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ORIGINAL ARTICLE

SAR study of tyrosine–chlorambucil hybrid regioisomers; synthesis and biological evaluation against breast cancer cell lines

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Abstract Amino acids were transformed and coupled to chlorambucil, a well-known chemotherapeutic agent, in an attempt to create new anticancer drugs with selectivity for breast cancer cells. Among the amino acids available, tyrosine was selected to act as an estrogenic ligand. It is hypothesized that tyrosine, which shows some structural similitude with estradiol, could possibly mimic the natural hormone and, subsequently, bind to the estrogen receptor. In this exploratory study, several tyrosine-drug conjugates have been designed. Thus, *ortho*-, *meta*- and *para*-tyrosine–chlorambucil analogs were synthesized in order to generate new anticancer drugs with structural diversity, more specifically in regards to the phenol group location. These new analogs were produced in good yield following efficient synthetic methodology. All the tyrosine–chlorambucil hybrids were more effective than the parent drug, chlorambucil. In vitro biological evaluation on estrogen receptor positive and estrogen receptor negative (ER^+ and ER^-) breast cancer cell lines revealed an enhanced cytotoxic activity for compounds with the phenol function located at position *meta*. Molecular docking calculations were performed for the pure L-*ortho*, L-*meta*- and L-*para*-tyrosine phenolic regioisomers. The synthesis of all tyrosine–chlorambucil hybrid regioisomers and their biological activity are reported herein. Possible orientations within the targeted protein [estrogen receptor alpha (ERα)] are discussed in relation to the biological activity.

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Introduction

Cancer is a devastating disease, which continues to grow in size worldwide. In recent years, much effort has been invested in developing new treatment for cancer. Usually, the most effective treatments are nonspecific to cancer site and produce undesirable side effects (Magrath 1994; Palmer and Wallace 2010). Hence, in order to overcome this lack of specificity, drug targeting is still a current and important research subject.

The estrogen receptor alpha (ERα) is a nuclear protein, which is present in breast cancer cells and becomes overexpressed in cancerous cells (Shanle and Xu 2010). Some research groups have already looked at the possibility of targeting this protein with known cytotoxic compounds (Gust et al. 2009). It has been discovered that the toxic moiety can concentrate only to female organs and thereby kill cancerous cells. Recently, the anticancer property of chlorambucil (CLL) (**1**) has been exploited by linking it to estradiol (**2**), the sex female hormone (Gupta et al. 2010) (Fig. 1). The new estradiol–chlorambucil hybrids (**3**) were made to target cells expressing the ERα. It is important to indicate that an estrogenic drug could, not only target the cells, but also induce some transcriptional activity as it was observed for other type of estrogen-cytotoxic derivatives (Van Themsche et al. 2009). Hence, non-steroidal drugs with structural similarities with the natural ligand could possibly avoid such estrogenic activity.

Natural products such as monoclonal antibodies, enzymes, proteins, peptides and amino acids have already

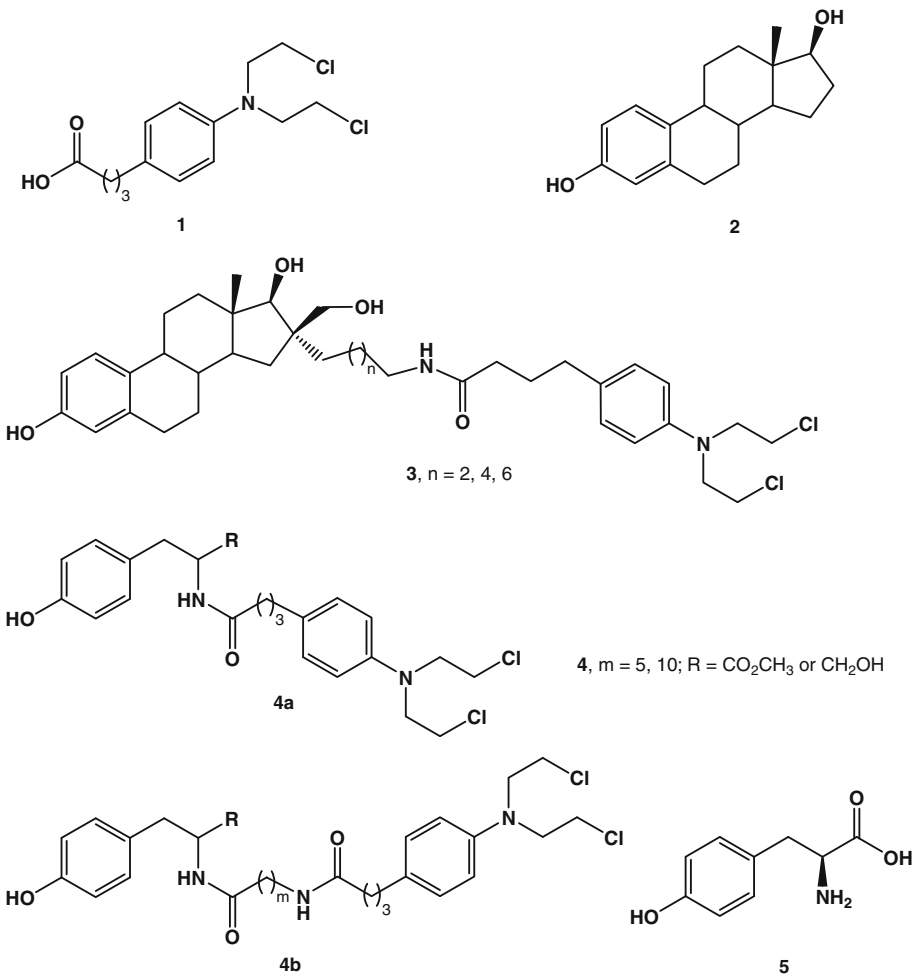


Fig. 1 Chemical structures of chlorambucil (**1**), 17β-estradiol (**2**), 17β-estradiol–chlorambucil hybrids (**3**), tyrosine–chlorambucil hybrids (**4a**, **4b**) and L-tyrosine (**5**)

been transformed and used to improve anticancer treatment (Sachdeva 1998). Drug delivery systems were conceived and developed based on these natural entities, making the new compounds well tolerated by the human body.

Amino acid-drug conjugates have also been designed. Several amino acids (proline, asparagine and glutamine) were linked to nitrogen mustard analogs (Chrzanowski et al. 2003; Gengrinnovitch and Izakovich 2005). These amino-acid drug conjugates were then used as prodrugs to treat cancer cells. Furthermore, the development of amino acid-antioxidant conjugates and amino acid-anti-angiogenic conjugates were also recently reported in the literature (Ortial et al. 2006; Durand et al. 2009; Contino-Pépin et al. 2009). Hence, amino-acid derivatives are a promising avenue, which must be further exploited in the search for new drug candidates.

Lately, we have reported the synthesis of D- and L-tyrosine–chlorambucil molecules (**4**) (Descoteaux et al. 2010). L-Tyrosine (**5**) was selected as ligand to reach the ERα as its structure shows likeness with the steroid backbone. These new tyrosine analogs showed potent activity against hormone-dependent breast cancer cells. Molecular modeling study predicted possible interactions with the ERα. However, despite the theoretical binding predictions and despite the interesting cytotoxicity observed, these molecules had very little in vitro affinity for the ERα when tested experimentally.

The study of interactions between the ERα and its potential ligands is a time consuming and difficult task. Structure activity relationship (SAR) studies have revealed that various compounds with diverse and unrelated structures can bind to the ERα (Demyttenaere-Kovatcheva et al. 2005). This can be explained by the motility and plasticity of the ERα ligand-binding cavity. Nevertheless, other studies have found some structural requirements for specific binding to the ERα (Muthyala et al. 2003). First of all, a phenol group, acting like the A-ring of estradiol, must be a part of the ligand. In addition, a second hydroxyl group or a phenol function must be placed approximately 11 Å from the A-ring phenol. These two polar entities act as contact points within the ERα binding cavity via hydrogen bonding network.

Keeping in mind the importance of the phenol group, and being aware that subtle structure discrepancies can

change the binding affinity to the ERα, we looked into the possibility of varying the position of the –OH group on the phenol portion of the tyrosine–chlorambucil hybrids. The aim of this study was to investigate how the phenol location could affect the cytotoxic activity of these molecules as well as their binding affinity.

In an attempt to study this, several tyrosine-based scaffold, DL-*ortho*- (DL-*o*-), L-*meta*- (L-*m*-) and L-*para*- (L-*p*-) tyrosine, were used as starting materials. It is noteworthy that the pure L-*o*-tyrosine was not commercially available. However, the mixture of isomers is adequate for this investigation, which was mainly aimed at studying the influence of the phenol location on the cytocidal activity of the tyrosine–chlorambucil hybrids. The chlorambucil moiety was either directly linked to the amino acid ligand or via an alkyl amide spacer. All of the new DL-*o*- (**6**, **7**), L-*m*- (**8**, **9**) and L-*p*-tyrosine–chlorambucil (**10**, **11**) (Fig. 2) hybrid molecules synthesized were evaluated for their in vitro cytotoxic activity on breast cancer cell lines (MCF-7, ER^+ and MDA-MB-231, ER^-). This manuscript gives a detailed description of the synthesis and cytotoxic activities of these novel tyrosine–chlorambucil hybrids. Finally, following molecular docking calculations, the orientation of L-*o*- (**12**, *ortho*-), L-*m*- (**12**, *meta*-) and L-*p*- (**12**, *para*-) tyrosine derivatives into the targeted protein, the ERα, was also investigated and the results are reported herein.

Materials and methods

Chemistry

All reactions were performed with ACS Fisher solvents. In some cases, solvent, as well as starting materials and reactants, were first purified and dried by standard means (Perrin and Armarego 1988). Anhydrous reactions required an inert atmosphere of dry nitrogen. DL-*o*-tyrosine was purchased from ABCR GmbH & Co.KG, Karlsruhe, Germany, L-*m*-tyrosine was purchased from PepTech Corporation, Burlington, MA, USA and L-*p*-tyrosine was purchased from Laboratoire Mat, Quebec, Canada. Of note, the pure L-*o*-tyrosine was not commercially available. The 6-aminohexanoic acid and 11-aminoundecanoic acid were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada. All reactions were monitored by UV fluorescence or staining with iodine on Sigma T 6145 commercial TLC plates (polyester silica gel 60 Å, 0.25 mm). Purifications were done using flash column chromatography according to the method of Still et al. (1978) on Silicycle UltraPure Flash Silica Gel, 40–63 µm mesh. Hexanes and acetone were distilled before their use as chromatography eluent.

The infrared spectra were taken on a Nicolet Impact 420 FT-IR and a Thermo Nicolet IS10 FT-IR with ATR.

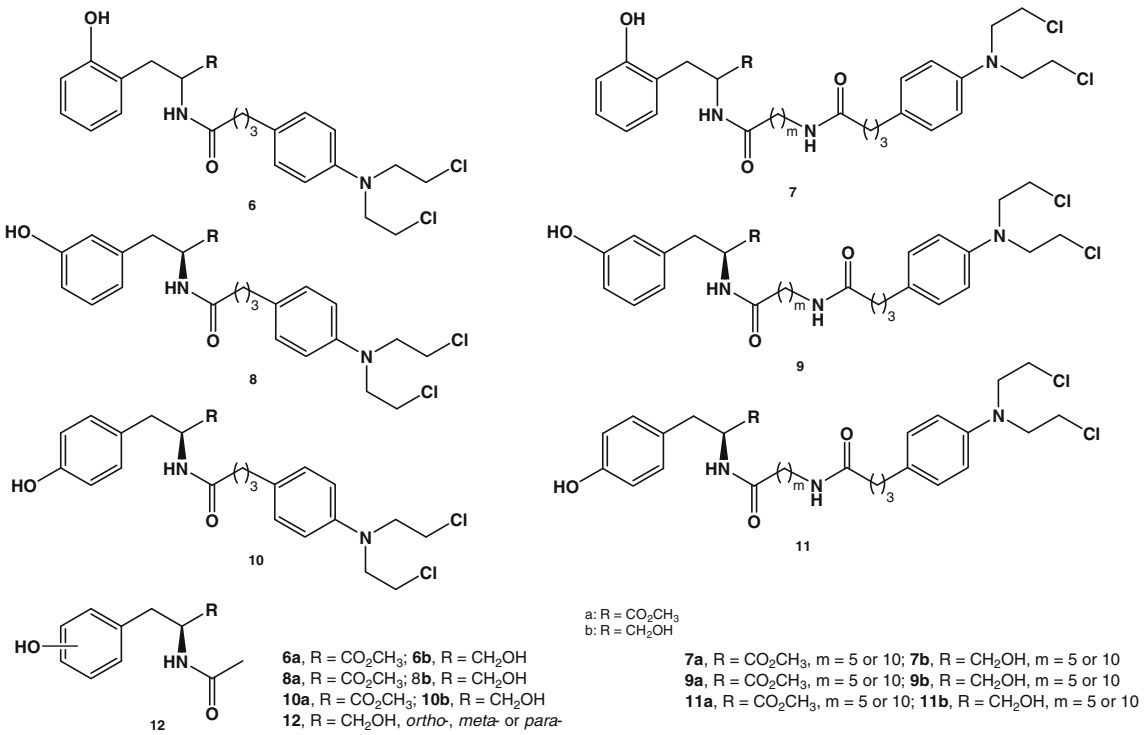


Fig. 2 DL-*o*- (**6**–**7**), L-*m*- (**8**–**9**), L-*p*-tyrosine–chlorambucil (**10**–**11**) hybrids and *N*-acetyl tyrosinols (**12**) regioisomers used as model for docking calculations

Sodium chloride, potassium bromide pellets or ATR diamond accessory were used for analysis. Mass spectral assays were obtained using a MS model 6210, Agilent technology instrument. The high-resolution mass spectra (HRMS) were obtained by TOF (time of flight) using ESI (electrospray ionization) using the positive mode (ESI^+) (analysis at Université du Québec à Montréal).

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 200 MHz NMR apparatus. Samples were dissolved in deuteroacetone (acetone-d_6) or deuterodimethylsulfoxide (DMSO-d_6) for data acquisition using tetramethylsilane as internal standard (TMS, δ 0.0 ppm for ^{1}H-NMR and ^{13}C-NMR). Chemical shifts (δ) are expressed in parts per million (ppm), the coupling constants (J) are expressed in hertz (Hz). Multiplicities are described by the following abbreviations: s for singlet, d for doublet, dd for doublet of doublets, dt for doublet of triplets, t for triplet, q for quartet, m for multiplet, #m for several multiplets, br s for broad singlet, br d for broad doublet and br t for broad triplet.

*General procedure for the preparation of the N-chlorambucil-aminoalkyl carboxylic acid (**14**, m = 5 or 10)*

The amino acid (**13**) (6.57 mmol) was dissolved in dichloromethane. Hexamethyldisilazane (21.69 mmol) and concentrated sulfuric acid (catalytic) were added. The mixture was stirred and heated to reflux under nitrogen atmosphere until complete dissolution. The solution was kept to reflux for 0.5 h. After cooling down, benzene, triethylamine (4.91 mmol) and chlorotrimethylsilane (4.91 mmol) were added. The resulting mixture was stirred at room temperature for 12 h. In another flask, chlorambucil (**1**) (3.29 mmol) was dissolved in dichloromethane at 0°C. Triethylamine (3.62 mmol) and isobutylchloroformate (3.62 mol) were added and the mixture was kept at 0°C for 1 h. Then, the chlorambucil solution was added to the activated amino acid solution and the mixture was stirred at room temperature for 4 h. Work-up was done by diluting with ethyl acetate and by washing the organic phase with chlorhydric acid 10% solution (2×) and with a saturated sodium chloride solution. The organic phase was dried with anhydrous magnesium sulfate, filtered and evaporated. The product was purified by flash chromatography (hexanes:acetone, 7:3) to give the desired material in 98% yield.

Spectral data for *N*-(6-chlorambucilamino)-hexanoic acid (**14**, $m = 5$). IR (NaCl, ν_{max}, cm^{-1}): 3300 (N–H), 3200–2600 (O–H), 1716 (C=O, COOH), 1622 (C=O, NHCOO), 1522 and 1255 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 7.26 (1H, br s, N**H**), 7.06 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.70 (2H, d, $J = 8.6$ Hz, 2-C**H** CLL), 3.73 (8H, m, 2× C$\mathbf{H}_2$Cl and 2× NC$\mathbf{H}_2$), 3.19 (2H, m, C$\mathbf{H}_2$NHCO), 2.51 (2H, t, $J = 7.4$ Hz, $CH_2C\mathbf{H}_2Ph$), 2.27 (2H, t, $J = 7.2$ Hz, C$\mathbf{H}_2$COOH), 2.18 (2H, t, $J = 7.4$ Hz, $CH_2NHCOC\mathbf{H}_2$), 1.78–1.96 (2H, m, $CH_2C\mathbf{H}_2CH_2Ph$), 1.28–1.67 (6H, m, 3× C$\mathbf{H}_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 174.3 (**C**OOH), 173.0 (**C**ONH), 144.8 (1-C CLL), 130.9 (4-C CLL), 129.7 (2C, 3-C CLL), 112.5 (2C, 2-C CLL), 53.3 (2C, 2× N**C**H_2CH_2Cl), 41.0 (2× C, 2× $NCH_2\mathbf{C}H_2Cl$), 39.1, 35.6, 34.3, 33.6, 29.4, 27.9, 26.5, 24.7. ESI + HRMS: $[M + H]^+$ calculated for $C_{20}H_{31}Cl_2N_2O_3 = 417.1706$; found = 417.1707.

Spectral data for *N*-(11-chlorambucilamino)-undecanoic acid (**14**, $m = 10$). IR (NaCl, ν_{max}, cm^{-1}): 3300 (N–H), 3200–2600 (O–H), 1714 (C=O, COOH), 1618 (C=O, NHCOO), 1526 and 1250 (C–N–H). ^{1}H-NMR (Acetone-d_6, δppm): 7.07 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 7.03 (1H, br s, N**H**), 6.72 (2H, d, $J = 9.0$ Hz, 2-C**H** CLL), 3.75 (8H, m, 2× C$\mathbf{H}_2$Cl and 2× NC$\mathbf{H}_2$), 3.17 (2H, m, C$\mathbf{H}_2$NHCO), 2.90 (1H, br s, COO**H**), 2.51 (2H, t, $J = 7.4$ Hz, $CH_2C\mathbf{H}_2Ph$), 2.27 (2H, t, $J = 7.4$ Hz, C$\mathbf{H}_2$COOH), 2.14 (2H, t, $J = 7.2$ Hz, $CH_2NHCOC\mathbf{H}_2$), 1.80–1.92 (2H, m, $CH_2C\mathbf{H}_2CH_2Ph$), 1.41–1.62 (4H, m, 2× C$\mathbf{H}_2$), 1.30 (12H, 6× C$\mathbf{H}_2$). ^{13}C-NMR (Acetone-d_6, δppm): 174.0 (**C**OOH), 172.1 (**C**ONH), 144.9 (1-C CLL), 131.0 (4-C CLL), 129.7 (2C, 3-C CLL), 112.5 (2C, 2-C CLL), 53.3 (2C, 2× N**C**H_2CH_2Cl), 41.0 (2C, 2× $NCH_2\mathbf{C}H_2Cl$), 39.0, 35.5, 34.2, 33.6, 29.8, 29.5 (2C), 29.3 (3C), 27.9, 27.0, 25.0. ESI + HRMS: $[M + H]^+$ calculated for $C_{25}H_{41}Cl_2N_2O_3 = 487.2489$; found = 487.2485.

*General procedure for the preparation of tyrosine-methyl ester hydrochloride regioisomers (**16**) (DL-o-tyr, L-m-tyr, L-p-tyr)*

A solution of tyrosine **15** (DL-*o*-, L-*m*- or L-*p*-, 1.67 mmol) in anhydrous methanol was stirred at 0°C. Then, thionyl chloride (5.00 mmol) was slowly added to the solution. The mixture was heated to reflux for 12 h. Then, the solvent was evaporated and the resulting crude compound was recristalized in diethyl ether. The final material was filtered, washed with cold ether and dried in a desiccator for one day. The crude hydrochloric acid salt, obtained in a 100% yield, was pure enough to be used as such in the next step.

Spectral data for DL-*o*-tyrosine methyl ester hydrochloride (**16**, DL-*o*-). IR (ATR, ν_{max}, cm^{-1}): 3258 (N–H), 3076 (O–H), 1734 (C=O ester), 1246 (CO–O–C). ^{1}H-NMR (DMSO-d_6, δppm): 8.74 (3H, br s, N$\mathbf{H}_3$), 7.05 (1H, t, $J = 10.9$ Hz, 5-C**H** tyr), 7.03 (1H, d $J = 6.6$ Hz, 3-C**H** tyr), 6.84 (1H, d, $J = 7.4$ Hz, 6-C**H** tyr), 6.71 (1H, t, $J = 6.9$ Hz, 4-C**H** tyr), 4.08 (1H, t, $J = 6.7$ Hz, C**H**NH_3), 3.58 (3H, s, O$\mathbf{CH}_3$), 3.02 (2H, d, $J = 6.7$ Hz, C$\mathbf{H}_2$CH), 2.48 (1H, s, O**H**). ^{13}C-NMR (DMSO-d_6, δ ppm): 170.0 (**C**OOCH_3), 156.2 (1-C tyr), 131.5 (3-C tyr), 129.1 (2-C tyr), 121.2 (5-C tyr), 119.4 (4-C tyr), 115.4 (6-C tyr), 52.9 (**C**HNH_3), 52.4 (O$\mathbf{CH}_3$), 32.0

(**C**H_2CH). ESI + HRMS: $[M + H]^+$ calculated for $C_{10}H_{14}NO_3 = 196.0968$; found = 196.0966.

Spectral data for L-*m*-tyrosine methyl ester hydrochloride (**16**, L-*m*-). IR (ATR, ν_{max}, cm^{-1}): 3243 (N–H), 3194 (O–H), 1745 (C=O ester), 1231 (CO–O–C). ^{1}H-NMR (DMSO-d_6, δppm): 9.53 (1H, br s, O**H**), 8.66 (3H, br s, N**H**$_3$), 7.08 (1H, t, $J = 7.7$ Hz, 5-C**H** tyr), 6.69–6.59 (2H, m, 4-C**H** tyr and 6-C**H** tyr), 6.62 (1H, s, 2-C**H** tyr), 4.16 (1H, t, $J = 6.25$ Hz, C**H**NH$_3$), 3.65 (3H, s, OC**H**$_3$), 3.14–2.92 (2H, m, C**H**$_2$CH). ^{13}C-NMR (DMSO-d_6, δppm): 170.0 (**C**OOCH$_3$), 158.0 (1-C tyr), 136.2 (3-C tyr), 130.0 (5-C tyr), 120.3 (4-C tyr), 116.7 (2-C tyr), 114.8 (6-C tyr), 53.7 (**C**HNH$_3$), 53.0 (O**C**H$_3$), 36.3 (**C**H$_2$CH). ESI + HRMS: $[M + H]^+$ calculated for $C_{10}H_{14}NO_3 = 196.0968$; found = 196.0964.

Spectral data for L-*p*-tyrosine methyl ester hydrochloride (**16**, L-*p*-). IR (KBr, ν_{max}, cm^{-1}): 3354 (N–H), 3078 (O–H), 1742 (C=O ester), 1224 (CO–O–C). ^{1}H-NMR (DMSO-d_6, δ ppm): 9.46 (1H, br s, O**H**), 8.60 (3H, br s, N**H**$_3$), 7.01 (2H, d, $J = 8.6$ Hz, 3-C**H** tyr), 6.73 (2H, d, $J = 8.60$ Hz, 2-C**H** tyr), 4.16 (1H, m, C**H**NH$_3$), 3.67 (3H, s, OC**H**$_3$), 3.03 (2H, m, C**H**$_2$CH). ^{13}C-NMR (DMSO-d_6, δ ppm): 170.1 (**C**OOCH$_3$), 157.4 (1-C tyr), 131.0 (2C, 3-C tyr), 125.0 (4-C tyr), 116.1 (2C, 2-C tyr), 54.1 (**C**HNH$_3$), 53.2 (O**C**H$_3$), 35.7 (**C**H$_2$CH). ESI + HRMS: $[M + H]^+$ calculated for $C_{10}H_{14}NO_3 = 196.0968$; found = 196.0968.

*General procedure for the preparation of N-chlorambucil-DL-o-, N-chlorambucil-L-m- and N-chlorambucil-L-p-tyrosine methyl ester (**6a**, **8a** and **10a**):*

The appropriate tyrosine methyl ester **16** (0.32 mmol) was dissolved in dimethylformamide and triethylamine (0.32 mmol) was added. At the same time, a solution of chlorambucil (**1**) (0.49 mmol) in dimethylformamide was activated using dicyclohexylcarbodiimide (DCC) (0.52 mmol) followed by *N*-hydroxybenzotriazole (HOBt) (0.52 mmol). The tyrosine solution was then added to the activated acid chlorambucil solution. The mixture was stirred at room temperature under nitrogen at room temperature for 24 h. The solution was diluted with ethyl acetate and water, and then washed with water (4×). The organic phase was dried with sodium sulfate, filtered and evaporated. The product was further purified by flash chromatography (hexanes: acetone, 4:1) to give a pure compound in 81% yield.

Spectral data for *N*-chlorambucil-DL-*o*-tyrosine methyl ester (**6a**). IR (ATR, ν_{max}, cm^{-1}): 3150–3400 (O–H and N–H), 1745 (C=O, COOCH$_3$), 1652 (C=O, NHCO), 1524 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 8.74 (1H, s, O**H**), 7.39 (1H, d, $J = 7.4$ Hz, CHN**H**CO), 7.01–7.13 (2H, m, 3-C**H** and 5-C**H** tyr), 7.03 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.67–6.88 (2H, m, 4-C**H** and 6-C**H** tyr), 6.69 (2H, d, $J = 9.0$ Hz, C**H** CLL), 4.75 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 4.7$ Hz and $J = 1.2$ Hz, C**H**$_2$Cl and NC**H**$_2$), 3.63 (3H, s, OC**H**$_3$), 2.92–3.20 (2H, m, C**H**$_2$CHNH), 2.46 (2H, t, $J = 7.7$ Hz, CH$_2$C**H**$_2$Ph), 2.17 (2H, t, $J = 7.4$ Hz, NHCOC**H**$_2$), 1.76–1.87 (2H, m, CH$_2$C**H**$_2$CH$_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 172.48 (NH**C**OCH$_2$), 172.3 (**C**OOCH$_3$), 155.4 (1-C tyr), 144.6 (1-C CLL), 131.1 (3-C tyr), 130.6 (C, 4-C CLL), 129.5 (2C, 3-C CLL), 128.0 (2-C tyr), 123.5 (5-C tyr), 119.5 (4-C tyr), 115.1 (C, 6-C tyr), 112.2 (2C, 2-C CLL), 53.0 (2C, 2× NCH$_2$CH$_2$Cl), 52.8 (**C**HNH), 51.2 (O**C**H$_3$), 41.8 (2× C, 2× NCH$_2$**C**H$_2$Cl), 34.9 (**C**H$_2$CHNH), 33.7 (CH$_2$**C**H$_2$Ph), 32.4 (NHCO**C**H$_2$), 27.4 (CH$_2$**C**H$_2$CH$_2$). ESI + HRMS: $[M + H]^+$ calculated for $C_{24}H_{31}Cl_2N_2O_4$ = 481.1655; found = 481.1652.

Spectral data for *N*-chlorambucil-L-*m*-tyrosine methyl ester (**8a**). IR (ATR, ν_{max}, cm^{-1}): 3100–3450 (O–H and N–H), 1748 (C=O, COOCH$_3$), 1643 (C=O, NHCO), 1519 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 8.31 (1H, s, O**H**), 7.28 (1H, d, $J = 8.2$ Hz, CHN**H**CO), 7.06 (1H, t, $J = 8.4$ Hz, 5-C**H** tyr), 7.03 (2H, d, $J = 9.0$ Hz, 3-C**H** CLL), 6.72 (1H, s, 2-C**H** tyr), 6.70 (4H, 3d, $J = 9.0$ Hz, 4-C**H** tyr, 6-C**H** tyr and 2-C**H** CLL), 4.72 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 4.7$ Hz and $J = 1.2$ Hz, C**H**$_2$Cl and NC**H**$_2$), 3.66 (3H, s, OC**H**$_3$), 2.85–3.12 (2H, m, C**H**$_2$CHNH), 2.46 (2H, t, $J = 7.7$ Hz, CH$_2$C**H**$_2$Ph), 2.18 (2H, t, $J = 7.4$ Hz, NHCOC**H**$_2$), 1.76–1.88 (2H, m, CH$_2$C**H**$_2$CH$_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 172.18 (NH**C**OCH$_2$), 172.10 (**C**OOCH$_3$), 157.4 (1-C tyr), 144.6 (1-C CLL), 138.7 (3-C tyr), 130.6 (4-C CLL), 129.5 (2C, 3-C CLL), 129.3 (5-C tyr), 120.2 (4-C tyr), 116.0 (2-C tyr), 113.7 (6-C tyr), 112.2 (2C, 2-C CLL), 53.4 (**C**HNH), 53.0 (2C, 2× NCH$_2$CH$_2$Cl), 51.3 (O**C**H$_3$), 40.8 (2× C, 2× NCH$_2$**C**H$_2$Cl), 37.3 (**C**H$_2$CHNH), 34.8 (CH$_2$**C**H$_2$Ph), 33.7 (NHCO**C**H$_2$), 27.4 (CH$_2$**C**H$_2$CH$_2$). ESI + HRMS: $[M + H]^+$ calculated for $C_{24}H_{31}Cl_2N_2O_4$ = 481.1655; found = 481.1650.

Spectral data for *N*-chlorambucil-L-*p*-tyrosine methyl ester (**10a**). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

*General procedure for the preparation of N-chlorambucil-DL-o-, N-chlorambucil-L-m- and N-chlorambucil-L-p-tyrosinol (**6b**, **8b** and **10b**)*

The *N*-chlorambucil-tyrosine methyl ester (**6a**, **8a** or **10a**) (0.15 mmol) was dissolved in diethyl ether and dichloromethane and was stirred under nitrogen atmosphere. The resulting solution was cooled down with an ice and water bath, afterwards lithium borohydride (0.90 mmol) was added. The mixture was kept at 0°C for 3 h. Afterwards sodium sulfate decahydrate (0.3 g) was added. Work-up was done by diluting with diethyl ether and the organic phase was washed with saturated ammonium chloride

solution (2×) and with water (4×). The organic phase was dried with anhydrous sodium sulfate, filtered and evaporated. The product was purified by flash chromatography (hexanes: acetone, 7:3) to give a pure compound in 88% yield.

Spectral data for *N*-chlorambucil-DL-*o*-tyrosinol (**6b**). IR (ATR, ν_{max}, cm^{-1}): 3100–3450 (O–H and N–H), 1638 (C=O, NHCO), 1517 and 1242 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 9.03 (1H, s, O**H**), 7.35 (1H, d, $J = 6.3$ Hz, CHN**H**CO), 7.02–7.11 (2H, m, 3-C**H** tyr and 5-C**H** tyr), 7.07 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.71 (2H, d, $J = 9.0$ Hz, 2-C**H** CLL), 6.69–6.85 (2H, m, 4-C**H** tyr and 6-C**H** tyr), 4.18 (1H, t, $J = 5.7$ Hz, C**H**NH), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C$\mathbf{H}_2$Cl and 2× NC$\mathbf{H}_2$), 3.58 (2H, dt, $J = 1.2$ Hz and $J = 5.5$ Hz, C$\mathbf{H}_2$OH), 2.92 (1H, s, CH$_2$O**H**), 2.78–2.91 (2H, m, C$\mathbf{H}_2$CHNH), 2.52 (2H, t, $J = 7.4$ Hz, CH$_2$C$\mathbf{H}_2$Ph), 2.25 (2H, t, $J = 7.3$ Hz, NHCOC$\mathbf{H}_2$), 1.83–1.94 (2H, m, CH$_2$C$\mathbf{H}_2$CH$_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 173.8 (NH**C**OCH$_2$), 156.0 (1-C tyr), 144.7 (1-C CLL), 130.9 (4-C CLL), 130.6 (3-C tyr), 129.5 (2C, 3-C CLL), 127.7 (5-C tyr), 124.6 (2-C tyr), 119.3 (4-C tyr), 115.8 (6-C tyr), 112.2 (2C, 2-C CLL), 62.3 (**C**H$_2$OH), 54.1 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 35.1 (**C**H$_2$CHNH), 33.9 (CH$_2$**C**H$_2$Ph), 31.9 (NHCO**C**H$_2$), 27.5 (CH$_2$**C**H$_2$CH$_2$). ESI + HRMS: $[M + H]^+$ calculated for $C_{23}H_{31}Cl_2N_2O_3$ = 453.1706; found = 453.1702.

Spectral data for *N*-chlorambucil-L-*m*-tyrosinol (**8b**). IR (ATR, ν_{max}, cm^{-1}): 3100–3450 (O–H and N–H), 1619 (C=O, NHCO), 1526 and 1254 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 8.34 (1H, s, O**H**), 7.08 (1H, t, $J = 7.4$ Hz, 5-C**H** tyr), 7.03 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.97–7.01 (1H, m, CHN**H**CO), 6.70 (2H, d, $J = 8.6$ Hz, 2-C**H** CLL), 6.64–6.79 (3H, #m, 2-C**H** tyr, 4-C**H** tyr and 6-C**H** tyr), 4.14 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 1.2$ Hz and $J = 4.7$ Hz, 2× C$\mathbf{H}_2$Cl and 2× NC$\mathbf{H}_2$), 3.53 (2H, d, $J = 4.7$ Hz, C$\mathbf{H}_2$OH), 2.67–2.91 (2H, m, C$\mathbf{H}_2$CHNH), 2.46 (2H, t, $J = 7.5$ Hz, CH$_2$C$\mathbf{H}_2$Ph), 2.16 (2H, t, $J = 7.4$ Hz, NHCOC$\mathbf{H}_2$), 1.77–1.88 (2H, m, CH$_2$C$\mathbf{H}_2$CH$_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 172.4 (NH**C**OCH$_2$), 157.4 (1-C tyr), 144.6 (1-C CLL), 140.6 (3-C tyr), 130.7 (4-C CLL), 129.5 (2C, 3-C CLL), 129.1 (5-C tyr), 120.3 (4-C tyr), 116.1 (2-C tyr), 113.1 (6-C tyr), 112.2 (2C, 2-C CLL), 63.2 (**C**H$_2$OH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 52.8 (**C**HNH), 40.8 (2C, 2× NCH$_2$**C**H$_2$Cl), 36.8 (**C**H$_2$CHNH), 35.3 (CH$_2$**C**H$_2$Ph), 33.9 (NHCO**C**H$_2$), 27.6 (CH$_2$**C**H$_2$CH$_2$). ESI + HRMS: $[M + H]^+$ calculated for $C_{23}H_{31}Cl_2N_2O_3$ = 453.1706; found = 453.1707.

Spectral data for *N*-chlorambucil-L-*p*-tyrosinol (**10b**). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

*General procedure for the preparation of N-[(N-chlorambucilamino)alcanoyl]- DL-o-, L-m- and L-p-tyrosine methyl ester (**7a**, **9a** and **11a**)*

The appropriate tyrosine methyl ester hydrochloride **16** (0.41 mmol) was dissolved in dimethylformamide and triethylamine (0.41 mmol). The latter was added in order to neutralize the hydrochloride salt. At the same time, a solution of *N*-chlorambucil-aminoalkyl carboxylic acid **14** ($m = 5$ or 10) (0.62 mmol) in dimethylformamide was activated using DCC (0.66 mmol) followed by HOBt (0.66 mmol). The tyrosine solution was then added to the activated *N*-chlorambucil-aminoalkyl carboxylic acid solution. The mixture was stirred at room temperature for 24 h. The solution was diluted with ethyl acetate and water, and then washed with water (4×). The organic phase was dried with sodium sulfate, filtered, and evaporated. The product was further purified by flash chromatography (hexanes: acetone, 7:3) to give a pure compound in 71% yield.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-DL-*o*-tyrosine methyl ester (**7a**, $m = 5$). IR (ATR, ν_{max}, cm^{-1}): 3100–3400 (O–H and 2× N–H), 1745 (C=O, COOCH$_3$), 1634 (2× C=O, 2× NHCO), 1520 and 1253 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm : 9.04 (1H, s, O**H**), 7.36–7.20 (2H, 2d apparent, CHN**H**CO and CH$_2$N**H**CO), 7.06–7.10 (2H, m, 3-C**H** and 5-C**H** tyr), 7.08 (2H, d, $J = 7.0$ Hz, 3-C**H** CLL), 6.75–6.92 (2H, 2d apparent, 4-C**H** and 6-C**H** tyr), 6.72 (2H, d, $J = 6.6$ Hz, 2-C**H** CLL), 4.67 (1H, m, C**H**NH), 3.75 (8H, s, 2× C$\mathbf{H}_2$Cl and 2× NC$\mathbf{H}_2$), 3.63 (3H, s, OC$\mathbf{H}_3$), 3.17 (2H, br m, C$\mathbf{H}_2$NHCO), 2.92–3.17 (2H, m, C$\mathbf{H}_2$CHNH), 2.53 (2H, t, $J = 7.0$ Hz, CH$_2$C$\mathbf{H}_2$Ph), 2.15 (4H, m apparent, CH$_2$NHCOC$\mathbf{H}_2$ and CHNHCOC$\mathbf{H}_2$), 1.84–1.95 (2H, m, CH$_2$C$\mathbf{H}_2$CH$_2$Ph), 1.28–1.58 (6H, #m, 3× C$\mathbf{H}_2$). ^{13}C-NMR (Acetone-d_6, δ ppm): 172.4 (**C**ONH), 172.2 (2C, **C**ONH and **C**OOCH$_3$), 155.5 (1-C tyr), 144.7 (1-C CLL), 131.1 (3-C tyr), 130.7 (4-C CLL), 129.5 (2C, 3-C CLL), 128.0 (2-C tyr), 123.6 (5-C tyr), 119.5 (4-C tyr), 115.1 (6-C tyr), 112.2 (2C, 2-C CLL), 53.2 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 51.2 (O**C**H$_3$), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 38.6, 35.4, 35.3, 34.0, 32.2, 29.0, 27.7, 25.8, 24.9. ESI + HRMS: $[M + H]^+$ calculated for $C_{30}H_{42}Cl_2N_3O_5$ = 594.2496; found = 594.2488.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-L-*m*-tyrosine methyl ester (**9a**, $m = 5$). IR (ATR, ν_{max}, cm^{-1}): 3150–3450 (O–H and 2× N–H), 1745 (C=O, COOCH$_3$), 1638 (2× C=O, 2× NHCO), 1517 and 1217 (C–N–H). ^{1}H-NMR (Acetone-d_6, δ ppm): 8.66 (1H, s, O**H**), 7.24 (1H, d, $J = 7.8$ Hz, CHN**H**CO), 7.16 (1H, m, CH$_2$N**H**CO), 7.09 (1H, t apparent, $J = 8.2$ Hz, 5-C**H** tyr), 7.07 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.71 (2H, d, $J = 8.6$ Hz, 2-C**H** CLL), 6.64–6.75 (3H, m apparent, 2-C**H** tyr, 4-CH tyr and 6-CH tyr), 4.71 (1H, m, C**H**NH),

3.75 (8H, dt, $J = 1.2$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.66 (3H, s, OC**H**$_3$), 3.17 (2H, m, C**H**$_2$NHCO), 2.84–3.10 (2H, m, C**H**$_2$CHNH), 2.52 (2H, t, $J = 7.7$ Hz, CH$_2$C**H**$_2$Ph), 2.18 (4H, m apparent, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.82–1.94 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.20–1.59 (6H, #m, 3× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 172.5 (**C**ONH), 172.1 (**C**ONH), 172.0 (**C**OOCH$_3$), 157.6 (1-C tyr), 144.6 (1-C CLL), 138.6 (3-C tyr), 130.6 (4-C CLL), 129.5 (2C, 3-C CLL), 129.2 (5-C tyr), 120.1 (4-C tyr), 116.3 (2-C tyr), 113.8 (6-C tyr), 112.2 (2C, 2-C CLL), 53.3 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 51.3 (O**C**H$_3$), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 38.7, 37.4, 35.4 (2C), 34.0, 27.7, 26.1, 25.1. ESI + HRMS: $[M + H]^+$ calculated for $C_{30}H_{42}Cl_2N_3O_5 = 594.2496$; found = 594.2491.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-L-*p*-tyrosine methyl ester (**11a**, $m = 5$). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-DL-*o*-tyrosine methyl ester (**7a**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3200–3400 (O–H and 2× N–H), 1749 (C=O, COOCH$_3$), 1645 and 1602 (2× C=O and 2× NHCO), 1520 and 1213 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 8.83 (1H, br s, O**H**), 7.31 (1H, d, $J = 7.4$ Hz, CHN**H**CO), 7.03–7.12 (3H, m, CH$_2$N**H**CO, 3-C**H** tyr and 5-C**H** tyr), 7.07 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.69–6.89 (2H, m, 4-C**H** tyr and 6-C**H** tyr), 6.71 (2H, d, $J = 9.0$ Hz, 2-C**H** CLL), 4.69 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.62 (3H, s, OC**H**$_3$), 3.19 (2H, q, $J = 5.9$ Hz, C**H**$_2$NHCO), 2.90–3.16 (2H, m, C**H**$_2$CHNH), 2.52 (2H, t, $J = 7.5$ Hz, CH$_2$C**H**$_2$Ph), 2.17 (4H, m, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.82–1.90 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.25–1.54 (16H, #m and s, 8× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 172.3 (2C, 2× **C**ONH), 172.1 (**C**OOCH$_3$), 155.4 (1-C tyr), 144.7 (1-C CLL), 131.1 (3-C tyr), 130.7 (4-C CLL), 129.4 (2C, 3-C CLL), 128.0 (2-C tyr), 123.6 (5-C tyr), 119.4 (4-C tyr), 115.1 (6-C tyr), 112.2 (2C, 3-C CLL), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 52.8 (**C**HNH), 51.1 (O**C**H$_3$), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 38.8, 35.5, 35.3, 34.0, 33.7, 32.4, 29.5, 29.2, 29.1, 29.0, 28.8, 27.7, 26.6. ESI + HRMS: $[M + H]^+$ calculated for $C_{35}H_{52}Cl_2N_3O_5 = 664.3279$; found = 664.3271.

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-L-*m*-tyrosine methyl ester (**9a**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3150–3400 (O–H and 2× N–H), 1745 (C=O, COOCH$_3$), 1642 and 1613 (2× C=O and 2× NHCO), 1517 and 1253 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 8.44 (1H, br s, O**H**), 7.26 (1H, d, $J = 7.8$ Hz, CHN**H**CO), 7.10 (1H, m, CH$_2$N**H**CO), 7.04–7.13 (1H, m5-C**H** tyr), 7.06 (2H, d, $J = 8.2$ Hz, 3-C**H** CLL), 6.71 (2H, d, $J = 9.0$ Hz, 2-C**H** tyr), 6.65–6.75 (3H, m,2-C**H** tyr, 4-C**H** tyr and 6-C**H** tyr), 4.69 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.65 (3H, s, OC**H**$_3$), 3.19 (2H, q, $J = 5.9$ Hz, C**H**$_2$NHCO), 2.83–3.10 (2H, m, C**H**$_2$CHNH), 2.51 (2H, t, $J = 7.6$ Hz, CH$_2$C**H**$_2$Ph), 2.16 (4H, 2t overlapped, $J = 7.2$ Hz, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.81–1.92 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.25–1.55 (16H, #m and s, 8× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 172.2 (**C**ONH), 172.1 (2C, **C**OOCH$_3$ and **C**ONH), 157.5 (1-C tyr), 144.6 (1-C CLL), 138.6 (3-C tyr), 130.7 (4-C CLL), 129.5 (2C, 3-C CLL), 129.2 (5-C tyr), 120.1 (4-C tyr), 116.1 (2-C tyr), 113.6 (6-C tyr), 112.2 (2C, 3-C CLL), 53.4 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 51.3 (O**C**H$_3$), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 38.8, 37.4, 35.5, 35.3, 34.0, 29.6, 29.2, 29.1, 28.9, 27.7, 26.7, 25.4. ESI + HRMS: $[M + H]^+$ calculated for $C_{35}H_{52}Cl_2N_3O_5 = 664.3279$; found = 664.3270.

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-L-*p*-tyrosine methyl ester (**11a**, $m = 10$). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

*General procedure for the preparation of N-[(N-chlorambucilamino)alcanoyl]- DL-o-, L-m- and L-p-tyrosinol (**7b**, **9b** and **11b**)*

The *N*-[(*N*-chlorambucilamino)alcanoyl)-tyrosine methyl ester (**7a**, **9a** or **11a**) (0.12 mmol) was dissolved in diethyl ether and dichloromethane and was stirred under nitrogen atmosphere. The resulting solution was cooled down with an ice and water bath; afterwards lithium borohydride (0.73 mmol) was added. The mixture was kept at 0°C for 3 h. Afterwards, sodium sulfate decahydrate (0.3 g) was added. Work-up was done by diluting with diethyl ether and washing the organic phase with saturated ammonium chloride solution (2×) and with water (4×). The organic phase was dried with anhydrous sodium sulfate, filtered and evaporated. The product was purified by flash chromatography (hexanes: acetone, 3:2) to give a pure compound in 82% yield.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-DL-*o*-tyrosinol (**7b**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3100–3400 (2× O–H and 2× N–H), 1638 (2× C=O, 2× NHCO), 1520 and 1242 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 9.16 (1H, br s, O**H**), 7.37 (1H, d, $J = 6.6$ Hz, CHN**H**CO), 7.17 (1H, br t, $J = 5.1$ Hz, CH$_2$N**H**CO), 7.02–7.11 (2H, m apparent, 3-C**H** tyr and 5-C**H** tyr), 7.06 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.70–6.88 (2H, m apparent, 4-C**H** tyr and 6-C**H** tyr), 6.71 (2H, d, $J = 8.5$ Hz, 2-C**H** CLL), 4.01 (1H, m, C**H**NH), 3.89 (1H, m, CH$_2$O**H**), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.51 (2H, d apparent, $J = 5.8$ Hz, C**H**$_2$OH), 3.19 (2H, dq, $J = 1.8$ Hz and $J = 6.8$ Hz, C**H**$_2$NHCO),

2.71–2.93 (2H, m, C**H**$_2$CHNH), 2.51 (2H, t, $J = 7.4$ Hz, CH$_2$C**H**$_2$Ph), 2.19 (4H, m apparent, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.78–1.93 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.27–1.67 (6H, #m, 3× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 173.8 (**C**ONH), 172.4 (**C**ONH), 156.0 (1-C tyr), 144.7 (1-C CLL), 131.0 (4-C CLL), 130.7 (3-C tyr), 129.5 (2C, 3-C CLL), 127.7 (5-C tyr), 124.7 (2-C tyr), 119.3 (4-C tyr), 115.7 (6-C tyr), 112.2 (2C, 2-C CLL), 62.6 (**C**H$_2$OH), 53.2 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 40.8 (2× C, 2× NCH$_2$**C**H$_2$Cl), 38.6, 35.7, 35.4, 34.0, 31.8, 29.2, 27.7, 26.1, 25.1. ESI + HRMS: $[M + H]^+$ calculated for $C_{29}H_{42}Cl_2N_3O_4 = 566.2547$; found = 566.2540.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-L-*m*-tyrosinol (**9b**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3100–3400 (2× O–H and 2× N–H), 1683 (2× C=O, 2× NHCO), 1520 and 1253 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 8.59 (1H, s, O**H**), 7.20 (1H, br t, $J = 5.3$ Hz, CH$_2$N**H**CO), 7.07 (1H, t, $J = 7.4$ Hz, 5-C**H** tyr), 7.06 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.98 (1H, d, $J = 8.5$ Hz, CHN**H**CO), 6.64–6.76 (3H, m, 2-C**H** tyr, 4-C**H** tyr and 6-C**H** tyr), 6.71 (2H, d, $J = 8.5$ Hz, 2-C**H** CLL), 4.13 (2H, m, C**H**NH and CH$_2$O**H**), 3.74 (8H, dt, $J = 1.2$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.52 (2H, t, $J = 5.1$ Hz, C**H**$_2$OH), 3.16 (2H, m, C**H**$_2$NHCO), 2.64–2.91 (2H, m, C**H**$_2$CHNH), 2.51 (2H, t, $J = 7.6$ Hz, CH$_2$C**H**$_2$Ph), 2.14 (4H, m apparent, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.82–1.89 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.20–1.58 (6H, #m, 3× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 172.51 (**C**ONH), 172.46 (**C**ONH), 157.5 (1-C tyr), 144.6 (1-C CLL), 140.5 (3-C tyr), 130.6 (4-C CLL), 129.5 (2C, 3-C CLL), 129.0 (5-C tyr), 120.3 (4-C tyr), 116.3 (2-C tyr), 113.1 (6-C tyr), 112.2 (2C, 2-C CLL), 63.4 (**C**H$_2$OH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 52.8 (**C**HNH), 40.8 (2× C, 2× NCH$_2$**C**H$_2$Cl), 38.7 (**C**H$_2$NHCO), 36.8 (**C**H$_2$CHNH), 35.9 (CHNHCO**C**H$_2$), 35.6 (CH$_2$**C**H$_2$Ph), 35.4 (CH$_2$NHCO**C**H$_2$), 34.0, 27.7, 26.2, 25.3. ESI + HRMS: $[M + H]^+$ calculated for $C_{29}H_{42}Cl_2N_3O_4 = 566.2547$; found = 566.2541.

Spectral data for *N*-[(6-*N*-chlorambucilamino)hexanoyl]-L-*p*-tyrosinol (**11b**, $m = 5$). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-DL-*o*-tyrosinol (**7b**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3100–3400 (2× O–H and 2× N–H), 1631 (2× C=O, NHCO), 1524 and 1246 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 9.11 (1H, s, O**H**), 7.36 (1H, d, $J = 5.9$ Hz, CHN**H**CO), 7.02–7.10 (3H, m, 3-C**H** tyr, 5-C**H** tyr and CH$_2$N**H**CO), 7.07 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.68–6.86 (2H, m, 4-C**H** tyr and 6-C**H** tyr), 6.71 (2H, d, $J = 8.6$ Hz, 2-C**H** CLL), 4.11 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.58 (2H,br d, $J = 5.1$ Hz, C**H**$_2$OH), 3.18 (2H, q, $J = 5.8$ Hz, C**H**$_2$NHCO), 2.70–2.93 (3H, m and br s, C**H**$_2$CHNH and CH$_2$O**H**), 2.52 (2H, t, $J = 7.6$ Hz, CH$_2$C**H**$_2$Ph), 2.22 (2H, t, $J = 7.2$ Hz, CH$_2$NHCOC**H**$_2$), 2.16 (2H, t, $J = 7.2$ Hz, CHNHCOC**H**$_2$), 1.81–1.93 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.29–1.59 (16H, #m and s, 8x C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 173.9 (**C**ONH), 172.0 (**C**ONH), 156.0 (1-C tyr), 144.7 (1-C CLL), 130.9 (4-C CLL), 130.7 (3-C tyr), 129.4 (2C, 3-C CLL), 127.7 (5-C tyr), 124.6 (2-C tyr), 119.2 (4-C tyr), 115.8 (6-C tyr), 112.2 (2C, 2-C CLL), 62.3 (**C**H$_2$OH), 53.1 (**C**HNH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 40.7 (2× C, 2× NCH$_2$**C**H$_2$Cl), 38.8, 35.7, 35.3, 34.0, 31.9, 29.6, 29.2 (3C), 29.0 (2C), 26.7. ESI + HRMS: $[M + H]^+$ calculated for $C_{34}H_{52}Cl_2N_3O_4 = 636.3329$; found = 636.3318.

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-L-*m*-tyrosinol (**9b**, $m = 10$). IR (ATR, ν_{max}, cm^{-1}): 3100–3400 (2× O–H and 2× N–H), 1634 (2× C=O, NHCO), 1520 and 1246 (C–N–H). ^{1}H-NMR (Acetone-d$_6$, δ ppm): 8.45 (1H, s, O**H**), 7.14 (1H, br t apparent, CH$_2$N**H**CO), 7.07 (1H, t, $J = 7.6$ Hz, 5-C**H** tyr), 7.06 (2H, d, $J = 8.6$ Hz, 3-C**H** CLL), 6.97 (1H, d, $J = 7.8$ Hz, CHN**H**CO), 6.65–6.77 (3H, m, 2-C**H** tyr, 4-C**H** tyr and 6-C**H** tyr), 6.71 (2H, d, $J = 8.6$ Hz, 2-C**H** CLL), 4.08 (1H, m, C**H**NH), 3.74 (8H, dt, $J = 1.6$ Hz and $J = 5.1$ Hz, 2× C**H**$_2$Cl and 2× NC**H**$_2$), 3.52 (2H, t, $J = 5.1$ Hz, C**H**$_2$OH), 3.19 (2H, q, $J = 6.5$ Hz, C**H**$_2$NHCO), 2.92 (1H, s, CH$_2$O**H**), 2.65–2.91 (2H, m, C**H**$_2$CHNH), 2.52 (2H, t, $J = 7.4$ Hz, CH$_2$C**H**$_2$Ph), 2.13 (4H, m apparent, CH$_2$NHCOC**H**$_2$ and CHNHCOC**H**$_2$), 1.82–1.93 (2H, m, CH$_2$C**H**$_2$CH$_2$Ph), 1.25–1.56 (16H, #m and s, 8× C**H**$_2$). ^{13}C-NMR (Acetone-d$_6$, δ ppm): 172.5 (**C**ONH), 172.2 (**C**ONH), 157.5 (1-C tyr), 144.6 (1-C CLL), 140.6 (3-C tyr), 130.7 (4-C CLL), 129.5 (2C, 3-C CLL), 129.0 (5-C tyr), 120.3 (4-C tyr), 116.2 (2-C tyr), 113.1 (6-C tyr), 112.2 (2C, 2-C CLL), 63.3 (**C**H$_2$OH), 53.0 (2C, 2× N**C**H$_2$CH$_2$Cl), 52.8 (**C**HNH), 40.7 (2C, 2× NCH$_2$**C**H$_2$Cl), 38.8, 36.8, 36.0, 35.4, 34.0, 29.6, 29.3, 29.2, 29.1, 29.0, 28.9, 27.7, 26.7, 25.6. ESI + HRMS: $[M + H]^+$ calculated for $C_{34}H_{52}Cl_2N_3O_4 = 636.3329$; found = 636.3326.

Spectral data for *N*-[(11-*N*-chlorambucilamino)undecanoyl]-L-*p*-tyrosinol (**11b**, $m = 10$). The spectral data for this particular compound were reported elsewhere and are in accordance with the literature (Descoteaux et al. 2010).

Biology

In vitro cytotoxic activity

The cytotoxicity of the tyrosine–chlorambucil regioisomers (**6a–11a** and **6b–11b**, $m = 5$ or 10) was evaluated on MCF-7 (ER$^+$) and MDA-MD-231 (ER$^-$) breast cancer cell lines. MTT (3-(4,5-dimethylthiazol-2-yl)-phenyl-tetrazolium bromide) assay, a standard colorimetric test, was used for measuring cellular proliferation (Carmichael et al. 1987). Briefly, tumor cell lines were added into 96-well

tissue culture plates in culture medium and incubated at 37°C in a 5% CO_2 atmosphere. Dilutions were done using cremophore:ethanol (1:1) solution. Cells were incubated with or without drugs for 72 h. Culture plates were processed using MTT for 3.5 h afterwards SDS solubilisation solution (HCl 0.010 M, sodium dodecyl sulfate solution 10%) was added. The absorbance was read using a scanning multiwell spectrophotometer (FLUOStar OPTIMA) at 565 nm. All measurements were carried in triplicates. The results were compared with those of a control reference plate fixed on the treatment day, and the growth inhibition percentage was calculated for each drug contact period.

Molecular modelling

17β-estradiol (**2**), L-*o*-*N*-acetyltyrosinol (**12**, *ortho*-), L-*m*–*N*-acetyltyrosinol (**12**, *meta*-) and L-*p*-*N*-acetyltyrosinol (**12**, *para*-), our model compounds, were docked into the ERα. This was done using ArgusDock docking engine, implemented in ArgusLab 4.0. This software is freely distributed for Windows platforms by Planaria Software (http://www.ArgusLab.com) (Thompson 2004). Although lagging behind in accuracy when compared to other docking programs, previous research proved that results from ArgusLab are biologically meaningful (Joy et al. 2006). The crystal structure of ERα in complexation with 17β-estradiol (1A52) was extracted from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (Tanenbaum et al. 1998; Berman et al. 2000). Ions, ligands and subunits not involved in the ligand binding were removed from the original structure file. The receptor input pdb file was then generated. Separatly, ligands files were prepared. The *N*-acetyl-tyrosinols (**12**) (Fig. 3) were used as a model for two main reasons: (1) smaller ligand involve less calculation time and more accurate results and (2) the *N*-acetyl-tyrosinol derivatives show similarities with our final tyrosine–chlorambucil hybrid molecules. For each ligand [17β-estradiol (**2**), L-*o*-, L-*m*- and L-*p*-*N*-acetyltyrosinol (**12**)], structural optimization was performed according to molecular mechanics (MM2) calculations with ChemBio3D Ultra 11.0 program. Even if the synthesis was achieved with a mixture of DL-*ortho*-tyrosine precursor, the pure L-*o*-tyrosinol enantiomer was chosen for docking calculations. The resulting ligand file was saved as a pdb file. Next, in ArgusLab, the binding site was defined. ArgusDock search docking engine was used with a grid resolution of 0.40 Å. Then, docking calculations were performed with a flexible ligand mode.

Results and discussion

Synthesis of tyrosine–chlorambucil hybrid regioisomers

The synthesis of regioisomers **6**–**11** was accomplished using the convergent synthesis previously reported for the *para*-tyrosine–chlorambucil series (Descoteaux et al. 2010) (Scheme 1). The ease of synthesis and the excellent global yields obtained in our previous study encourage us to use this methodology for the two other commercially available regioisomers of tyrosine (*ortho*- and *meta*-). Moreover, as proved in our earlier study, the initial stereochemistry of the amino acids (with D- or L-*para*-tyrosine) was maintained all along the synthesis, giving optically pure final compounds (Descoteaux et al. 2010). For this study, DL-*ortho*-tyr and L-*meta*-tyr are used as the starting material. So, the stereochemistry of the final hybrids made from L-*meta*-tyrosine was maintained during the course of the synthesis.

6-Aminohexanoic acid (**13**, $m = 5$) and 11-aminoundecanoic acid (**13**, $m = 10$) were first coupled to chlorambucil (**1**). Thus, chlorambucil (**1**) was activated [with isobutylchloroformate ($ClCO_2^iBu$)] and then coupled to the amino acid function using hexamethyldisilazane and trimethylsilyl chloride in acidic conditions. Separately, DL-*o*-tyrosine (**15**, *ortho*-), L-*m*-tyrosine (**15**, *meta*-) and L-*p*-tyrosine (**15**, *para*-) were transformed into the

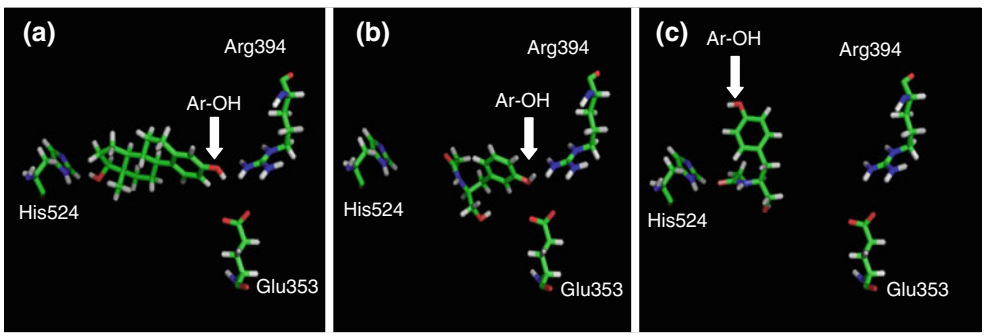
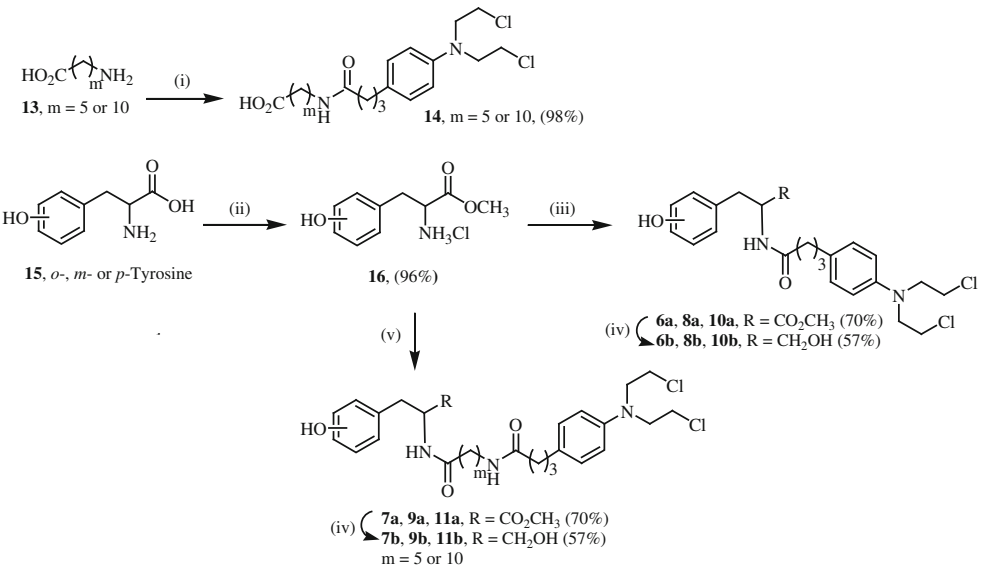


Fig. 3 17β-estradiol (**2**), L-*m*- (**12**, *meta*-) and L-*p*- (**12**, *para*-) *N*-acetyltyrosinol within the active site of the ERα. The main interactions are occurring on ring A of the steroid nucleus (**a**) or on the aromatic hydroxy group (Ar–OH) of the tyrosinol derivatives (**b**, **c**)

Scheme 1 Synthesis of tyrosine–chlorambucil hybrid regioisomers (**6a–11a** and **6b–11b**)



Reagents: i) 1. HMDS, H_2SO_4 cat., DCM, triethylamine, TMSCl; 2. CLL, HOBt, DCC, triethylamine, DMF; (ii) MeOH, $SOCl_2$,; (iii) CLL, HOBt, DCC, triethylamine, DMF; (iv) $LiBH_4$, Et_2O, 0°C to 22°C; (v) **14**, m = 5 or 10, HOBt, DCC, triethylamine, DMF.

corresponding methyl ester hydrochloride salt (**16**) with thionyl chloride in methanol. The corresponding tyrosine methyl ester hydrochloride salts were obtained in 96 to 100% yield. Then, under standard reaction conditions (HOBt and DCC), the resulting tyrosine esters were coupled to chlorambucil (**1**) via an amide function giving the tyrosine–chlorambucil hybrids without spacer. Purification gave compounds **6a**, **8a** and **10a** in 68%, 81% and 81% yield, respectively. The tyrosine methyl esters (**16**) were also linked to chlorambucil (**1**) via an alkyl chain spacer. The six carbon atoms and the eleven carbon atoms derivatives prepared earlier (**14**, $m = 5$ or 10) were coupled to the tyrosine ester (**16**) moiety. Again, standard reaction conditions (HOBt and DCC) were used for the coupling reaction. Subsequently, purification gave compounds **7a** ($m = 5$, 10), **9a** ($m = 5$, 10) and **11a** ($m = 5$, 10) in good yields (about 72%). Finally, all tyrosine–chlorambucil methyl ester derivatives (**6a**, **7a**, **8a**, **9a**, **10a**, and **11a**) were submitted to selective reduction of the ester function with lithium borohydride in dry diethyl ether. The corresponding tyrosinol-chlorambucil hybrids (**6b**, **7b**, **8b**, **9b**, **10b** and **11b**) were obtained in good to excellent yields (36–88%). All the new compounds were fully characterized by their respective IR, ^{1}H-NMR, ^{13}C-NMR and mass spectra.

In vitro biological activity

All the DL-*o*-, L-*m*- and L-*p*-tyrosine–chlorambucil hybrid regioisomers (**6–11**) were evaluated for their cytotoxic activity on estrogen-receptor positive (MCF-7, ER^+) and estrogen-receptor negative (MDA-MB-231, ER^-) tumor cell lines using the MTT colorimetric assay (Carmichael et al. 1987). Chlorambucil (**1**) was used as the control on both human mammary carcinomas.

As shown by the MTT assays, all the derivatives tested were more cytotoxic than the parent drug, chlorambucil (from 1.7 to 7.4 times), on both breast cancer cell lines (Tables 1, 2). The methyl ester (**6a–11a**) ($m = 5$, 10) as well as the tyrosinol (**6b–11b**) ($m = 5$, 10) derivatives presented comparable biological activity. Chlorambucil is a known bis-alkylation agent which forms covalent adducts with DNA (Armitage 1993). It can initiate apoptosis regardless of the presence or absence of the ER protein. However, the new tyrosine–chlorambucil hybrids show greater activity than chlorambucil (**1**) itself, which is already used for solid breast cancer treatments. This could reveal a possible beneficial role of the tyrosine ligand to target the drug where it is needed. Thus, the objective to increase the activity of chlorambucil by using an estrogen-like ligand was achieved. This particular molecular combination could possibly diversify the use for this type of nitrogen-mustard anticancer agent in the future.

The new tyrosine–chlorambucil hybrids were active on both breast cancer cell lines (ER^+ and ER^-). This can be explained by the complexity of the protein contents of these cell types. The hormone-independent MDA-MB-231 cancer cell line does not express the ERα protein, unlike the MCF-7 cell line. However, other similar proteins, which can be ER subtypes, are also present in ER^- cell lines. A possible interaction of the tyrosine with one (or more) of these proteins could explain the activity observed

Table 1 Cytocidal activity of tyrosine–chlorambucil regioisomers (**6a–11a** and **6b–11b**, $m = 5$, 10) on estrogen receptor positive (ER^+) MCF-7 breast cancer cell lines

Compound	DL-*o*		Compound	L-*m*		Compound	L-*p*	
	IC_{50}, μM[a]	CLL ratio		IC_{50}, μM[a]	CLL ratio		IC_{50}, μM[a]	CLL ratio
6a	52.63 ± 4.68	2.48	**8a**	17.72 ± 1.27	7.36	**10a**	31.92 ± 0.89	4.08
6b	20.54 ± 1.97	6.35	**8b**	23.42 ± 2.94	5.57	**10b**	34.11 ± 2.04	3.82
7a, $m = 5$	44.97 ± 3.93	2.90	**9a**, $m = 5$	18.63 ± 2.32	7.00	**11a**, $m = 5$	25.43 ± 2.06	5.13
7b, $m = 5$	43.71 ± 5.37	2.98	**9b**, $m = 5$	48.31 ± 1.36	2.70	**11b**, $m = 5$	62.16 ± 5.50	2.10
7a, $m = 10$	NR	–	**9a**, $m = 10$	41.00 ± 3.97	3.18	**11a**, $m = 10$	67.90 ± 8.68	1.92
7b, $m = 10$	47.73 ± 3.61	2.73	**9b**, $m = 10$	21.68 ± 2.63	6.01	**11b**, $m = 10$	19.39 ± 2.66	6.72
1	130.36 ± 2.92							

NR not reached

[a] Inhibitory concentration (IC_{50}, μM) as obtained by the MTT assay. Experiments were performed in duplicates and the results represent the mean ± SEM of three independent experiments. The cells were incubated for a period of 72 h

Table 2 Cytocidal activity of tyrosine–chlorambucil regioisomers (**6a–11a** and **6b–11b**, $m = 5$, 10) on estrogen receptor negative (ER^-) MDA-MB-231 breast cancer cell lines

Compound	DL-*o*		Compound	L-*m*		Compound	L-*p*	
	IC_{50}, μM[a]	CLL ratio		IC_{50}, μM[a]	CLL ratio		IC_{50}, μM[a]	CLL ratio
6a	57.45 ± 5.38	2.38	**8a**	32.24 ± 2.85	4.24	**10a**	37.83 ± 1.52	3.62
6b	26.47 ± 2.09	5.17	**8b**	28.71 ± 3.51	4.77	**10b**	39.57 ± 2.40	3.46
7a, $m = 5$	38.18 ± 2.77	3.58	**9a**, $m = 5$	34.01 ± 3.37	4.02	**11a**, $m = 5$	36.81 ± 3.97	3.72
7b, $m = 5$	33.97 ± 3.19	4.03	**9b**, $m = 5$	45.07 ± 5.04	3.04	**11b**, $m = 5$	55.09 ± 5.33	2.48
7a, $m = 10$	NR	–	**9a**, $m = 10$	63.03 ± 4.00	2.17	**11a**, $m = 10$	NR	–
7b, $m = 10$	79.37 ± 6.54	1.72	**9b**, $m = 10$	71.83 ± 5.65	1.91	**11b**, $m = 10$	NR	–
1	136.85 ± 6.79							

NR not reached

[a] Inhibitory concentration (IC_{50}, μM) as obtained by the MTT assay. Experiments were performed in duplicates and the results represent the mean ± SEM of three independent experiments. The cells were incubated for a period of 72 h

on ER^- cells with the tyrosine derivatives. Moreover, the new tyrosine–chlorambucil molecules, being bis-alkylating agents, show the same pattern of activity in vitro than chlorambucil. They are active on both cell types. It is important to indicate that the desired selectivity towards ER^+ cancer cells might be expressed more clearly in vivo as it was previously reported for other anticancer agents (Otto et al. 1991; Karl et al. 1988).

Aforementioned, this study was undertaken in order to verify the influence of the position of the phenol hydroxyl group on the biological activity. Interestingly, among all the regioisomers tested, compound **8a**, with the phenol group located in *meta* position, showed the most significant cytocidal activity [7.36 times more active than chlorambucil (**1**)]. Generally, all the L-*m*-tyrosine methyl ester derivatives are more active than their corresponding DL-*o*- and their L-*p*- analogs. Furthermore, in most cases, the L-*m*-tyrosine–chlorambucil hybrids reveals to be slightly more specific (more active) on hormone-dependent breast cancer cells compared to hormone-independent breast cancer cells. This specificity could be explained by the phenol group in *meta* position, which could possibly adopt a structure which favorises better orientation and stronger interactions within the ERα compared to the other two regioisomers (*ortho* and *para*). Otherwise, the location of the phenol group itself (*ortho*-, *meta*- or *para*-) might play a role in the overall cytocidal activity observed with these various hybrids. Indeed, it is reported that *m*-tyrosine is significantly more phytotoxic than its structural isomers *o*- and *p*-tyrosine (Bertin et al. 2007). Furthermore, it is reported that phenolic compounds exhibiting antioxidant properties can contribute to the destruction of cancer cells (Durand et al. 2009). The slightly greater activity observed for the *m*-tyrosine regioisomer might be explained by these factors being more important for the *meta* isomer compared to the *ortho* and *para* isomers.

Some molecules (**9a**, **9b**, **11a** and **11b**) ($m = 5$) have been submitted to estrogen receptor alpha affinity evaluation. Even if these specific molecules presented non negligible cytotoxic activity, only very little affinity was

observed experimentally. Consequently, further investigations will be done to elucidate the exact mode of action of these novel tyrosine–chlorambucil hybrids.

Docking

To the best of our knowledge, to date, no study have been performed in order to verify if tyrosine can theorically bind into the ERα pocket. In attempt to explain the mode of binding, preliminary molecular docking calculations have been done using Arguslab program. This was done in order to verify if the amino acid squeletton could assume an orientation close to specific residue (Arg394, Glu353 and His524) within the ERα and, by doing so, establish H bonding interactions as estradiol. Such interactions could allow the tyrosine moiety reach cells expressing ERα and then allow chlorambucil to act on cancer cells more specifically.

In light of our molecular modeling results, the three regioisomers [L-*o*-, L-*m*- and L-*p*-*N*-acetyltyrosinol, (**12**)] seem to be oriented in different ways into the ERα binding pocket (Fig. 3). Figure 3a shows the native ligand, 17β-estradiol (**2**), within the active site of the ERα. The *m*-tyrosinol derivative (**12**, *meta*-) (Fig. 3b) adopts a conformation similar to that of estradiol (Fig. 3a). The docking calculations demonstrate that the phenol group of the *m*-tyrosinol is located in the A-ring region of estradiol, near residues Arg394 and Glu353. Beside, the same docking calculations were performed with the *para* regioisomer (**12**, *para*-). This time, the same phenol group appears to be close to the His524 residue (Fig. 3c) an orientation completely opposite to that observed with *m*-tyrosinol. The L-*o*-*N*-acetyltyrosinol (**12**, *ortho*-) behaves as its *para* regioisomer (figure not shown). Hence, these results demonstrate that the *meta*-tyrosine derivative mimics the estradiol ligand much better than its *ortho* and *para* analogs. Also, all *N*-acetyltyrosine methyl esters were docked into the ERα. Each molecule behaves as its corresponding *ortho*, *meta* or *para* tyrosinol analog (**12**) where, in each cases, the phenol group is oriented similarly into the ERα pocket (figure not shown). The *m*-tyrosine ester derivative still positions itself into the ERα like the natural ligand. Of note, the docking of the various tyrosinol and tyrosine ester derivatives led repeatedly to the same results.

Furthermore, the tyrosine ligand is much smaller than estradiol. It is already known that the hydroxyl functions on the estrogenic nucleus are involved in the receptor recognition (Gabano et al. 2005). It is also known that the optimal distance between the two polar regions (3-OH and 17β-OH) should be approximately 11Å to achieve suitable binding (Muthyala et al. 2003). Probably, the distance between the two polar regions of the tyrosine unit studied (between Ar–OH and $-CO_2CH_3$ for the tyrosine methyl ester analogs or between Ar–OH and $-CH_2OH$ for the tyrosinol analogs) are not sufficiently distant to allow H bonding interactions being formed between the ligand and all the known ERα binding sites (Arg394, Glu353 and His524). So, molecules with polar regions more distant from each other would possibly fit much better into the ERα binding cavity.

Conclusion

Regiosomers of tyrosine were coupled to chlorambucil (**1**), directly or via a 5 carbon or a 10 carbon atoms spacer. Eighteen compounds were synthesized in 38 to 67% overall yield using an efficient synthetic methodology. The association of tyrosine to chlorambucil gave compounds with higher cytotoxicity than chlorambucil itself when tested on human breast cancer cell lines. One goal of this study was to verify the influence of the phenol group location on the biological activity of the new compounds. The *m*-tyrosine–chlorambucil hybrids (**6** and **8**, $m = 5, 10$) showed greater cytotoxicity compared to the other regioisomers. Moreover, the *m*-tyrosine molecules were slightly more specific for hormone-dependent cancer cells. Docking calculations gave us insight to the mode of binding of the *m*-tyrosine derivatives. The *m*-tyrosine regioisomer seems to mimic closely the 3-OH group of estradiol into the ERα pocket when compared to the two other regioisomers. This can explain, in part, the greater biological activity observed for the *meta* regioisomer. Also, the antioxidant properties of the *meta* phenolic compounds could contribute to the overall cytotoxicity. This exploratory study also suggests that larger ligands could fit more adequately into the ERα binding cavity. Hence, a second series of bulkier tyrosine–chlorambucil hybrids are presently designed in our laboratory in order to obtain ligand with enhanced affinity for the ERα.

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