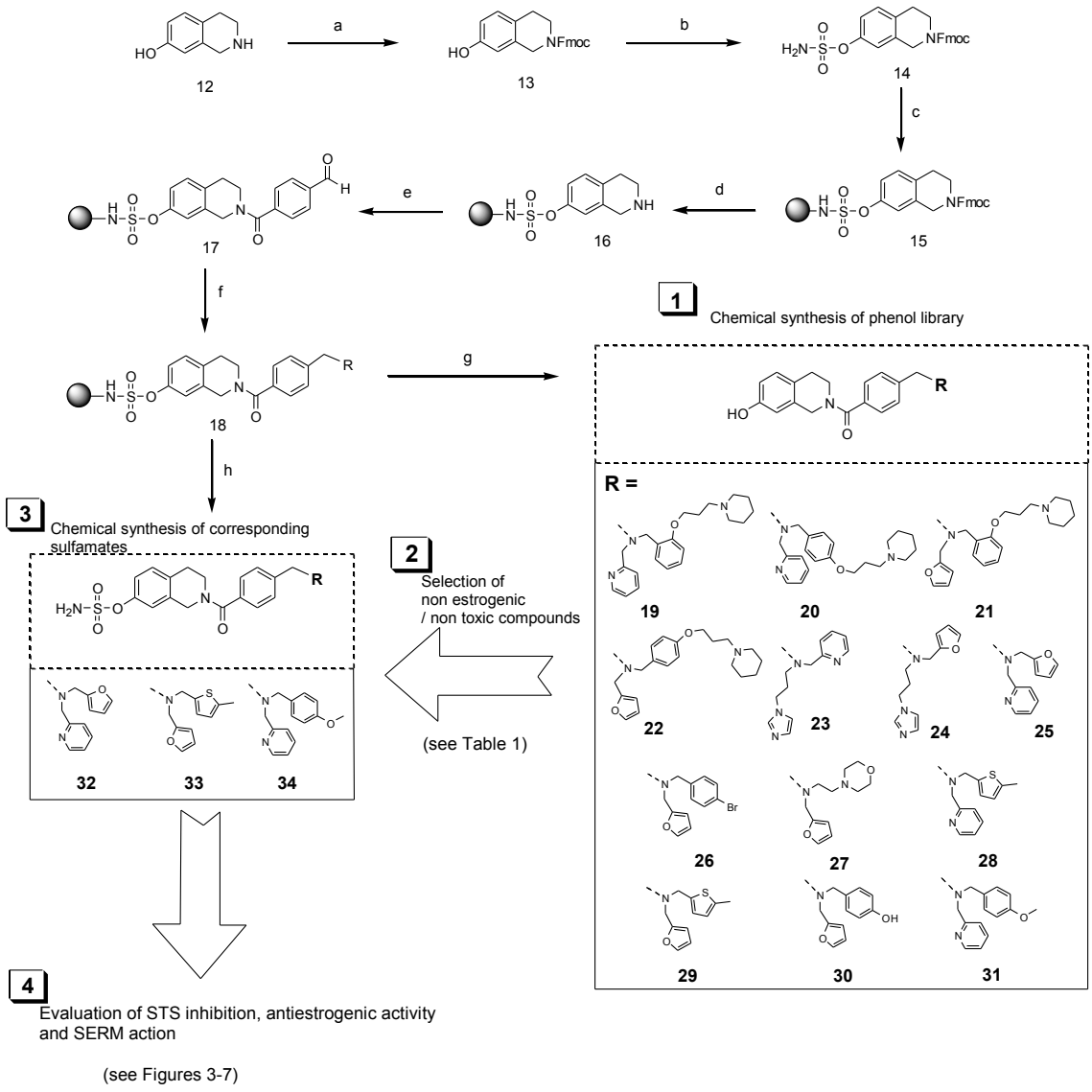
solid-phase bounded compound **15**. Removal of the Fmoc protecting group provided resin **16** with a free NH, which was acylated with carboxybenzaldehyde to give resin **17**. The diversification of **17** was obtained by performing a reductive amination with various secondary amines that yielded resin **18**. Finally, the phenol derivatives **19-31** were obtained by a nucleophilic cleavage from resin **18**. The released compounds were found sufficiently pure to proceed to the estrogenicity test on T-47D (ER+) cells. The compounds that showed no trace of estrogenicity and toxicity (compounds **25**, **29** and **31**) were then purified by flash chromatography and next submitted to a series of biological assays.

*Chemical synthesis of sulfamates **32-34***

The corresponding sulfamates **32-34** of phenols **25**, **29** and **31** were synthesized using the same solid phase chemical synthesis route as for phenol derivatives. In that case, however, an acid cleavage using HFIP was used at the end of the synthesis to release the sulfamate compounds **32-34**. The compounds were then purified by flash chromatography.



Scheme 1. Preparation of building blocks **(**secondary amines **1a-b**, **2a-b**, **3c** and **4c)** and structures of all commercially available secondary amines **5-11** used for the synthesis of targeted tetrahydroisoquinoline phenolic derivatives.



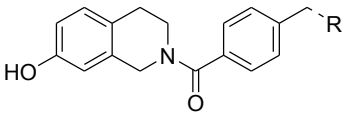
Scheme 2. Reagents and conditions for the chemical synthesis of phenol library (compounds **19-31**) and sulfamate compounds (**32-34**). Representation of the four steps (1-4) behind the identification of dual-action compounds. (a) Fmoc-*O*-succinimide, $NaHCO_3$, H_2O; (b) NH_2SO_2Cl, 2,6-di-*tert*-butyl-4-methylpyridine, DCM; (c) trityl chloride resin, DIPEA, DMA/DCM; (d) 20% piperidine in DMF; (e) carboxybenzaldehyde, DIPEA, HOBt, PyBOP, DMF; (f) secondary amine (R_1R_2NH), $NaBH(OAc)_3$, 10% AcOH in NMP; (g) 30% DEA in THF, 60°C; (h) 30% HFIP in DCM.

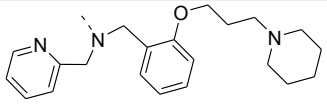
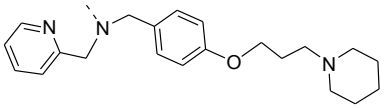
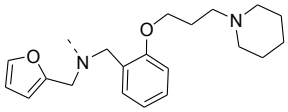
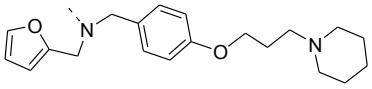
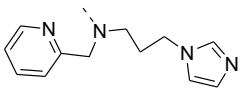
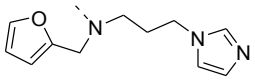
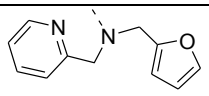
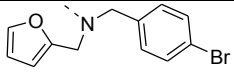
Biological assays

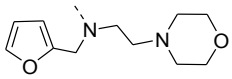
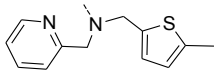
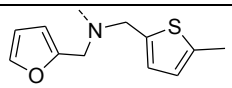
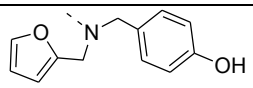
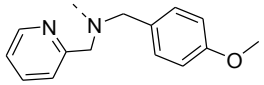
Initial screening

The library of phenolic compounds described above was tested on estrogen-sensitive breast cancer T-47D cells (Table 1).

Table 1. Structure of phenolic compounds and their capacity (estrogenicity) to induce the proliferation (%) of breast cancer T-47D (ER+) cells.



ID	R	Proliferation of T-47D cells (%)		
		0.01 µM	**0.1 µM**	**1 µM**
19		102.8 ± 6.9	99.8 ± 4.2	37.1 ± 4.2
20		105.9 ± 1.0	104.5 ± 4.8	60.7 ± 6.4
21		96.8 ± 1.2	105.9 ± 5.6	0 ± 9.1
22		95.6 ± 1.8	119.7 ± 4.8	133.7 ± 3.0
23		102.8 ± 2.2	112.9 ± 3.7	39.2 ± 2.9
24		94.9 ± 1.3	98.5 ± 4.5	114.6 ± 5.9
25		104.1 ± 2.3	103.2 ± 2.5	103.5 ± 6.5
26		106.3 ± 0.6	109.4 ± 1.2	59.2 ± 3.1

27		108.2 ± 5.5	105.9 ± 1.5	118.8 ± 9.6
28		100.6 ± 3.8	105.4 ± 4.4	78.9 ± 6.8
29		97.1 ± 2.6	103.2 ± 1.8	96.8 ± 2.2
30		108.2 ± 5.5	138.0 ± 1.2	200.3 ± 4.9
31		105.6 ± 0.6	104.6 ± 7.4	100.9 ± 7.9

Cell proliferation of control cells is set to 100%. Results are expressed as mean ± SD of one experiment in triplicate.

Compounds were discarded if they stimulated the proliferation of T-47D cells (estrogenic activity) or if they showed some cytotoxicity by reducing the cell number. Amongst the library of phenolic compounds, only compounds **25**, **29** and **31** showed interesting results. In fact, they showed neither estrogenicity nor cytotoxicity on T-47D cells at the concentrations tested (0.01 μM, 0.1 μM and 1 μM). Such results suggest that these three compounds do not possess the ability to activate ERα in breast cancer cells and consequently to induce unwanted cell proliferation.

Steroid sulfatase inhibition

Following the interesting results obtained with phenolic derivatives **25**, **29** and **31** in the initial screening, we synthesized the sulfamate analogues (compounds **32**, **33** and **34**) and evaluated their capacity to inhibit the steroid sulfatase (STS) activity. To test STS inhibition, we used homogenated HEK-293 cells overexpressing STS and evaluated the capacity of our compounds to inhibit the conversion of [^{3}H]-E1S into [^{3}H]-E1. In the test we used EM-1913, a potent steroidal STS inhibitor, as a reference compound.[32] As reported previously in the literature, phenolic inhibitors of STS are less potent than their sulfamoylated analogues.[22,34-35] Our results confirm such affirmations since only the

sulfamate compounds inhibited STS (Fig. 3). Effectively, the phenolic compounds showed no significant inhibition while the sulfamate compounds showed good inhibition of STS with IC_{50} values of 16.6 ± 2.7 nM for **32**, 8.9 ± 1.2 nM for **33** and 3.9 ± 1.1 nM for **34**. Interestingly, these three compounds demonstrated a higher STS inhibition potency than our previous generation of dual-action compounds, where one of the best STS inhibitor had an IC_{50} of 300 nM.[15] Therefore, only our sulfamate compounds can efficiently inhibit STS in estrogen-sensitive breast cancer cells.

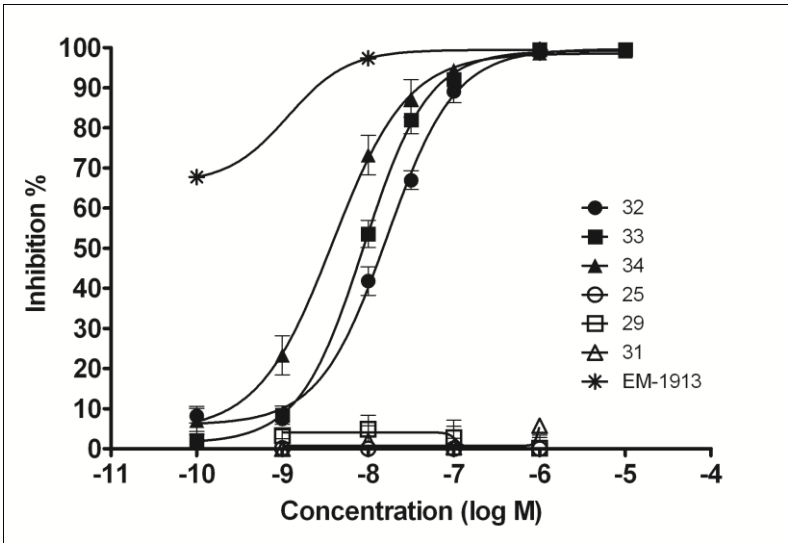


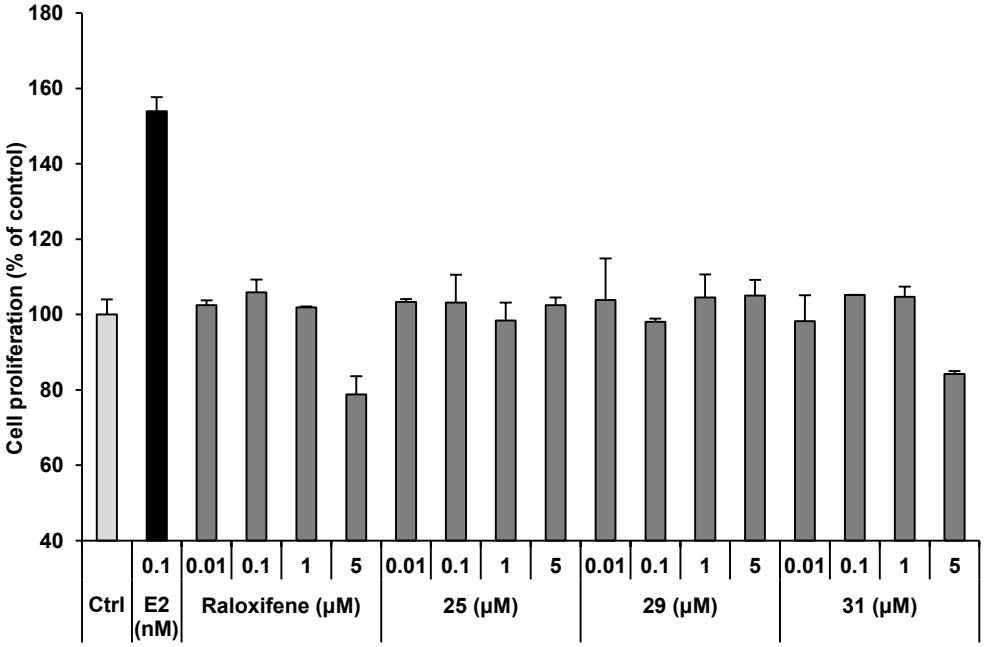
Fig. 3 Effect of sulfamate compounds **32**, **33** and **34** and their respective phenolic analogues **25**, **29** and **31** on steroid sulfatase activity in homogenates of transfected HEK-293 cells. The three sulfamate derivatives clearly inhibited STS while the three phenolic analogues showed no significant inhibition of STS. Results are expressed as % inhibition of [^{3}H]-E1S conversion into [^{3}H]-E1 by STS. Each point represents the mean ± SD of triplicate measurements in one experiment.

Effect on breast cancer T-47D cells

As the three sulfamate compounds are good inhibitors of STS, we wanted to know if they were also devoid of estrogenic activity. Along with the three phenolic analogues, to reconfirm the results of the initial screening, we tested the compounds on estrogen-sensitive

T-47D cells (Fig. 4A and 4B). We used the reference compound E2, as positive control of cell proliferation stimulation, and raloxifene, a SERM with no estrogenicity in breast tissue. E2, at a concentration of 0.1 nM, induced approximately 160% cell proliferation while raloxifene showed no estrogenic activity. Our six compounds showed good results because none of them stimulated the proliferation of T-47D cells. On the other hand, we observed cytotoxicity at 5 µM for some of the compounds. This should not be a problem as 5 µM is a high dose hardly achievable *in vivo* and that even raloxifene, a SERM used in clinic, induced some cytotoxicity at this concentration. As a result, both sulfamate and phenolic compounds can be used on breast cancer cells without stimulating their proliferation.

A



B

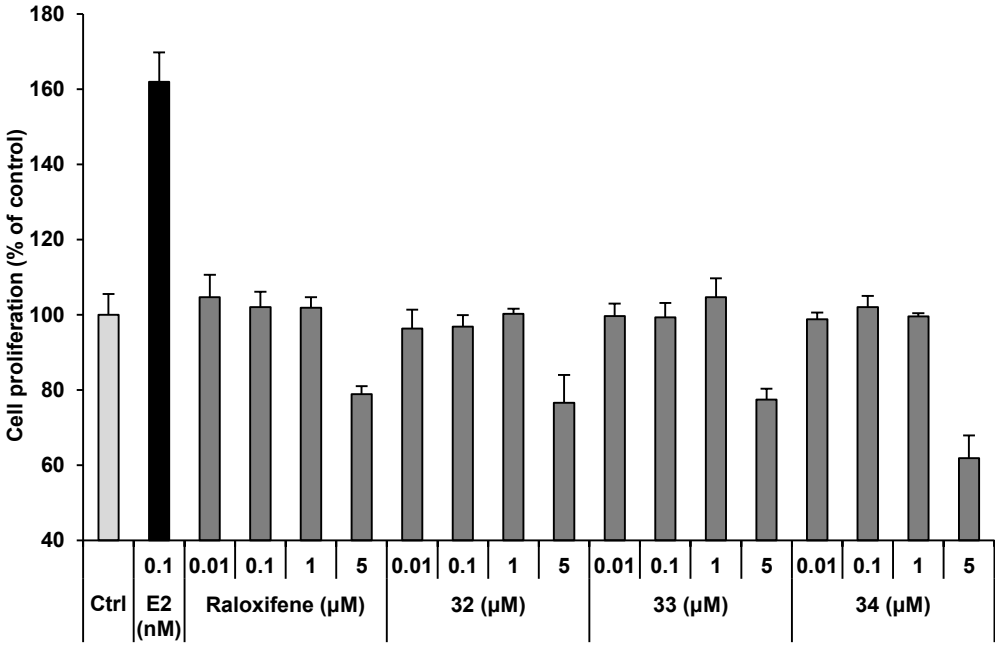
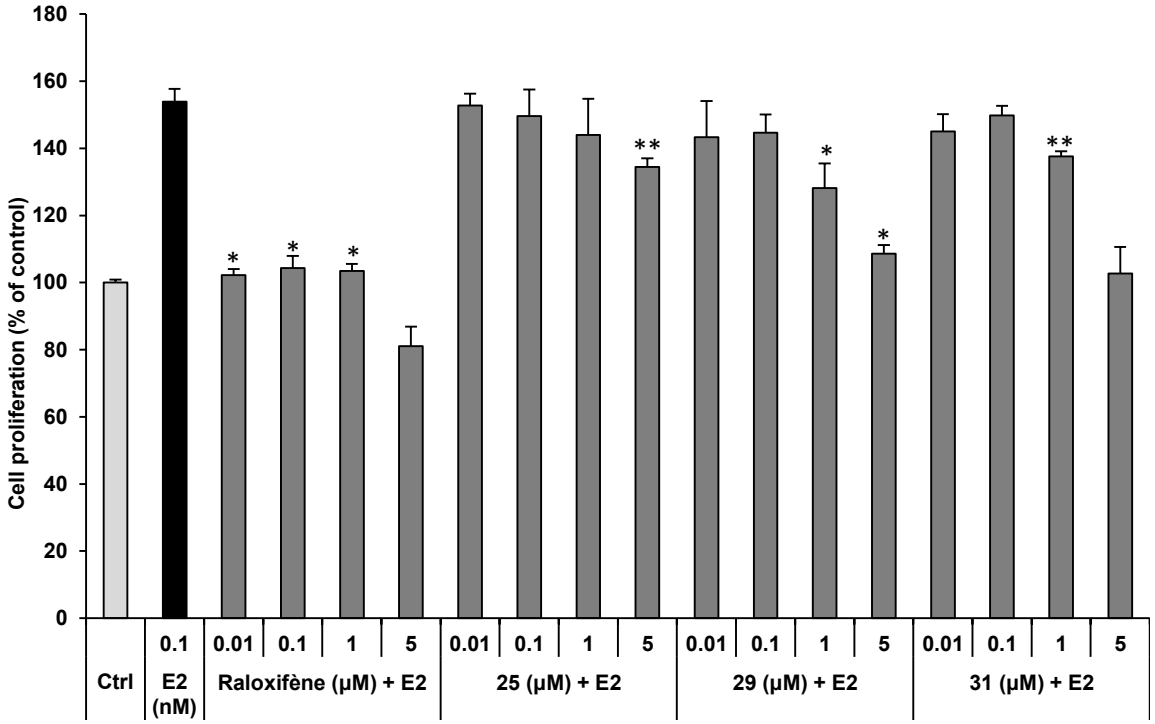


Fig. 4 Effect of phenolic (**A**) and sulfamate (**B**) compounds on T-47D cell proliferation after 7 days of treatment. The estrogenicity of phenol derivatives **25**, **29** and **31** as well as sulfamate derivatives **32**, **33** and **34** was evaluated by incubating the cells with different concentrations (0.01 μM to 5 μM) of each compound. The proliferation of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements.

We next wanted to evaluate the capacity of compounds **25**, **29**, **31-34** to block the stimulation of proliferation in T-47D cells induced by E2 (Fig. 5A and 5B). The cells were incubated with our six compounds and raloxifene at different concentrations in presence of E2 (0.1 nM). Raloxifene was able to block the E2 stimulation at concentrations of 0.01 μM to 1 μM and still showed some cytotoxicity at 5 μM. For our six compounds, the results show that they all possess some antiestrogenic activity in T-47D cells. Because some compounds showed cytotoxicity at 5 μM in the previous test, their antiestrogenicity at this concentration was not considered. The phenol derivative **29** possesses the best antiestrogenic activity since it blocked approximately 84% (at 5 μM) of the stimulation induced by E2. Even if compound **29** is not as good antiestrogenic as raloxifene, it still got the properties we were looking for in breast tissue.

A


Cell proliferation (% of control)
180
160
140
120
100
80
60
40
20
0
0.1
0.01
0.1
1
5
0.01
0.1
1
5
0.01
0.1
1
5
0.01
0.1
1
5
Ctrl
E2 (nM)
Raloxifène (µM) + E2
25 (µM) + E2
29 (µM) + E2
31 (µM) + E2

B

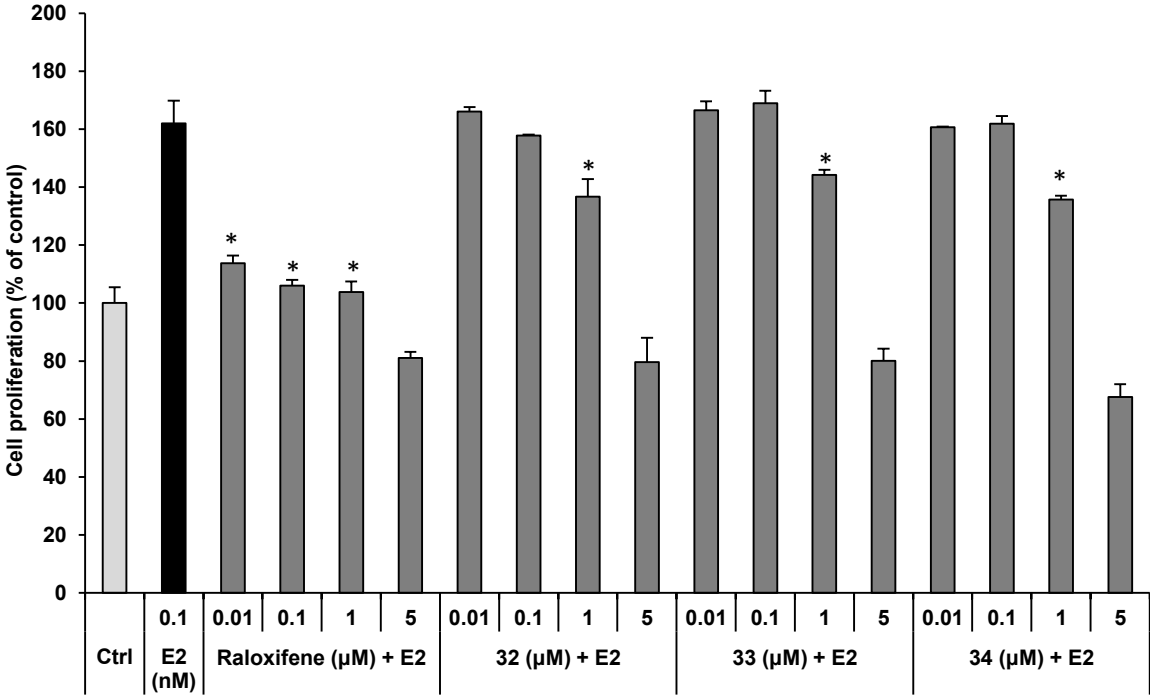

Cell proliferation (% of control)
200
180
160
140
120
100
80
60
40
20
0
0.1
0.01
0.1
1
5
0.01
0.1
1
5
0.01
0.1
1
5
0.01
0.1
1
5
Ctrl
E2 (nM)
Raloxifene (µM) + E2
32 (µM) + E2
33 (µM) + E2
34 (µM) + E2

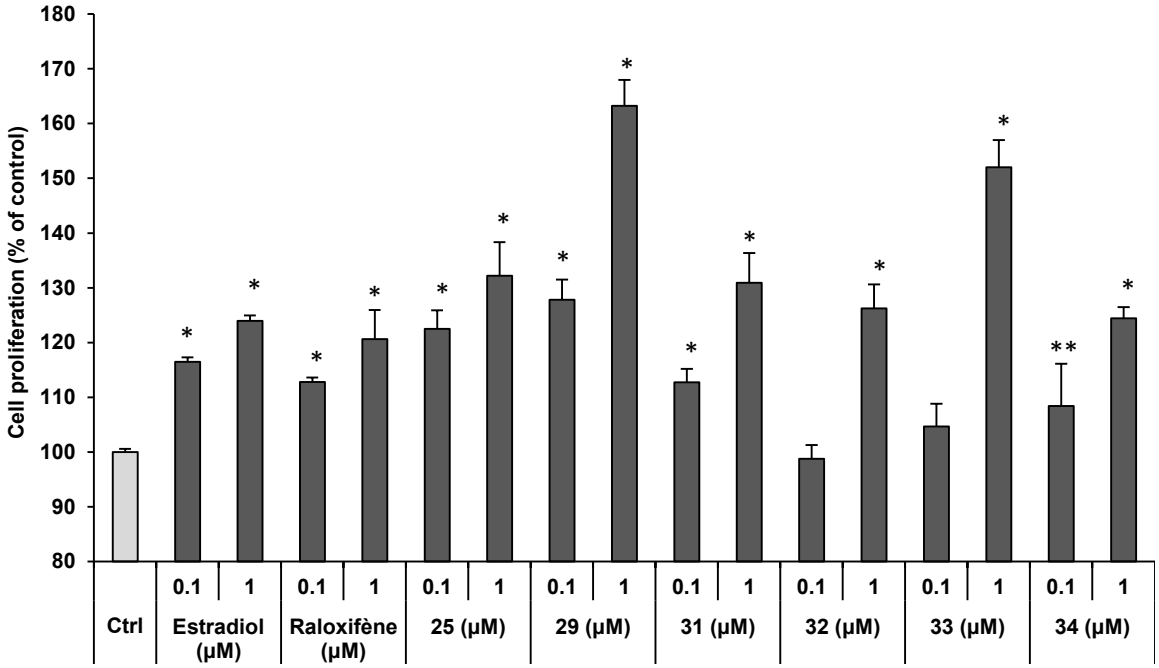
Fig. 5 Effect of phenolic (**A**) and sulfamate (**B**) compounds on T-47D cell proliferation after 7 days of treatment. The antiestrogenicity of phenol derivatives **25**, **29** and **31** as well as sulfamate derivatives **32**, **33** and **34** was evaluated by incubating the cells with different concentrations (0.01 μM to 5 μM) of each compound and 0.1 nM of E2. The proliferation of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. E2, ** $p \leq 0.05$ vs. E2.

Effect on osteoblast-like Saos-2 cells

Cell proliferation

As SERMs possess the ability to block ERα in breast tissue and to activate ERα in other tissues, such as bone tissue, we investigated the action of compounds **25**, **29**, **31-34** on the osteoblast-like Saos-2 cells. First, we wanted to evaluate their effect on cell proliferation. To do so, we incubated Saos-2 cells for 7 days with E2 or raloxifene, as a reference compound, and our six compounds of interest at concentrations of 0.1 μM and 1 μM (Fig. 6A). All compounds, including E2 and raloxifene, induced cell proliferation of Saos-2 significantly at 1 μM. However, the induced proliferation is quite low with the exception of phenolic compound **29** and its sulfamate analogue **33**, which both induced cell proliferation approximately by 150%. The slight cell proliferation observed is probably due to the osteoblast cells in themselves. One of the main roles of osteoblasts in bone tissue is to construct a calcified extracellular matrix.[15] As such, bone formation is not principally due to osteoblasts proliferation but rather osteoblasts maturation into mature osteocytes. To further investigate the high cell proliferation induced by compounds **29** and **33** and to confirm previous results, Saos-2 cells were incubated 7 days with **29** or **33** at a wider range of concentration (Fig. 6B). We observed an apparent dose-dependent response from 1 nM to 1 μM for raloxifene, **29** and **33**. At higher concentrations, all three compounds induced cytotoxicity in Saos-2 cells. Even if the mechanism by which **29** and **33** induced a higher cell proliferation remains unclear, all our compounds clearly induced a desirable effect in osteoblasts.

A


Cell proliferation (% of control)
180
170
160
150
140
130
120
110
100
90
80
0.1
1
Ctrl
Estradiol (μM)
Raloxifène (μM)
25 (μM)
29 (μM)
31 (μM)
32 (μM)
33 (μM)
34 (μM)

B

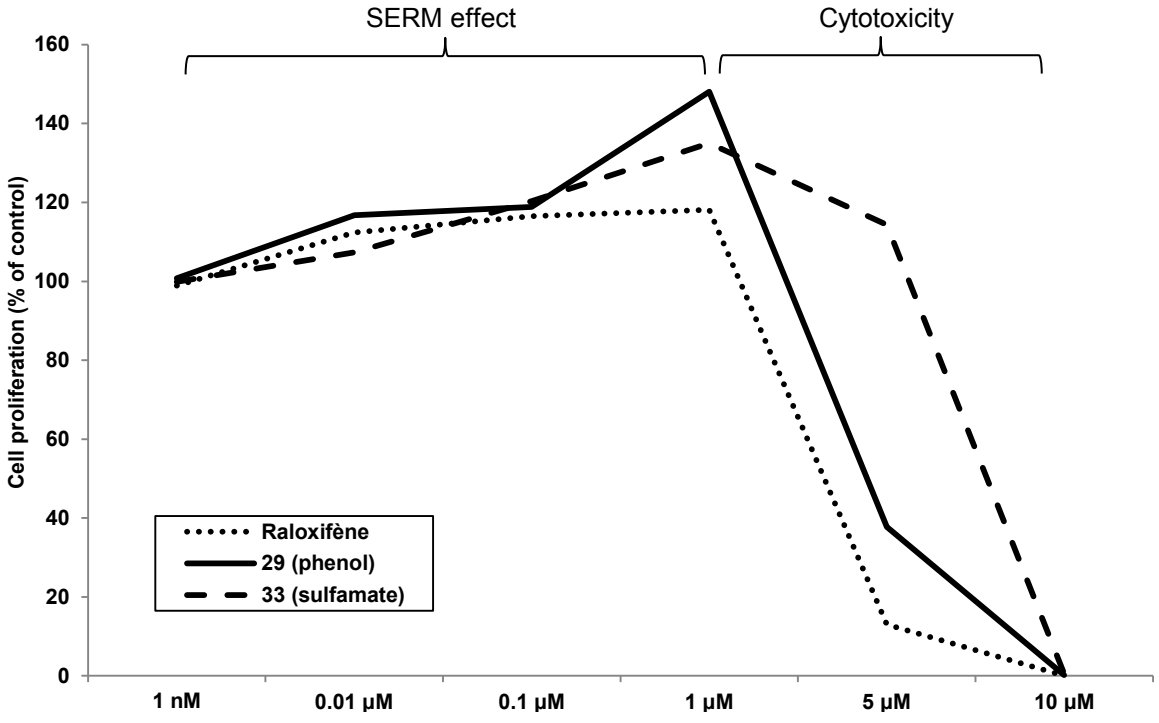

SERM effect
Cytotoxicity
Cell proliferation (% of control)
160
140
120
100
80
60
40
20
0
Raloxifène
29 (phenol)
33 (sulfamate)
1 nM
0.01 μM
0.1 μM
1 μM
5 μM
10 μM

Fig. 6 Effect of phenolic and sulfamate compounds on saos-2 cell proliferation after 7 days of treatment. **A)** Saos-2 cells were incubated with E2, raloxifene, **25**, **29**, **31**, **32**, **33** and **34** at 0.1 μM and 1 μM. **B)** The cells were incubated with raloxifene, phenolic compound **29** or sulfamate compound **33** at concentrations ranging from 1 nM to 10 μM. The proliferation of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. control, ** $p \leq 0.05$ vs. control.

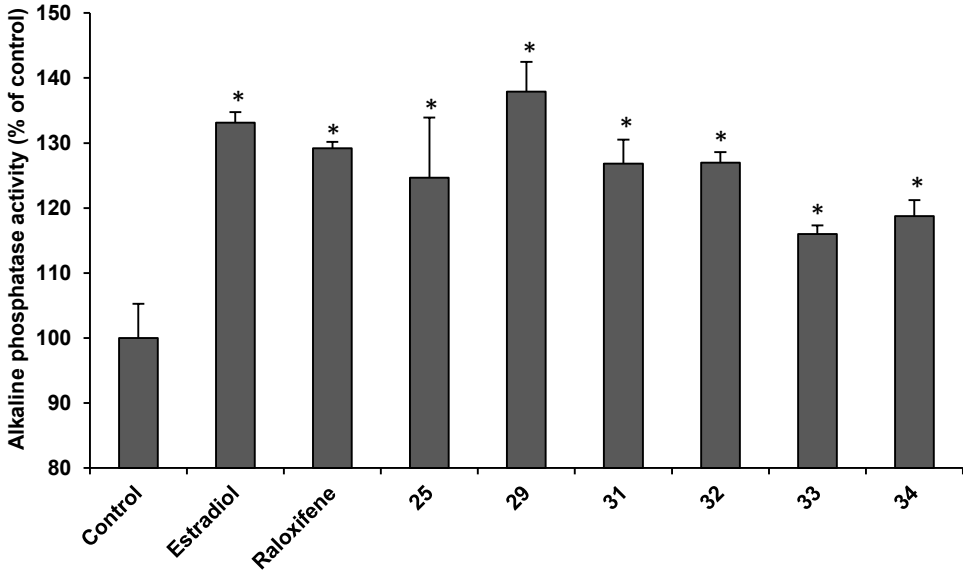
Alkaline phosphatase activity

Compounds **25**, **29**, **31**, **32**, **33** and **34** had an apparently beneficial effect on osteoblasts. Since they induced only a slight stimulation on Saos-2 cells proliferation, we decided to investigate the activity of a differentiation marker, the alkaline phosphatase (ALP). ALP is an enzyme that releases inorganic phosphate from different intracellular substrates such as pyrophosphate and pyridoxal 5'-phosphate.[25] The inorganic phosphate is used in the formation of hydroxyapatite crystals which are then inserted in the extracellular protein scaffold to form the solid part of bone tissue. In osteoblasts cells, ALP is regulated by estrogens and is a good indicator of osteoblast differentiation.[26] We tested our compounds, raloxifene or E2 on Saos-2 cells and investigated their effect on ALP activity (Fig 7A). All compounds significantly increased ALP activity in Saos-2 cells, bu it is interesting to see that **29** induced the highest ALP activity (138%) just like obtained in the cell proliferation assay.

To confirm that the observed stimulation of ALP activity induced by our compounds is mediated by estrogen receptors. We used MPP, an ERα antagonist, PHTPP, an ERβ antagonist, and ICI 164,384, a pure antiestrogen.[27-28] We incubated Saos-2 cells 3 days with E2, raloxifene or compound **29**, which compounds induced the highest ALP activity, in presence of 1 μM of either MPP, PHTPP, a combination of both ER subtype antagonists or with ICI 164,384 (Fig. 7B). First, E2, raloxifene and **29** stimulated ALP activity similarly to the previous test and the three antagonists used had no effect on ALP in Saos-2 cells. The results also demonstrate that E2, raloxifene and **29** stimulated ALP activity in Saos-2 cells via estrogen receptors as the use of either ICI-164,384 or the combination of both

MPP and PHTPP completely blocked the stimulation. When the antagonist MPP or PHTPP was used alone, we observed some differences in the mechanism by which E2, raloxifene and **29** stimulated ALP activity. Effectively, it seems that the stimulation induced by E2 is mediated more by ERα than by ERβ since MPP had a greater impact than PHTPP on the decrease of ALP activity. At the opposite, raloxifene and **29** seems to mediate their effect on ALP much more via ERβ than by ERα. This result is quite interesting since we want to obtain compounds that possess SERM properties. Phenolic derivative **29** seems to act similarly and even better than the SERM raloxifene on Saos-2 cells and as such it is a promising dual-action compound.

A



B

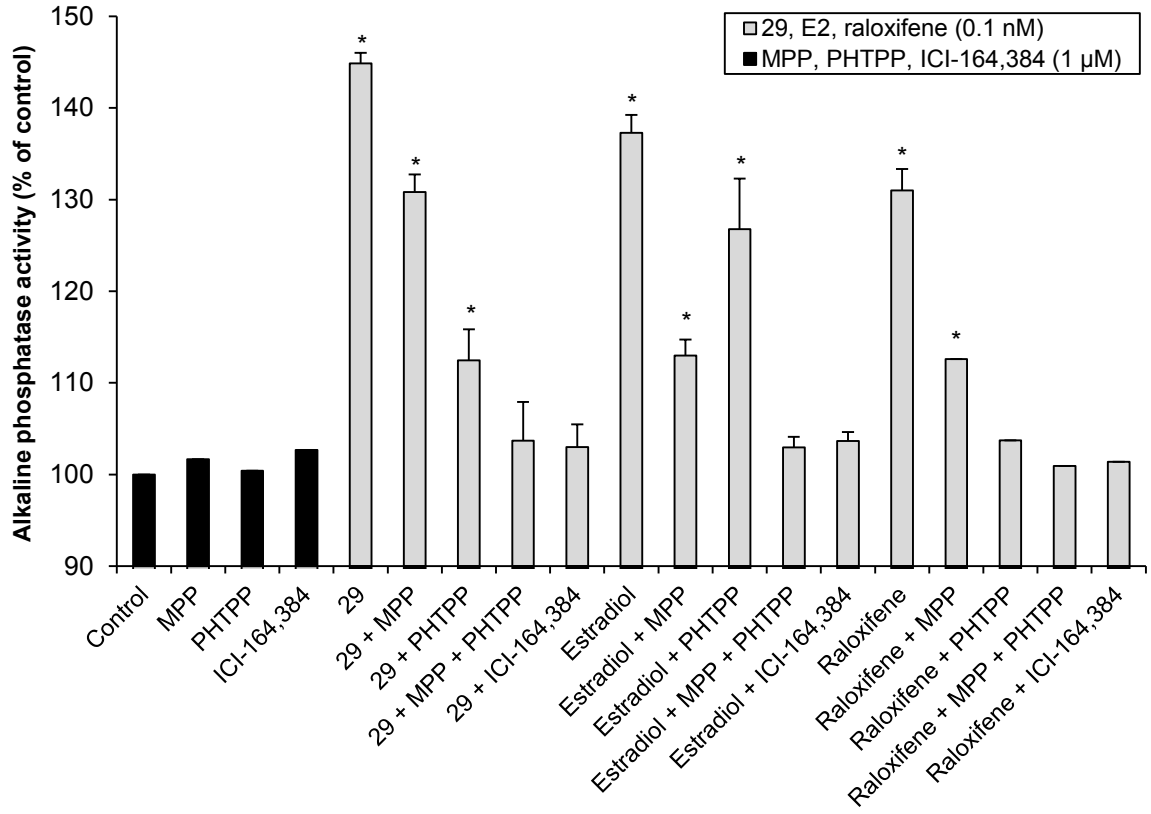


Fig. 7 Effect of phenolic and sulfamate compounds on alkaline phosphatase (ALP) activity in Saos-2 cells after 3 days of treatment. **A)** Saos-2 cells were incubated with E2, raloxifene or compounds of interest at a concentration of 0.1 nM. **B)** Saos-2 cells were treated with 0.1 nM of E2, raloxifene or **29** in presence of MPP (ERα antagonist), PHTPP (ERβ antagonist) or ICI-164,384 (pure antiestrogen) at a concentration of 1 µM. The ALP activity of control cells is set to 100%. Results are expressed as means ± SD of triplicate measurements. * $p \leq 0.01$ vs. control.

3D modelization

*Superimposition of compound **32** with reference compounds*

In the goal to visualize and better understand the structural determinants that could be important in the STS inhibition and SERM activity observed, we have proceeded to a 3D molecular modelization of one of the best compound identified in this study (compound

32). The modelization as well as related superimposition of compound **32** with a STS inhibitor (EM-1913) and a SERM (Raloxifene) was performed using Chem3D software (Fig. 8).[29] The two sulfamate compounds (**32** and EM-1913) were first submitted to an automated MM2 energy minimization followed by a step of manual iteration to find the best minimum energy conformation possible. For raloxifene, the minimum conformation was converted from PDB file of Raloxifene-ER complex to a Chem3D structure.[30]

Compound **32** was first superimposed to 3-*O*-sulfamate 17α-benzyl-2-methoxy-estra-1,3,5(10)-trien-17β-ol (EM-1913), a potent STS inhibitor that possess a benzyl substituent at the position 17α of the E2 core.[31-32] This 17α-benzyl group was found to be an important pharmacophore that interact favorably with the hydrophobic pocket of the STS. The superimposition of the minimized structures showed a good recovery of the tetrahydroisoquinoline core of **32** with steroid scaffold of EM-1913 and a good recovery of the N-substituted moiety of compound **32** with the 17α-benzyl group of EM-1913 (Fig. 8A). This observation comfort us that the N-substituted hydrophobic chain could certainly be in a good orientation to favor STS inhibition.

On the other side, the compound **32** was superimposed with raloxifene with an acceptable recovery following a pivotal of the N-dialkyl substituent moiety by a rotation of 180° (Fig. 8B). The resulting global value of steric energy for this new conformation was very close to energy conformation found for STS inhibition (18.7 Kcal / mol vs. 18.3 Kcal / mol). In this conformation targeting SERM action, we observed that the pyridine group was reasonably close (5.4 Å) to the piperidine group of raloxifene to potentially form an H-bond with key amino acid Asp351. Compound **32** could also make hydrogen bonds with the key amino acids Glu353, Arg394 and His524 present in the ligand binding domain of ERα.

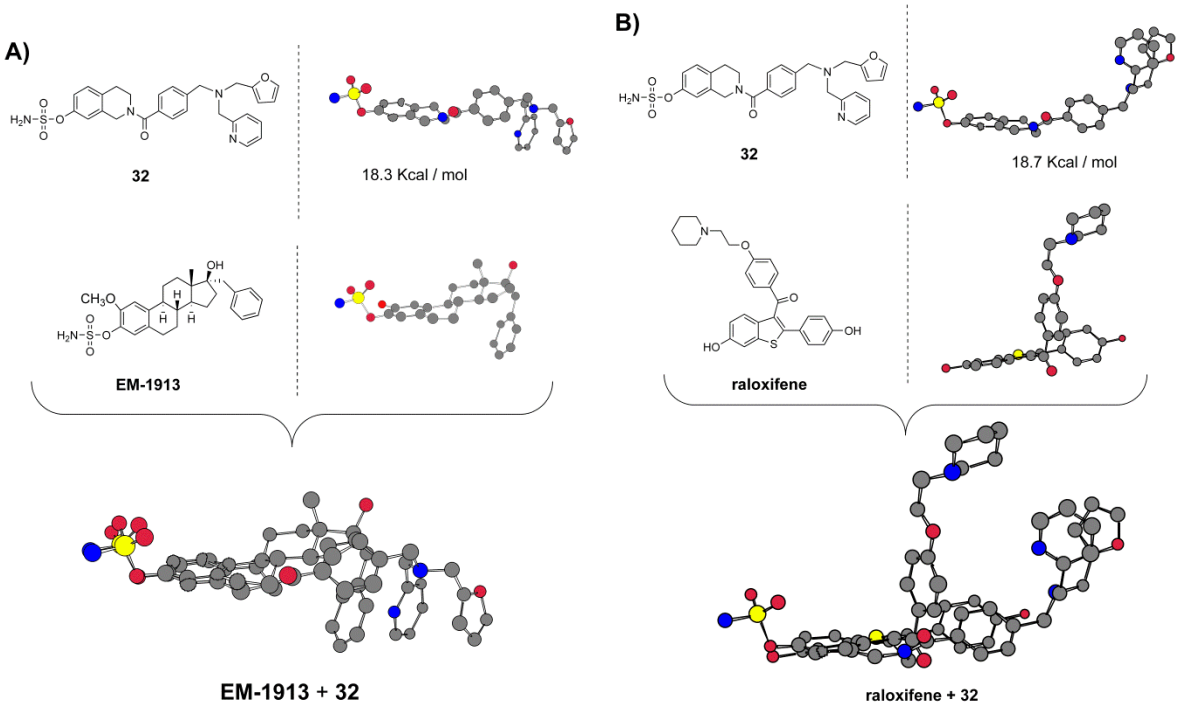


Figure 8. **A)** Minimized conformation energies of compound **32** and EM-1913 and superimposition of the two compounds; **B)** Minimized conformation energies of compound **32** and raloxifene and superimposition of the two compounds.

Conclusion

Here we described the synthesis and the *in vitro* assays of tetrahydroisoquinoline derivatives designed to inhibit STS and act as SERMs. The library of phenolic compounds and some sulfamate analogues were all synthesized by parallel solid-phase chemistry using a multidetachable sulfamate linker. The library of phenols was tested on estrogen sensitive breast cancer T-47D cells to discard compounds bearing estrogenicity or cytotoxicity at concentrations tested. Amongst the library, phenolic derivatives **25**, **29** and **31** showed no such undesirable activity and were selected for further testing. Their sulfamate analogues **32**, **33** and **34** were tested on homogenated HEK-293 cells overexpressing STS and demonstrated to be good STS inhibitors with IC_{50} values of 16.6 nM, 8.9 nM and 3.9 nM, respectively. All six compounds were tested on T-47D cells and showed no estrogenicity and even some antiestrogenic activity. Following this, we tested our compounds on

osteoblast-like Saos-2 cells as SERM compounds must be active in tissues where estrogenic activity is beneficial. All compounds stimulated Saos-2 cell proliferation with sulfamate compound **29** being the best stimulator. As the slight stimulation of cell proliferation was not enough convincing, we evaluated the effect of our compounds on the ALP activity of Saos-2 cells. The six molecules significantly increased the ALP activity with **29** being again the best inducer. To make sure that the observed stimulation was mediated by estrogen receptors, we used the ERα antagonist MPP, the ERβ antagonist PHTPP and the pure antiestrogen ICI 164,384 and investigated their effect on the stimulation induced by E2, raloxifene and compound **29**. For compound **29**, the induced stimulation is mediated by both estrogen subtypes with ERβ mediating the activation a bit more than ERα. Finally, we made a 3D modelization of one of the six compounds and superimposed its structure over a very potent STS inhibitor (EM-1913) and a SERM (raloxifene). The superimposition shows that our molecule could make interactions with the hydrophobic cavity in STS active site as well as key interactions with key amino acids important for the binding and the SERM activity on ERα. The results obtained in this study are promising and suggest that selected compounds, such as **32-34**, could be used in future *in vivo* tests as dual-action compounds for the treatment of estrogen-dependent breast cancer.

Experimental section

Chemistry

General informations

Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA) and solvents were obtained from Fisher Scientific (Montréal, QC, Canada) and VWR (Ville Mont-Royal, QC, Canada). Trityl chloride resin was supplied by EMD Biosciences (Novabiochem, La Jolla, CA, USA). Flash chromatography was performed on Silicycle 60 230-400-mesh silica gel (Québec, QC, Canada). Thin-layer chromatography (TLC) was

performed on Whatman 0.25-mm silica gel 60 F_{254} plates (Fisher Scientific, Nepean, ON, Canada) and compounds were visualized by exposure to UV light (254 nm), a solution of ammonium molybdate/sulphuric acid/ethanol (plus heating). Infrared (IR) spectra were recorded on a ABB MB3000 spectrometer (Québec, QC, Canada) and obtained from a thin film of the solubilized compound on NaCl pellets (usually in CH_2Cl_2 or acetone). Only significant bands are reported (in cm^{-1}). ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, using a Bruker AVANCE 400 spectrometer (Billerica, MA, USA). The chemical shifts (δ) are expressed in ppm and referenced to chloroform (7.26 and 77.0 ppm), acetone (2.05 and 29.8 ppm) or methanol (3.31 and 49.0 ppm) for ^1H and ^{13}C, respectively. The multiplicity signal are designed as s (singulet) d (doublet), t (triplet), q (quadruplet), p (pentaplet), m (multiplet). Low-resolution mass spectra (LRMS) were recorded on a Shimadzu Prominence apparatus (Kyoto, Japan) equipped with an atmospheric pressure chemical ionization (APCI) source on positive mode.

Synthesis of aldehydes building blocks

Aldehydes **3** and **4** were commercially available and aldehydes **1** and **2** were synthesized as follow:

To a solution of 2- or 4-hydroxy-benzaldehyde (1.64 mmol) in anhydrous acetone (25 mL) was added cesium carbonate (4.92 mmol) and the solution was stirred at room temperature for 10 min under an argon atmosphere. Chloropropylpiperidine hydrochloride (2.46 mmol) and sodium iodide (0.82 mmol) was added to the solution and the mixture was heated at reflux overnight. The resulting solution was filtered and evaporated to dryness. The crude compound was diluted with EtOAc, washed successively with a saturated carbonate solution and water, dried over $MgSO_4$, filtered and evaporated under reduce pressure. Purification by flash chromatography (hexanes/acetone/TEA: 80:19:1 to 70:29:1) yielded the desired compound **1** (360 mg, 89%), or **2** (400 mg, 99%) respectively.

2-[3-(piperidin-1-yl)propoxy]benzaldehyde (**1**)

^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.45 (q, *J* = 6.0 Hz, 2H), 1.59 (p, *J* = 5.6 Hz, 4H), 2.05 (m, 2H), 2.40 (broad s, 4H), 2.50 (t, *J* = 7.4 Hz, 2H), 4.14 (t, *J* = 6.3 Hz, 2H), 7.01 (t, J = 8.1 Hz, 2H), 7.53 (m, 1H), 7.83 (dd, *J* = 1.8, 7.8 Hz, 1H), 10.51 (s, 1H). APCI-MS for $C_{15}H_{22}O_2N$ $[M + H]^+$: 248.3 *m/z*.

4-[3-(piperidin-1-yl)propoxy]benzaldehyde (**2**)

^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.45 (m, 2H), 1.59 (q, *J* = 5.6 Hz, 4H), 2.00 (m, 2H), 2.40 (broad s, 4H), 2.47 (t, *J* = 7.4 Hz, 2H), 4.10 (t, *J* = 6.4 Hz, 2H), 7.00 (d, *J* = 8.7 Hz, 2H), 7.82 (d, *J* = 8.7 Hz, 2H), 9.88 (s, 1H). APCI-MS for $C_{15}H_{22}O_2N$ $[M + H]^+$: 248.3 *m/z*.

Synthesis of secondary amines **1a**, **2a**, **1b**, **2b**, **3c** and **4c** (General procedure)

To a solution of aldehyde **1**, **2**, **3** or **4** (1.21 mmol) in absolute ethanol (12 mL) was added the appropriate amine **a** (1-(pyridin-2-yl)methanamine), **b** (1-(furan-2-yl)methanamine) or **c** (3-(1*H* imidazol-1-yl)propan-1-amine) (1.45 mmol) and 4A° molecular sieves. The solution was stirred at room temperature for 2.5 h and filtered to remove molecular sieves. Sodium borohydride (2.90 mmol) was then added in small portion to the resulting ethanol solution at 0°C and allowed to return at room temperature and stirred overnight. Water (15 mL) was added and the solution stirred for 15 min before to be concentrated under reduced pressure. The aqueous layer was extracted with DCM (5 x 5 mL), and the combined extracts was washed with brine, dried with $MgSO_4$, filtered and evaporated to dryness. Purification by flash chromatography (DCM/MeOH:TEA (98:1:1 to 90:9:1) yielded **1a** (370 mg, 90%), **2a** (318 mg, 78%), **1b** (320 mg, 70%), **2b** (347 mg, 76%), **3c** (400 mg, 99%) or **4c** (400 mg, 97%) according to the aldehyde and primary amine used.

1-{2-[3-(piperidin-1-yl)propoxy]phenyl}-*N*-(pyridin-2-ylmethyl)methanamine (**1a**)

^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.44 (m, 2H), 1.59 (p, *J* = 5.6 Hz, 4H), 1.89 (broad s, NH), 2.01 (m, 2H), 2.38 (broad s, 4H), 2.48 (t, *J* = 7.5 Hz, 2H), 3.87 (s, 2H), 3.92 (s, 2H), 4.03 (t, *J* = 6.2 Hz, 2H), 6.85 (d, *J* = 8.0 Hz, 2H), 6.90 (t, *J* = 7.4 Hz, 1H), 7.14 (m, 1H), 7.21 (m,

1H), 7.27 (m, 1H), 7.37 (d, *J* = 6.8 Hz, 1H), 7.63 (td, *J* = 1.8, 7.7 Hz, 1H), 8.54 (ddd, *J* = 0.9, 1.8, 4.9 Hz, 1H). APCI-MS for $C_{21}H_{30}ON_3$ $[M + H]^+$: 340.3 *m/z*.

1-{4-[3-(piperidin-1-yl)propoxy]phenyl}-*N*-(pyridin-2-ylmethyl)methanamine (**2a**)
^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.45 (m, 2H), 1.60 (p, *J* = 5.6 Hz, 4H), 1.98 (m, 2H), 2.42 (broad s, 4H), 2.49 (t, *J* = 7.5 Hz, 2H), 3.78 (s, 2H), 3.91 (s, 2H), 4.00 (t, *J* = 6.4 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 7.16 (ddd, *J* = 1.2, 4.9, 7.5 Hz, 1H), 7.25 (d, J = 8.5 Hz, 2H), 7.31 (d, *J* = 7.8Hz, 1H), 7.64 (td, *J* = 1.8, 7.7 Hz, 1H), 8.56 (ddd, *J* = 1.0, 1.9, 4.9 Hz, 1H). APCI-MS for $C_{21}H_{30}ON_3$ $[M + H]^+$: 340.3 *m/z*.

1-(furan-2-yl)-*N*-{2-[3-(piperidin-1-yl)propoxy]benzyl}methanamine (**1b**)
^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.45 (m, 2H), 1.59 (p, *J* = 5.6 Hz, 4H), 1.99 (m, 2H), 2.40 (broad s, 4H), 2.48 (t, J = 7.5 Hz, 2H), 3.76 (s, 2H), 3.81 (s, 2H), 4.03 (t, *J* = 6.2 Hz, 2H), 6.18 (dd, *J* = 1.0, 3.2 Hz, 1H), 6.31 (dd, *J* = 1.8, 3.1 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, 1H), 6.90 (t, *J* = 7.4 Hz, 1H), 7.22 (m, 2H), 7.36 (dd, *J* = 0.6, 1.6 Hz, 1H). APCI-MS for $C_{20}H_{29}O_2N_2$ $[M + H]^+$: 329.3 *m/z*.

1-(furan-2-yl)-*N*-{4-[3-(piperidin-1-yl)propoxy]benzyl}methanamine (**2b**)
^{1}H NMR (400 MHz, $CDCl_3$) δ : 1.44 (m, 2H), 1.59 (p, *J* = 5.6 Hz, 4H), 1.97 (m, 2H), 2.40 (broad s, 4H), 2.47 (t, *J* = 7.5 Hz, 2H), 3.72 (s, 2H), 3.77 (s, 2H), 4.00 (t, *J* = 6.4 Hz, 2H), 6.18 (dd, *J* = 0.91, 3.2 Hz, 1H), 6.32 (dd, *J* = 1.9, 3.2 Hz, 1H), 6.85 (d, *J* = 8.6 Hz, 2H), 7.22 (d, *J* = 8.6 Hz, 2H), 7.37 (dd, *J* = 0.8, 1.9 Hz, 1H). APCI-MS for $C_{20}H_{29}O_2N_2$ $[M + H]^+$: 329.3 *m/z*.

3-(1*H*-imidazol-1-yl)-*N*-(pyridin-2-ylmethyl)propan-1-amine (**3c**)
^{1}H NMR (400 MHz, $CDCl_3$) δ 1.97 (p, *J* = 6.8 Hz, 2H), 2.64 (t, *J* = 6.7 Hz, 2H), 3.88 (s, 2H), 4.06 (t, *J* = 6.9 Hz, 2H), 6.91 (s, 1H), 7.04 (s, 1H), 7.18 (ddd, *J* = 1.2, 4.9, 7.6 Hz, 1H), 7.26 (d, *J* = 7.8 Hz, 1H), 7.47 (s, 1H), 7.65 (td, *J* = 1.8, 7.7 Hz, 1H), 8.56 (m, 1H). APCI-MS for $C_{12}H_{17}N_4$ $[M + H]^+$: 217.3 *m/z*.

N-(furan-2-ylmethyl)-3-(1H-imidazol-1-yl)propan-1-amine (**4c**)

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.92 (p, *J* = 6.9 Hz, 2H), 2.59 (t, *J* = 6.7 Hz, 2H), 3.75 (s, 2H), 4.04 (t, *J* = 6.9 Hz, 2H), 6.15 (dd, *J* = 0.6, 3.2 Hz, 1H), 6.32 (dd, *J* = 1.9, 3.2 Hz, 1H), 6.89 (t, *J* = 1.3 Hz, 1H), 7.05 (s, 1H), 7.37 (dd, *J* = 0.8, 1.8 Hz, 1H), 7.45 (s, 1H). APCI-MS for $C_{11}H_{16}ON_3$ $[M + H]^+$: 206.3 *m/z*.

Synthesis of phenol library

The compounds **19-31** were synthesized in good quantity (28 to 39 mg) following the strategy we previously developed and published for similar phenolic derivatives.[15] All these compounds were purified by reverse phase on LC-MS preparative system (Model Prominence, Shimadzu, Kyoto, Japan) equipped with a photodiode detector (SPD M 20A) and mass analyser (MS 2020) with atmospheric-pressure chemical positive ionisation (APCI) systems with a synergi C18 column (250 x 21.2 mm x 4 µM). These compounds were purified in 50 min using a solvent gradient already established (70-100% MeOH, 30-0% water) at flow rate of 10 mL/min at room temperature.

(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)(4-{[{2-[3-(piperidin-1-yl)propoxy]benzyl}(pyridin-2-ylmethyl)amino]methyl}phenyl)methanone (**19**) ^{1}H NMR (400 MHz, CD_3OD) δ : 1.48 (broad s, 2H), 1.60 (q, *J* = 4.7, 5.3 Hz, 4H), 1.97 (m, 2H), 2.47 (broad s, 4H), 2.54 (t, *J* = 7.6 Hz, 2H), 2.76 (broad s, 1H), 2.85 (broad s, 1H), 3.58 (broad s, 1H), 3.70 (s, 2H), 3.71 (s, 2H), 3.76 (s, 2H), 3.91 (broad s, 1H), 4.02 (t, *J* = 6.1 Hz, 2H), 4.50 (s, 1H), 4.74 (s, 1H), 6.63 (d, *J* = 7.5 Hz, 2H), 6.94 (m, 2H), 6.97 (d, *J* = 7.8 Hz, 1H), 7.18 (td, *J* = 1.5, 7.7 Hz, 1H), 7.26 (m, 1H), 7.38 (d, *J* = 6.4 Hz, 2H), 7.46 (d, *J* = 6.3 Hz, 1H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.70 (dt, *J* = 1.2, 8.0 Hz, 1H), 7.79 (td, *J* = 1.80, 7.7 Hz, 1H), 8.40 (d, *J* = 4.4 Hz, 1H). APCI-MS for $C_{38}H_{45}O_3N_4$ $[M + H]^+$: 605.4 *m/z*. HPLC purity of 91.0 %.

(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)(4-{[{4-[3-(piperidin-1-yl)propoxy]benzyl}(pyridin-2-ylmethyl)amino]methyl}phenyl)methanone (**20**)

^{1}H NMR (400 MHz, CD_3OD) δ : 1.53 (d, *J* = 6.6 Hz, 2H), 1.67 (p, *J* = 5.8 Hz, 4H), 2.02 (p, *J* = 6.1 Hz, 2H), 2.68 (m, 6H), 2.76 (broad s, 2H), 2.85 (broad s, 1H), 3.56 (s, 2H), 3.59 (m, 1H), 3.63 (s, 2H), 3.71 (s, 2H), 3.91 (s, 1H), 4.05 (t, *J* = 6.0 Hz, 2H), 4.50 (s, 1H), 4.75 (s, 1H), 6.63 (d, *J* = 6.3 Hz, 2H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.97 (d, *J* = 7.2 Hz, 1H), 7.29 (m, 3H), 7.40 (d, *J* = 7.3 Hz, 2H), 7.50 (d, *J* = 7.8 Hz, 2H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.82 (td, *J* = 1.8, 7.7 Hz, 1H), 8.41 (d, *J* = 4.4 Hz, 1H). APCI-MS for $C_{38}H_{45}O_3N_4$ $[M + H]^+$: 605.4 *m/z*. HPLC purity of 90.0 %.

4-{[(furan-2-ylmethyl){2-[3-(piperidin-1-yl)propoxy]benzyl}amino]methyl}phenyl)(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**21**)

^{1}H NMR (400 MHz, CD_3OD) δ : 1.67 (broad s, 2H), 1.85 (broad s, 4H), 2.11 (dq, *J* = 5.88, 11.4 Hz, 2H), 2.77 (broad s, 1H), 2.86 (broad s, 1H), 3.14 (m, 2H), 3.61 (broad s, 1H), 3.67 (s, 2H), 3.68 (s, 2H), 3.70 (s, 2H), 3.93 (broad s, 1H), 3.93 (t, *J* = 6.2 Hz, 1H), 4.05 (t, *J* = 5.8 Hz, 2H), 4.51 (s, 1H), 4.77 (s, 1H), 6.28 (d, *J* = 3.0 Hz, 2H), 6.38 (d, *J* = 3.1 Hz, 2H), 6.63 (d, *J* = 6.6 Hz, 2H), 6.95 (m, 4H), 7.23 (td, *J* = 1.6, 7.7 Hz, 1H), 7.40 (d, *J* = 7.5 Hz, 2H), 7.49 (m, 4H); APCI-MS for $C_{37}H_{44}O_4N_3$ $[M + H]^+$: 594.4 *m/z*. HPLC purity of 89.9 %.

(4-{[(furan-2-ylmethyl){4-[3-(piperidin-1-yl)propoxy]benzyl}amino]methyl}phenyl)(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**22**)

^{1}H NMR (400 MHz, CD_3OD) δ : 1.51 (d, *J* = 5.9 Hz, 2H), 1.64 (p, *J* = 5.6 Hz, 4H), 2.00 (dq, *J* = 6.05, 11.9 Hz, 2H), 2.61 (m, 6H), 2.77 (broad s, 2H), 2.86 (broad s, 1H), 3.54 (s, 2H), 3.60 (s, 2H), 3.62 (m, 3H), 3.92 (s, 1H), 4.01 (t, *J* = 6.1 Hz, 2H), 4.52 (s, 1H), 4.76 (s, 1H), 6.24 (dd, *J* = 0.9, 3.3 Hz, 1H), 6.36 (dd, *J* = 1.9, 3.2 Hz, 1H), 6.63 (d, *J* = 6.2 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 6.98 (d, *J* = 8.0 Hz, 1H), 7.28 (d, *J* = 8.6 Hz, 2H), 7.40 (d, *J* = 6.7 Hz, 2H), 7.49 (m, 3H). APCI-MS for $C_{37}H_{44}O_4N_3$ $[M + H]^+$: 594.5 *m/z*. HPLC purity of 92.0 %.

(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)[4-({[3-(1*H*-imidazol-1-yl)propyl](pyridin-2-ylmethyl)amino}methyl)phenyl]methanone (**23**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.01 (p, *J* = 6.9 Hz, 2H), 2.51 (t, *J* = 6.8 Hz, 2H), 2.77 (broad s, 1H), 2.86 (broad s, 1H), 2.86 (t, *J* = 6.1 Hz, 1H), 3.60 (br t, *J* = 5.0 Hz, 1H), 3.68 (s, 2H), 3.74 (s, 2H), 3.92 (br t, *J* = 5.0 Hz, 1H), 4.02 (t, *J* = 6.9 Hz, 2H), 4.50 (s, 1H), 4.76 (s, 2H), 6.63 (d, *J* = 6.4 Hz, 2H), 6.88 (s, 1H), 6.98 (m, 2H), 7.30 (ddd, *J* = 1.3, 5.0, 7.5 Hz, 1H), 7.40 (d, J = 6.0 Hz, 2H), 7.49 (m, 2H), 7.59 (dt, *J* = 1.1, 7.9 Hz, 1H), 7.82 (td, *J* = 1.8, 7.7 Hz, 1H), 8.44 (ddd, *J* = 0.9, 1.8, 5.1 Hz, 1H). APCI-MS for $C_{29}H_{32}O_2N_5$ $[M + H]^+$: 482.3 *m/z*. HPLC purity of 99.0 %.

[4-({(furan-2-ylmethyl)[3-(1*H*-imidazol-1-yl)propyl]amino}methyl)phenyl](7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**24**)

^{1}H NMR (400 MHz, CD_3OD) δ : 1.99 (p, *J* = 6.8 Hz, 2H), 2.47 (t, *J* = 6.7 Hz, 2H), 2.77 (broad s, 1H), 2.86 (broad s, 1H), 3.65 (m, 5H), 3.93 (broad s, 1H), 4.07 (t, *J* = 6.8 Hz, 2H), 4.52 (s, 1H), 4.77 (s, 1H), 6.23 (d, *J* = 3.2 Hz, 1H), 6.35 (dd, *J* = 1.9, 3.2 Hz, 1H), 6.63 (d, J = 5.2 Hz, 2H), 6.93 (s, 1H), 7.00 (m, 1H), 7.03 (s, 1H), 7.46 (m, 5H), 7.59 (s, 1H). APCI-MS for $C_{28}H_{31}O_3N_4$ $[M + H]^+$: 471.3 *m/z*. HPLC purity of 90.0 %.

(4-{[(furan-2-ylmethyl)(pyridin-2-ylmethyl)amino]methyl}phenyl)(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**25**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.77 (broad s, 1H), 2.86 (broad s, 1H), 3.60 (s, 1H), 3.70 (s, 2H), 3.71 (s, 2H), 3.80 (s, 2H), 3.91 (s, 1H), 4.51 (s, 1H), 4.75 (s, 1H), 6.26 (d, *J* = 3.1 Hz, 1H), 6.35 (dd, *J* = 1.8, 3.2 Hz, 1H), 6.63 (d, *J* = 4.6 Hz, 2H), 6.98 (d, *J* = 7.1 Hz, 1H), 7.28 (m, 1H), 7.40 (d, *J* = 6.1 Hz, 2H), 7.47 (s, 1H), 7.51 (d, *J* = 7.9 Hz, 2H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.81 (td, *J* = 1.8, 7.7 Hz, 1H), 8.42 (d, *J* = 4.6 Hz, 1H). APCI-MS for $C_{28}H_{28}O_3N_3$ $[M + H]^+$: 454.2 *m/z*. HPLC purity of 94.7 %.

(4-{[(4-bromobenzyl)(furan-2-ylmethyl)amino]methyl}phenyl)(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**26**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.77 (t, *J* = 5.6 Hz, 1H), 2.86 (s, 1H), 3.55-3.95 (m, 8H), 4.51 (s, 1H), 4.76 (s, 1H), 6.33 (s, 1H), 6.40 (s, 1H), 6.63 (d, *J* = 4.4 Hz, 2H), 6.98 (d, *J* = 7.9 Hz, 1H), 7.33 (d, *J* = 8.2 Hz, 2H), 7.40 – 7.55 (m, 7H). APCI-MS for $C_{29}H_{28}BrO_3N_2$ $[M + H]^+$: 531.5 and 533.5 *m/z.* HPLC purity of 97.1 %.

[4-({(furan-2-ylmethyl)[2-(morpholin-4-yl)ethyl]amino}methyl)phenyl](7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**27**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.44 (s, 4H), 2.53 (dd, *J* = 5.7, 8.2 Hz, 2H), 2.66 (dd, *J* = 5.8, 8.4 Hz, 2H), 2.77 (broad s, 1H), 2.86 (s, 1H), 3.65 (m, 4H), 3.70 (s, 4H), 3.92 (broad s, 1H), 4.53 (s, 1H), 4.65 4.77 (s, 1H), 6.27 (d, *J* = 3.2 Hz, 1H), 6.36 (d, *J* = 2.9 Hz, 1H), 6.63 (d, *J* = 6.1 Hz, 2H), 6.98 (d, *J* = 7.1 Hz, 2H), 7.39 – 7.54 (m, 6H). APCI-MS for $C_{28}H_{34}O_4N_3$ $[M + H]^+$: 476.4 *m/z*. HPLC purity of 91.0%.

(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)[4-({[(5-methylthiophen-2-yl)methyl](pyridin-2-ylmethyl)amino}methyl)phenyl]methanone (**28**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.44 (d, *J* = 1.2 Hz, 3H), 2.76 (t, *J* = 5.7 Hz, 1H), 2.85 (broad s, 1H), 3.60 (broad s, 1H), 3.69 (s, 2H), 3.75 (2s, 4H), 3.90 (broad s, 1H), 4.50 (s, 1H), 4.75 (s, 1H), 6.62 (m, 3H), 6.74 (d, *J* = 3.3 Hz, 1H), 6.97 (d, *J* = 7.5 Hz, 1H), 7.28 (ddd, *J* = 1.3, 5.0, 7.4 Hz, 1H), 7.40 (d, *J* = 6.5 Hz, 2H), 7.54 (d, *J* = 7.8 Hz, 2H), 7.73 (dt, *J* = 1.1, 7.9 Hz, 1H), 7.83 (td, *J* = 1.8, 7.7 Hz, 1H), 8.42 (ddd, *J* = 0.89, 1.7, 5.1 Hz, 1H). APCI-MS for $C_{29}H_{30}O_2N_3S$ $[M + H]^+$: 484.2 *m/z*. HPLC purity of 99.4 %.

[4-({(furan-2-ylmethyl)[(5-methylthiophen-2-yl)methyl]amino}methyl)phenyl](7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**29**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.44 (s, 3H), 2.78 (broad s, 1H), 2.86 (broad s, 1H), 3.65 (m, 5H), 3.74 (s, 2H), 3.92 (s, 1H), 4.53 (s, 1H), 4.76 (s, 1H), 6.26 (d, *J* = 3.1 Hz, 1H), 6.37 (dd, *J* = 1.9, 3.2 Hz, 1H), 6.60 (m, 3H), 6.73 (d, *J* = 3.4 Hz, 1H), 6.98 (d, *J* = 7.4 Hz, 1H), 7.44 (m, 3H), 7.53 (d, *J* = 7.7 Hz, 2H). APCI-MS for $C_{28}H_{29}O_3N_2S$ $[M + H]^+$: 473.3 *m/z*. HPLC purity of 98.1 %.

(4-{[(furan-2-ylmethyl)(4-hydroxybenzyl)amino]methyl}phenyl)(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)methanone (**30**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.77 (t, *J* = 5.7 Hz, 2H), 2.86 (broad s, 1H), 3.49 (s, 2H), 3.62 (3s, 6H), 3.92 (broad s, 1H), 4.52 (s, 1H), 4.76 (s, 1H), 6.23 (d, *J* = 3.0 Hz, 1H), 6.36 (dd, *J* = 1.9, 3.1 Hz, 1H), 6.63 (m, 2H), 6.74 (d, *J* = 8.0 Hz, 2H), 6.97 (d, *J* = 7.1 Hz, 1H), 7.19 (d, *J* = 8.3 Hz, 2H), 7.40 (d, *J* = 6.9 Hz, 2H), 7.49 (t, *J* = 8.1 Hz, 3H). APCI-MS for $C_{29}H_{29}O_4N_2$ $[M + H]^+$: 469.2 *m/z*. HPLC purity of 89.3 %.

(7-hydroxy-3,4-dihydroisoquinolin-2(1*H*)-yl)(4-{[(4-methoxybenzyl)(pyridin-2-ylmethyl)amino]methyl}phenyl)methanone (**31**)

^{1}H NMR (400 MHz, CD_3OD) δ : 2.76 (broad s, 1H), 2.85 (broad s, 1H), 3.56 (s, 2H), 3.59 (broad s, 1H), 3.64 (s, 2H), 3.71 (s, 2H), 3.76 (s, 3H), 3.91 (broad s, 1H), 4.50 (s, 1H), 4.75 (s, 1H), 6.62 (broad s, 2H), 6.87 (d, *J* = 8.6 Hz, 2H), 6.98 (d, *J* = 8.1 Hz, 1H), 7.29 (m, 3H), 7.40 (m, 2H), 7.51 (d, *J* = 7.8 Hz, 2H), 7.69 (d, *J* = 7.9 Hz, 1H), 7.82 (td, *J* = 1.78, 7.7 Hz, 1H), 8.40 (d, *J* = 4.4 Hz, 1H). APCI-MS for $C_{31}H_{32}O_3N_3$ $[M + H]^+$: 494.3 *m/z*. HPLC purity of 98.3 %.

*Synthesis of sulfamate **32-34***

General procedure: The compounds **32-34** were synthesized following the strategy we previously developed and published for similar sulfamate derivatives.[15] The compounds were purified by flash chromatography.

2-[(4-{[(furan-2-ylmethyl)(pyridin-2-ylmethyl)amino]methyl}phenyl)carbonyl]-1,2,3,4-tetrahydroisoquinolin-7-yl sulfamate (**32**)

^{1}H NMR (400 MHz, $CDCl_3$) δ : 2.88 (m, 2H), 3.70 (m, 6H), 3.82 (s, 2H), 3.98 (broad s, 1H), 4.60 (broad s, 1H), 4.88 (broad s, 1H), 5.12 (broad s, 2H), 6.22 (dd, *J* = 0.9, 3.2 Hz, 1H), 6.33 (dd, *J* = 1.8, 3.2 Hz, 1H), 7.18 (m, 4H), 7.44 (m, 5H), 7.58 (d, *J* = 7.8 Hz, 1H),

7.69 (td, *J* = 1.8, 7.7 Hz, 1H), 8.53 (dd, *J* = 1.5, 4.7 Hz, 1H). APCI-MS for $C_{28}H_{29}O_5N_4S$ $[M + H]^+$: 533.3 *m/z*. HPLC purity of 79.9%.

2-{[4-({(furan-2-ylmethyl)[(5-methylthiophen-2-yl)methyl]amino}
methyl)phenyl]carbonyl}-1,2,3,4-tetrahydroisoquinolin-7-yl sulfamate (**33**)
^1H NMR (400 MHz, $CDCl_3$) δ : 2.47 (s, 3H), 2.84 (m, 2H), 3.66 (s, 2H), 3.69 (s, 2H), 3.77 (s, 2H), 3.98 (broad s, 1H), 4.64 (broad s, 1H), 4.89 (broad s, 2H), 5.00 (m, 2H), 6.22 (dd, *J* = 0.83, 3.1 Hz, 1H), 6.35 (dd, *J* = 1.8, 3.2 Hz, 1H), 6.59 (dd, *J* = 1.4, 3.3 Hz, 1H), 6.72 (d, *J* = 3.4 Hz, 1H), 7.15 (m, 3H), 7.42 (d, J = 8.0 Hz, 3H), 7.50 (d, *J* = 8.0 Hz, 2H). APCI-MS for $C_{28}H_{30}O_5N_3S_2$ $[M + H]^+$: 552.3 *m/z*. HPLC purity of 90.9%.

2-[(4-{[(4-methoxybenzyl)(pyridin-2-ylmethyl)amino]methyl}phenyl)carbonyl]-1,2,3,4-tetrahydroisoquinolin-7-yl sulfamate (**34**)
^1H NMR (400 MHz, $CDCl_3$) δ : 2.89 (m, 2H), 3.57 (s, 2H), 3.64 (m, 3H), 3.74 (s, 2H), 3.80 (s, 3H), 3.91 (broad s, 1H), 4.59 (s, 1H), 4.8-5.2 (m, 3H), 6.87 (d, *J* = 8.6 Hz, 2H), 7.17 (m, 4H), 7.31 (d, *J* = 8.6 Hz, 2H), 7.39 (m, 2H), 7.46 (m, 2H), 7.58 (d, *J* = 7.9 Hz, 1H), 7.68 (td, *J* = 1.8, 7.7 Hz, 1H), 8.51 (dt, *J* = 1.3, 5.0 Hz, 1H). APCI-MS for $C_{31}H_{33}O_5N_4S$ $[M + H]^+$: 573.3 *m/z*. HPLC purity of 83.0%.

Biological assays

Chemicals and Reagents

17β-estradiol, Tris, EDTA, glycerol, insulin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Canada Ltd (Oakville, ON). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt (MTS) was purchased from Promega (Madison, WI). Radiolabeled [6,7-^3H] estrone sulfate (54.3 Ci/mmol) was purchased from Perkin Elmer (Woodbridge, ON, Canada). Raloxifene hydrochloride was bought from Cayman Chemical (Ann Harbor, MI). Biodegradable Counting Scintillant was purchased from Amersham Biosciences. The two selective

estrogen receptor antagonists, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H pyrazole dihydrochloride (MPP) and 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), were purchased from Tocris Biosciences (Minneapolis, MN). Penicillin/streptomycin mix, L-glutamine, non-essential amino acids, sodium pyruvate, normal and charcoal-stripped Fetal Bovine Serum (FBS) and geneticin (G418 sulfate) were purchased from Wisent, Inc. (St-Bruno, QC, Canada). All cell culture medias were purchased from Life Technologies (Grand Island, NY) except for phenol-red free McCoy's 5A medium that was purchased from PromoCell (Heidelberg, Germany). STS inhibitor EM-1913 and pure antiestrogen ICI-164,384 (EM-100) were synthesized in our Laboratory of Medicinal Chemistry using published procedure.[32-33]

For the purpose of *in vitro* assays, all chemicals tested (inhibitors and reference compounds) were first dissolved in DMSO and subsequent dilutions were done in the proper buffer or cell culture media. The final concentration of DMSO in the culture medium was 0.1% or less.

Cell Culture

The ER^+ breast cancer cell line T-47D and the osteoblast-like Saos-2 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). The HEK-293 cell line overexpressing STS was obtained from Dr. Van Luu-The (CHUQ-CHUL Research Center).[31] All cell lines were maintained in culture flasks (175 cm^2 growth area, BD Falcon) at 37°C in a 5% CO_2 humidified atmosphere. The T-47D cells were grown in phenol red free RPMI 1640 medium supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL), L-glutamine (2 mM) and 17β-estradiol (1 nM). The Saos-2 cells were grown in phenol red free McCoy's 5A medium supplemented with 10% FBS, penicillin (100 IU/mL) and streptomycin (100 µg/mL). The HEK-293 cells transfected with STS were maintained in Minimum Essential Medium supplemented with 10% FBS,

penicillin (100 IU/mL), streptomycin (100 µg/mL), L-glutamine (2 mM), non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and geneticin (G418 sulfate) (700 µg/mL).

Steroid sulfatase inhibition assay

An enzymatic assay previously described was used for the inhibition of the transformation of estrone sulfate (E1S) to estrone (E1) by STS.[34] Briefly, the transfected HEK-293 cells were homogenized by repeated (5 times) cycles of freezing (-80°C) and thawing on ice (4°C). The homogenates were then incubated for 2 h at 37°C (shaking water bath) with or without inhibitors (0.01 µM – 1 µM) in presence of [^{3}H]-E1S (9 nM), adjusted to 1 µM with E1S, in a Tris-acetate buffer (pH 7.4) containing 5 mM EDTA and 10% glycerol. After the incubation, 1 mL of xylene was added to each tube and the solutions were then centrifuged at 3000 rpm for 20 min to separate the organic ([^{3}H]-E1) and aqueous ([^{3}H]-E1S) phases. Once 500 µL of each phase was added to 10 mL of Biodegradable Counting Scintillant, the radioactivity of samples was recorded using a Wallac 1411 Liquid Scintillation Counter. The percentage of inhibition was determined by comparison with the control (buffer + homogenate + [^{3}H]-E1S) which was set to 0% of inhibition. IC_{50} value was obtained using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Cell proliferation

CellTitter 96® Aqueous One Solution Cell Proliferation Assay was used as an indirect colorimetric measurement of cell proliferation according to the manufacturer's instructions. Briefly, after the treatments, 20 µL of MTS solution was added to each well (100 µL) of the plates and incubated at 37°C for 2 h (Saos-2) or 4 h (T-47D). The absorbance at 490 nm was then measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of cell proliferation.

T-47D cells

T-47D cells were suspended in RPMI supplemented with insulin (50 ng/ml), instead of 17β-estradiol, and 5% charcoal-stripped FBS to deprive the media of estrogens. The cells were plated in 96-well plates at a density of 3 000 cells/well and allowed to attach for 48 h. After this pre-incubation, the inhibitors and the reference compounds diluted in fresh culture media were added to the wells and replaced every 2 days for 7 days of treatment.

Saos-2 cells

Saos-2 cells were suspended in phenol-red free McCoy's 5A medium supplemented with 10% charcoal-stripped FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL). The cells were seeded in 96-well plates at a density of 3 000 cells/well and allowed to attach. After 24 h, the inhibitors and the reference compounds diluted in fresh culture media were added to the wells and replaced every 2 days for 7 days of treatment.

Alkaline phosphatase activity

Saos-2 cells were treated similarly as reported in the cell proliferation assay. The cells were seeded at a density of 2 000 cells/well and were treated with the inhibitors and the reference compounds for 3 days. The alkaline phosphatase (ALP) activity was measured using Sensolyte® pNPP Alkaline Phosphatase Assay Kit *Colorimetric* (AnaSpec, Fremont, CA) following the manufacturer's protocol. Briefly, after the 3 days of treatment, the cells were washed twice with washing buffer (provided with the kit) and lysed with 0.2% Triton X-100. The cell lysates were centrifuged and the supernatants were used to determine the ALP activity. The supernatants were deposed in a 96-well plate and incubated 30 min with a *p*-nitrophenyl phosphate solution (provided with the kit). The absorbance at 405 nm was measured with a Thermo max microplate reader (Molecular Devices, Sunnyvale, CA). The control (culture media + DMSO) is set to 100% of alkaline phosphatase activity.

Acknowledgments

We thank the Fondation du Cancer du Sein du Québec for granting this study. We also thank Jenny Roy for the HPLC purification of all compounds. Careful reading of the manuscript by Micheline Harvey is greatly appreciated.

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Conclusion

Dans les trois chapitres de ce mémoire, j'ai présenté la synthèse et l'évaluation biologique *in vitro* de nouveaux dérivés sulfamates non-stéroïdiens. Le but de ce projet était de développer des composés à double-action pour le traitement des cancers du sein hormonodépendants. Cette double-action résulte de la combinaison de deux approches différentes pour bloquer la stimulation des cellules cancéreuses du sein, soit par l'inhibition d'une enzyme impliquée dans la biosynthèse des estrogènes et par le blocage du récepteur nucléaire induisant la réponse aux estrogènes. Effectivement, les composés devaient être en mesure d'inhiber la stéroïde sulfatase (STS) et de moduler le récepteur des estrogènes alpha (REα) selon le tissu (effet SERM).

Dans le 1er chapitre, j'ai rapporté la synthèse ainsi que les résultats initiaux d'une première génération de composés à double-action. Un noyau commun tétrahydroisoquinoline fut utilisé pour créer sur support solide en parallèle une variété de composés (54 sulfamates et 54 phénols). La diversité moléculaire des librairies de composés provient du clivage final pour obtenir un sulfamate ou un composé phénolique, des différents « spacers » utilisés et leur orientation *para* ou *meta*, ainsi que des divers groupements attachés à l'amine tertiaire (voir introduction Fig. 14). Les premiers résultats pour l'inhibition de la STS montrèrent que l'utilisation du groupement « spacer » benzamide en position *para* permettait d'obtenir de bons inhibiteurs de la STS. Les composés phénoliques analogues utilisant ce « spacer » furent testés sur des cellules MCF-7 pour s'assurer qu'ils ne possédaient pas une estrogénicité indésirable. Le criblage initial permit d'identifier plusieurs composés sulfamates qui inhibaient bien la STS et des analogues phénoliques qui n'induisaient pas une prolifération indésirable des cellules MCF-7 sensibles aux estrogènes. En effet, certains composés sulfamates démontrèrent plus de 90% d'inhibition de la STS à 1 μM tandis que certains composés phénoliques ne démontrèrent aucune activité estrogénique ou cytotoxique sur les cellules MCF-7 aux concentrations testées (0.01 μM - 1 μM). Certains de ces composés ont également été en mesure de stimuler la prolifération et l'activité alkaline phosphatase (ALP) des cellules ostéoblastiques Saos-2. Ces premiers essais ont donc permis d'identifier des inhibiteurs de la STS qui semblent posséder l'effet SERM

désiré, soit une action antagoniste dans le tissu mammaire et une action agoniste dans le tissu osseux. De plus, les résultats obtenus nous ont permis d'identifier certains groupements des chaînes latérales qui permettraient de meilleures interactions avec la STS et REα.

Dans le 2e chapitre, j'ai rapporté l'évaluation biologique *in vitro* approfondie d'un composé sulfamate et son analogue phénolique qui avaient produit de bons résultats préliminaires au chapitre 1. Le composé 6-EO-14 (Fig. 1), nommé 3-EO-14 dans le manuscrit du chapitre 1, s'est avéré être un très bon inhibiteur de la STS en inhibant 88% de l'activité enzymatique à 1 μM. Son analogue phénolique 8-EO-14, nommé 3'-EO-14 dans le manuscrit du chapitre 1, n'a pas stimulé de la prolifération des cellules MCF-7. Également, 8-EO-14 est un des composés ayant le plus stimulé la prolifération et l'activité ALP des cellules Saos-2. Pour ces raisons, nous avons décidé d'évaluer plus en profondeur l'activité et le mécanisme d'action des composés 6-EO-14 et 8-EO-14.

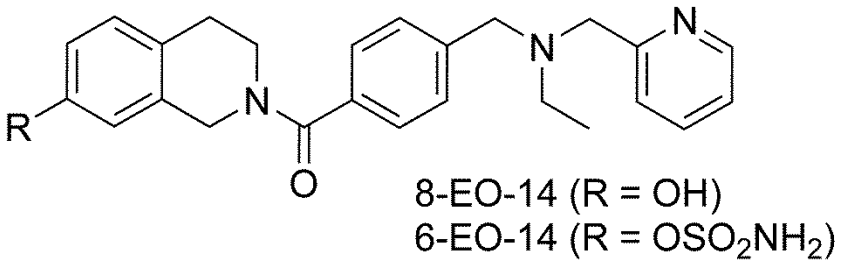

Figure 1. Structure des composés 8-EO-14 et 6-EO-14

Pour avoir une quantité suffisante et pure des composés pour les tests biologiques, les composés 6-EO-14 et 8-EO-14 furent resynthétisés et purifiés par HPLC. Le composé sulfamate 6-EO-14 fut testé à nouveau comme inhibiteur de la STS, ce qui permit d'obtenir une valeur d'IC_{50} de 300 ± 50 nM. Les deux composés ont été retestés sur les cellules Saos-2 et démontrèrent une faible stimulation de la prolifération cellulaire. L'effet du composé sulfamate et phénolique sur l'activité ALP des cellules Saos-2 fut investigué et permis de déterminer que la stimulation de l'activité était uniquement médié par REα. Suite à cela, j'ai mis au point un test utilisant le REα humain pour évaluer l'affinité des deux composés

pour ce récepteur. Étonnamment, le dérivé sulfamate 6-EO-14 (IC_{50} = 3.1 μM) a une meilleure affinité que le dérivé phénolique 8-EO-14 (IC_{50} = 24.6 μM) pour REα, contrairement à ce qui a été observé avec nos molécules de référence, soit l'estrone sulfamate (EMATE) et l'estrone. Même si leur affinité est faible, nous avons testé les deux composés sur les cellules T-47D, une autre lignée cellulaire de cancer du sein sensible aux estrogènes, pour nous assurer qu'ils ne possédaient pas d'activité estrogénique. Malheureusement, le 6-EO-14 et le 8-EO-14 ont stimulé la prolifération cellulaire et n'ont pas été en mesure de bloquer la stimulation induite par l'estradiol. L'absence d'estrogénicité observé lors du criblage initial du composé 8-EO-14 est probablement due à la plus faible expression de REα dans les cellules MCF-7 comparativement aux cellules T-47D. Malgré le fait que ces deux composés ne sont plus intéressants pour traiter les cancers du sein hormonodépendants, l'évaluation biologique permit tout de même de voir que nos composés de 1^{e} génération inhibent bien la STS et qu'ils sont en mesure de se lier à REα.

Dans le 3^{e} chapitre, j'ai abordé la synthèse et l'évaluation biologique *in vitro* d'une 2^{e} génération de composés à double action. Les résultats obtenus avec les composés de première génération nous ont permis de sélectionner certains éléments importants à inclure dans nos composés à double-action. Une librairie de 13 composés phénoliques fut synthétisée en utilisant le groupement « spacer » *para*-benzamide et en utilisant divers groupements sur l'amine tertiaire qui permettraient de faire des liaisons hydrogènes avec certains acides aminés de REα.

Un criblage initial des composés phénoliques sur les cellules T-47D permit d'identifier trois composés qui n'avaient aucune activité estrogénique ou cytotoxique. Leurs analogues sulfamates furent synthétisés, purifiés par HPLC et testés sur les cellules HEK-293 surexprimant la STS. Pour ces trois composés, des valeurs d'IC_{50} de 3.9 nM, 8.9 nM et 16.6 nM ont été calculées, ce qui est nettement supérieur à celle de 6-EO-14 et des autres inhibiteurs de 1^{ere} génération. Par la suite, les trois composés sulfamates ainsi que les trois composés phénoliques furent testés sur les cellules T-47D pour s'assurer qu'ils ne possèdent pas d'activité estrogénique et pour évaluer leur potentiel antiestrogénique. Les

six composés n'ont pas stimulé la prolifération aux concentrations testées (0.01 μM - 5 μM) et certains composés ont démontré de la cytotoxicité à la plus forte concentration. Également, les six composés ont été en mesure de bloquer en partie la prolifération des cellules T-47D induite par l'estradiol, ce qui démontre un caractère antiestrogénique. Ensuite, des cellules Saos-2 furent incubées avec nos composés pour évaluer leur potentiel SERM. Les tests de prolifération montrèrent que nos six molécules stimulent significativement les cellules Saos-2. Le composé phénolique **29** ainsi que le composé sulfamate **33** (Fig. 2) ont démontré des résultats intéressants car ils ont induit une stimulation de la prolifération de plus de 50% à 1 μM, ce qui est supérieur à celles du raloxifène (21%) et de l'estradiol (24%) à la même concentration. Les six composés ont également été testés sur les cellules Saos-2 pour évaluer leur effet sur l'activité ALP. Similairement au test de prolifération, les composés ont tous stimulé significativement l'activité ALP et les composés **29** et **33** ont induit la meilleure stimulation. Afin de mieux comprendre le mécanisme d'action, les cellules Saos-2 furent incubées avec le composé phénolique **29** et des antagonistes spécifiques pour les 2 types de récepteurs estrogéniques (α et β). Les résultats montrèrent que le composé **29** augmente l'activité ALP un peu plus via REβ que REα. Ces résultats sont très intéressants car le SERM raloxifène a démontré le même type d'induction.

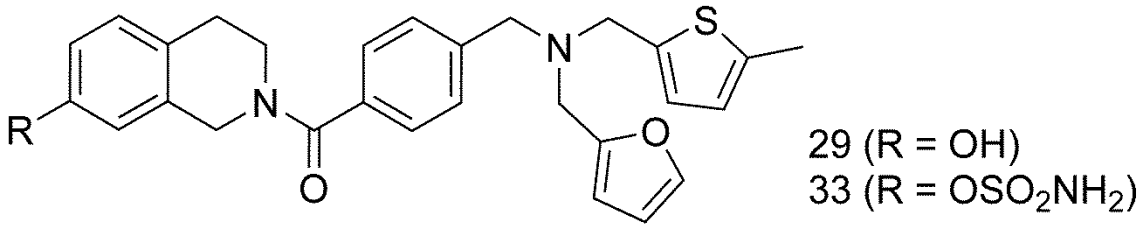

Figure 2. Structure des composés **29** et **33**

Le composé sulfamate **33** serait un bon candidat pour de futurs tests *in vivo*. En effet, il pourrait être injecté à des rats pour évaluer son effet sur le tissu osseux ou pourrait être injecté à des souris immunodéficientes porteuses de tumeurs cancéreuses du sein afin d'évaluer son potentiel anticancéreux. Ces deux tests cruciaux permettront de bien évaluer

le potentiel de ce produit et de l'approche novatrice d'un inhibiteur de la STS à effet SERM.

En résumé, nous avons réussi à développer des composés à double-action fort intéressants pour le traitement des cancers du sein hormonodépendants. Les composés synthétisés résultent d'une approche unique n'ayant jamais été rapportée auparavant. En effet, tous les composés ont été synthétisés chimiquement à l'aide de la chimie parallèle sur support solide, une méthode permettant la création d'une variété d'inhibiteurs de la STS ayant un effet SERM potentiel. Du point de vue de la STS, nos composés sulfamates de 2^{e} génération ont un potentiel d'inhibition supérieur aux composés de 1re génération. En ce qui a trait à l'effet SERM recherché, on peut voir que les composés de 1re génération ne possèdent pas cette caractéristique mais semblent tout de même posséder la capacité de se lier à REα. Pour les six composés de 2^{e} génération sélectionnés, les résultats sont très prometteurs. Ils ne stimulent pas les cellules cancéreuses du sein sensibles aux estrogènes, possèdent un caractère antiestrogénique et stimulent la prolifération et la différentiation de cellules ostéoblastiques. Ces composés seraient donc une bonne alternative de traitement des cancers du sein hormonodépendants sans entrainer certains effets secondaires associés à la diminution des estrogènes dans le sang tel que l'ostéoporose.

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