Synthesis, Analysis and Biological Evaluation of Novel Steroidal Estrogenic Prodrugs for the Treatment of Breast Cancer

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Abstract

Breast cancer is the most prevalent type of cancer in pre- and postmenopausal women in most Western countries. In the treatment of metastatic breast cancer, doxorubicin has the broadest spectrum of antitumor activity of any drug currently available but produces a dose-dependent cardiomyopathy that limits its clinical usefulness. The aim of this research project was to target the affected tissues, which contain estrogen receptors (ERs). Initially, a series of estrogen derivatives with side chains linked at the 3- and 17-positions of estrone were synthesized, and then novel anticancer prodrugs were obtained from these by further linking these compounds to doxorubicin by means of various alkyl spacer groups. These estrogenic prodrugs were designed to target tumor cells containing ERs, found in human breast cancer cells, and to release the active anticancer moiety when internalized. The estrogenic prodrugs were then biologically evaluated using in vitro chemosensitivity assays against human ER-positive (MCF-7) and ER-negative (MCF-7ADR and MT-1) breast tumor cells and a leukemia (K562) cell line. The results showed that estrone derivatives with substituted aminoalcohol side chains of various lengths (2–6 carbons) linked to the 17-position of estrone were mostly inactive. Estrone-doxorubicin prodrugs containing doxorubicin at the 3-position of estrone (CCRL 1042 and CCRL 1036) were relatively inactive and nonselective against all cell lines tested. However, when doxorubicin was linked to the 17-position of estrone, these prodrugs had at least an order greater activity than their 3-linked counterparts. Using a short aminooxy-spacer group (2 carbons) at this position produced CCRL 1035, which had a lower activity against all cell lines tested compared to doxorubicin. In contrast, the prodrug incorporating doxorubicin at the 17-position of estrone via a long spacer group (12 carbons, CCRL 1033) was both potent and selective against ER-positive cells MCF-7. These studies have shown that linking doxorubicin to the 17-position of estrone via a long alkyl spacer group conferred selectivity of cytotoxic action against ER-positive breast cancer tumor cells.

Keywords: Breast cancer, cancer chemotherapy, doxorubicin, drug targeting, estrogenic prodrugs, estrogen receptor, K562, MCF-7, MCF-7ADR, MT-1, spacer group, steroids.

Introduction

Breast cancer is the most prevalent type of cancer in pre- and postmenopausal women in industrialized nations except Japan. It is estimated that nearly 1 in every 10 in the United States (Cooper, 1992) and 1 in every 12 women in the United Kingdom will develop breast cancer at some point in life (Baum et al., 1991). In the treatment of breast cancer, there are three basic strategies: surgery, radiotherapy, and chemotherapy that may be used alone or in combination. Surgery (mastectomy) is an unattractive option for women; and treatment by radiotherapy as local-regional therapy is limited in controlling primary breast cancer and nonmetastatic forms of disease. Therefore, systemic chemotherapy is mainly used to treat metastatic forms of breast cancer and prevent local recurrence of disease. Alkylating agents (e.g., nitrogen mustard) as a largest group of DNA-damaging drugs, antimetabolites [e.g., 5-fluorouracil (5-FU) and methotrexate], natural products such as Vinca alkaloids and taxol, anthracycline antibiotics such as...
doxorubicin (DOX), hormonal products, antiestrogens (e.g., tamoxifen, etc.) are the category of drugs used in systemic chemotherapy. Among these, tamoxifen, DOX (Fig. 1), and their derivatives are widely used as adjuvant therapy in patients with all stages of breast cancer (Kuo & Runowicz, 1995).

DOX, an anthracycline antibiotic with a broad spectrum of antitumor activity against human malignancies, has been in clinical use for the treatment of metastatic breast cancer for more than 25 years (Ferguson et al., 1993). However, the anthracycline drugs are associated with a dose-related, cumulative, and potentially fatal cardiomyopathy (Bielack et al., 1989), which is a limitation to prolonged clinical use. At least 55–65% of primary breast tumors and 45–55% of all advanced breast cancers are estrogen receptor (ER) positive (contain more than 10 fmol/mg cytosolic ER protein) and also contain progesterone receptors (PRs), which may respond to endocrine therapy (Wittliff, 1984).

Because DOX is relatively nonselective against hormone-dependent tumors, and resistance to tamoxifen and DOX by different molecular mechanisms is a major cause of treatment failure in breast cancer (Pasman & Schouten, 1993; Osborne et al., 1995), the design of tamoxifen and DOX prodrugs, which enhance target selectivity, have previously been investigated by this research group and others. Here we describe prodrugs in which DOX was covalently coupled to steroid carrier molecules in an attempt to achieve greater selectivity of action against hormone-dependent tumors. The type of carrier molecule, location of linkage (Eisenbrand et al., 1988), and spacer groups and their lengths (Manns et al., 1993) are important factors in prodrug design.

For a molecule to be considered as a good carrier for cell-specific targeting factors, such as distribution, selectivity, slow clearance, carrier capacity, and binding between carrier and potent drug, immunological response and toxicity of that molecule should be considered (Albert, 1985). So far, several synthetic and natural products including liposomes, antibodies, DNA, and hormones have been assessed as potential carrier molecules.

The aims of this study were the synthesis of steroidal prodrugs in which DOX is linked to 3- or 17-positions of estrone moiety via an alkyl spacer group, determination of the cytotoxicity profiles of these prodrugs against a panel of human ER-positive and ER-negative breast cancer cells plus a human leukemia cell line, and investigation of the structure-activity relationships of these ER-affinic prodrugs toward hormone-dependent tumor cells.

**Materials and Methods**

All chemicals were obtained from Aldrich Chemical Company Ltd. (Dorset, UK) except for DOX, which was obtained from David Bull Labs., UK, and all solvents were

![Figure 1. Structures of doxorubicin and its steroidal prodrugs: (I) doxorubicin and its amide analog, (II) CCRL 1035, (III) CCRL 1024 if n = 10 and CCRL 1036 if n = 4, (IV) CCRL 1033.](image-url)
purchased from Fisons (Loughborough, UK). Reagents were used as purchased, and solvents were dried using type 4-A molecular sieves.

Proof of chemical purity was established by normal spectral (IR, NMR, MS) and analytical (TLC and chemical analysis) techniques. Polyester coated by silica gel GF254 as thin-layer chromatography (TLC) plates (Aldrich) were pre-washed with methanol/dichloromethane (50:50) and activated in an oven at 110°C for 1 h before use and visualized by UV light at 254 nm developed by 50% (conc.) H2SO4 in methanol or by iodine vapor.

Melting points (m.p.) were determined on an Electro thermal apparatus, IA9000 series (Essex, UK), and are uncorrected.

IR spectra were obtained in a 1% KBr disk using a Perkin Elmer (USA) 297 infrared spectrophotometer.

1H and 13C NMR were determined at 270 MHz and 67 MHz, respectively, on a JEOL JNM-GX270 Fourier transform nuclear magnetic resonance (FT-NMR) spectrometer, with 5-mm tubes.

Mass spectra were recorded on a AEI (Kratos, University of Newcastle, UK) MS902 updated with a MSS Console and data system, accelerating voltage 8kV, 70 eV, 300μA emission; fast atom bombardment (FAB) spectra were obtained using a Kratos MS50 spectrometer.

Elemental analysis for C, H, and N were obtained using a CE Elantech (Lakewood, NJ, USA) 1106 Elemental Analyzer and weighed using a Mettler MT 5 microbalance.

Compound names are identified initially using the IUPAC nomenclature and then by a product registration specific number for the Cancer Chemotherapy Research Laboratory (CCRL) in the University of Bradford.

Synthesis of prodrugs

Synthesis of 17 aminooxy-estrone-doxorubicin prodrugs (CCRL 1035, II)

Estrone (0.5 g, 1.85 mmol) and carboxymethoxylamine hemichloride (0.8 g, 3.7 mmol) were dissolved in dry pyridine (20 ml) and refluxed at 65°C for 3 h. After the completion of reaction, the reaction mixture was cooled to room temperature and diluted with 150 ml of chilled water and extracted with ethyl acetate (4 × 200 ml) after acidification with dilute 2 M HCl (pH 5.5). The organic extracts were combined, washed with water (300 ml), dried over anhydrous Na2SO4, and the solvent was removed by rotary evaporator to give 550 mg of product. Recrystallization from methanol (50 ml) gave 450 mg (71% yield) of product so-called CCRL 1035 (Rf, 0.8; m.p., 186–188°C). CCRL 1035 (20 mg, 60μmol) and DOX HCl (20 mg, 36.8μmol) were reacted following a modification method of Hartman et al. (1988) to give 24 mg of CCRL 1035 (Fig. 1). The Rf of the compound was 0.72 using normal phase TLC with a mobile phase of CHCl3: methanol (95:5 v/v).

m.p.: 207–209°C, IR: 3625–3417 cm−1 (OH and NH), 1723–1644 cm−1 (C=O), 1616 and 1502 cm−1 (aromatic ring), 1230 and 1018 cm−1 (C–N), 1169–1066 cm−1 (C–O).

13C NMR (DMSO): The 13C NMR spectrum showed the following resonance in ppm: 125.9 (C1-O), 112.7 (C2-O), 154.9 (C3-O), 114.8 (C4-O), 136.9 (C5-O), 37.7 (C6-O), 26.7 (C7-O), 28.9 (C8-O), 43.3 (C9-O), 129.9 (C10-O), 24.4 (C11-O), 33.7 (C12-O), 44.1 (C13-O), 52.1 (C14-O), 22.4 (C15-O), 35.6 (C16-O), 207.6 (C17-O), 16.8 (C18-O), 43.8 (C1-S), 227.8 (C2-S), 168.0 (C1-X), 120.7 (C2-X), 136.9 (C3-X), 120.6 (C4-X), 171.9 (C5-X), 154.9 (C6-X), 33.7 (C7-X), 83.7 (C8-X), 38.5 (C9-X), 67.8 (C10-X), 152.6 (C11-X), 203.8 (C12-X), 218.4 (C13-X), 65.3 (C14-X), 130.5 (C4a-X), 112.7 (C5a-X), 136.8 (C6a-X), 136.9 (C10a-X), 112.6 (C11a-X), 117.5 (C12a-X), 90.9 (C1’-X), 28.7 (C2’-X), 44.1 (C3’-X), 71.9 (C4’-X), 67.9 (C5’-X). (“O” refers to estrone carbon, “X” refers to doxorubicin carbon, and “S” refers to spacer group carbon.)

Mass spectrum: m/z (relative intensity), 868 (M+, 5%), 773 (M–C6H10O2), 740 (M–C6H9NO2, daunomycin sugar), 712 (M–C6H9NO2), 601 (M–C5H2NO), 214 (M–C1H2O), 167 (base peak, 100%).

Elemental analysis: Calculated for C17H19NO4S, found: C, 58.57%; H, 5.73%; N, 2.77%. Requires: C, 64.97%; H, 5.99%; N, 3.22%. Calculated for C17H19NO4S, 5H2O requires: C, 58.80%; H, 6.47%; N, 2.92%.

Synthesis of 3-estrone dodecanedioyl ester-DOX prodrug (CCRL 1024, III if n = 10)

Estrone (756 mg, 2.8 mmol) and dodecanedioyl chloride (2.1 ml, 4.8 mmol) were reacted in dry pyridine (20 ml) at room temperature for 6–8 h under N2 gas. When the reaction was completed, pyridine was evaporated using N2 gas, and pure CCRL 1022 product was obtained by column chromatography (420 mg, 31% yield) using a mobile phase of dichloromethane: methanol (95:5); m.p., 139–141°C.

DOX (15 mg, 20μmol) was dissolved in 15 ml dichloromethane and stirred for 20 min. CCRL 1022 (15 mg, 30μmol) and N,N-dicyclohexylcarbodiimide (DCC, 30 mg) were then added and stirred at RT for 24 h. The product was removed by extraction from 2 M phosphate buffer, pH 5.0 into dichloromethane (3 × 20 ml). The organic phases were combined, dried over anhydrous sodium sulfate, and solvent was removed under vacuum to give a pink solid (10 mg, 40% yield) as CCRL 1024 with an Rf value of 0.9, using normal phase TLC with a mobile phase of n-butanol:water:acetic acid (60:25:15).

m.p.: 165–166°C, IR: 3635–3434 cm−1 (OH and NH), 3031 cm−1 (= C–H), 2960–2876 cm−1 (aliphatic CH2), 1720–1656 cm−1 (C = O), 1619 and 1514 cm−1 (aromatic rings), 1235 and 1016 cm−1 (C–N), 1173–1074 cm−1 (C–O).

13C NMR (DMSO): The 13C NMR spectrum showed the following resonance in ppm: 119.9 (C1-O), 110.7 (C2-O), 155.4 (C3-O), 118.9 (C4-O), 135.0 (C5-O), 38.4 (C6-O), 27.5 (C7-O), 38.7 (C8-O), 40.3 (C9-O), 134.0 (C10-O), 27.4 (C11-O), 38.7 (C12-O), 40.3 (C13-O), 56.5 (C14-O), 18.8
(C15-O), 39.7 (C16-O), 213.7 (C17-O), 16.9 (C18-O), 186.5 (C1-S), 38.4 (C2-S), 38.7–40.3 (C3-C11-S), 40.0 (C12-S), 160.7 (C13-S), 160.7 (C1-X), 119.7 (C2-X), 134.6 (C3-X), 120.0 (C4-X), 186.5 (C5-X), 155.4 (C6-X), 38.4 (C7-X), 75.0 (C8-X), 38.7 (C9-X), 69.5 (C10-X), 156.0 (C11-X), 186.5 (C12-X), 213.7 (C13-X), 66.6 (C14-X), 134.6 (C4a-X), 110.7 (C5a-X), 134.6 (C6a-X), 134.5 (C10a-X), 110.7 (C11a-X), 120.0 (C12a-X), 56.5 (CH2O-X), 100.2 (C1'-X), 27.5 (C2'-X), 40.3 (C3'-X), 66.6 (C4'-X), 66.5 (C5'-X), 16.9 (C H3 -5'X). ("O" refers to estrone carbon, "X" refers to doxorubicin carbon, and "S" refers to spacer group carbon.)

Mass spectrum: m/z (relative intensity), 1007 (M+', 5%), 958 (M' -CH2O2), 904 (M' -C6H2O3, daunosamine sugar), 838 (M' -CH4O2, estrone moiety minus CH3), 752 (M' -C2H4O2 estrone moiety minus CH2), 529 (M' -C6H5O2, 486 (M'-C3H6O2 estrone moiety plus spacer group), 311 (M' -C8H17O2), 193 (M' -C5H3NO3, spacer group).

Elemental analysis: Calculated for C37H49 NO14, found: C, 67.18%; H, 6.52%; N, 1.57%. Requires: C, 67.92%; H, 6.85%; N, 1.39%.

Synthesis of DOX-dodecanedioyl ester (CCRL 1023, I)

DOX (6 mg) was reacted with dodecanediolyl chloride (6 µl, 200 µmol) in dichloromethane (10 ml) using n,N-dicyclohexyl Carbodiimide (DCC) (14 mg, 70 µmol) and triethylamine (250 µl) at RT overnight. Any unreacted DOX was removed by distillation. The reaction mixture was stirred at RT for 8 h under N2. After completion of reaction (checked by TLC), pyridine was removed, using N2 gas; pure product was obtained by column chromatography (toluene : ethyl acetate 5 : 1 was used as mobile phase). Evaporation of solvent gave 80 mg (22% yield) of analytically pure compound called CCRL 1020; melting point (178–180°C). Other analysis experiments confirmed the synthesis of desired compound. CCRL 1020 (40 mg, 0.11 mmol) was dissolved in 5 ml of dry dimethyl formamide (DMF) and stirred under N2 gas at ~10°C. Ten milligrams (14 µl, 0.1 mmol) of triethylamine and (20 µl, 0.15 mmol) of isobutyl chloroformate were added and stirred for 20 min. A mixture of DOX, HCl (20 mg, 34.5 µmol), and triethylamine (20 µl) in 5 ml of DMF was then added and stirred at ~10°C for 1 h and at RT for 30 min. Distilled water (20 ml) was added and extracted with dichloromethane (4 × 20 ml). The organic phase was washed with 4% aqueous sodium hydrogen carbonate (2 × 50 ml), followed by a solution of saturated NaCl (2 × 50 ml), and then air-dried over anhydrous sodium sulfate and removed under vacuum. The residue was crystallized from dry anhydrous diethyl ether (10 ml). The pinkish crystals were recrystallized from methanol (30 ml) to produce 20 mg (43% yield) of CCRL 1036 as product. Rf, 0.65; m.p., 168–170 °C (decomp).

IR: 3625–3434 cm⁻¹ (OH and NH), 3040 cm⁻¹ (= C-H), 2960–2875 cm⁻¹ (aliphatic CH2), 1722–1658 cm⁻¹ (C=O), 1619 and 1503 cm⁻¹ (aromatic ring), 1235 cm⁻¹ (C-N), 1016–1174 cm⁻¹ (C-O).

13C NMR (DMSO): The 13C-NMR spectrum showed the following resonance in ppm: 119.9 (C1-O), 110.7 (C2-O), 155.4 (C3-O), 118.9 (C4-O), 135.0 (C5-O), 38.4 (C6-O), 27.5 (C7-O), 38.7 (C8-O), 40.3 (C9-O), 134.0 (C10-O), 27.4 (C11-O), 38.7 (C12-O), 40.3 (C13-O), 56.5 (C14-O), 18.8 (C15-O), 39.7 (C16-O), 213.7 (C17-O), 16.9 (C18-O), 186.5 (C1-S), 38.4 (C2-S), 38.7–40.2 (C3,4-S), 40.0 (C5-S), 155.4 (C6-S), 160.7 (C1-X), 119.7 (C2-X), 134.6 (C3-X), 120.0 (C4-X), 186.5 (C5-X), 155.4 (C6-X), 38.4 (C7-X), 75.0 (C8-X), 38.7 (C9-X), 69.5 (C10-X), 156.0 (C11-X), 186.5 (C12-X), 213.7 (C13-X), 66.6 (C14-X), 134.6 (C4a-X), 110.7 (C5a-X), 134.6 (C6a-X), 134.5 (C10a-X), 110.7 (C11a-X), 120.0 (C12a-X), 56.5 (CH2O-X), 100.2 (C1'-X), 27.5 (C2'-X), 40.3 (C3'-X), 66.6 (C4'-X), 66.5 (C5'-X), 16.9 (CH3-5'X). ("O" refers to estrone carbons, "X" refers to doxorubicin carbons, and "S" refers to spacer group carbon.)

Mass spectrum: m/z (relative intensity), 923 (M'), 767 (M' -C5H13O, 5%), 666 (M' -C4H9O2, 45%), 414 (M' -C3H6O3, DOX aglycone, 50%), 321, 289, 230, 154 (M'-767, base peak, 100%), 136 (daunosamine sugar, 80%).

Elemental analysis: Calculated for C51H57NO15, found: C, 65.92%; H, 6.07%; N, 1.67%. Requires: C, 66.30%; H, 6.17%; N, 1.51%.

Synthesis of estrone 17-amino-dodecane-DOX prodrug (CCRL 1033, IV)

Estrone (1.6 g, 5.9 mmol) was dissolved in dry pyridine (15 ml), and hydroxylamine HCl (840 mg, 12 mmol) was
dissolved in dry absolute ethanol (12 ml). The two solutions were combined and stirred at RT for 12 h. After completion, the reaction mixture was concentrated by rotary evaporator, filtered, and vacuum dried. Recrystallization from methanol (50 ml) gave 1.5 g (91% yield) of product called CCRL 1030. R<sub>r</sub> 0.1; m.p., 230–233 °C.

CCRL 1030 (0.8 g, 2.8 mmol) was dissolved in 60 °C n-butanol (70 ml), and dry metallic sodium (2 g, 87 mmol) was added in small pieces and refluxed until dissolved. The solvent was removed, and residue was redissolved in 50 ml water. This aqueous solution was purged with gaseous CO<sub>2</sub> to precipitate the product, which was filtered and washed with 3 × 100 ml warm water (60 °C). Recrystallization from methanol (30 ml) gave 0.4 g (yield 53%) compound called CCRL 1031. R<sub>r</sub> 0.24; m.p., 225–228 °C.

CCRL 1031 (500 mg, 1.85 mmol) was dissolved in 50 ml of methanol, and 1,10-dodecanedicarboxylic acid (500 mg, 2.17 mmol) was added and stirred at RT. 1-Ethyl-3-(3′-dimethylaminopropyl) carbodiimide (EDAC, 500 mg, 2.6 mmol) was then added to the mixture and stirred under N<sub>2</sub> gas at RT overnight. Methanol was removed under vacuum, and residue was dissolved in aqueous solution of HCl (1 M, 60 ml) and extracted with dichloromethane (4 × 50 ml). The organic phase was washed with 100 ml of distilled water and concentrated to 50 ml, extracted with NaOH (2 M, 4 × 40 ml). The aqueous extract was then acidified using dilute HCl (4 M) and then extracted with ether (4 × 100 ml). Organic layer dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Recrystallization from methanol (30 ml) gave 0.4 g (yield 53%) compound called CCRL 1032. R<sub>r</sub> 0.2; m.p., 166–168 °C.

CCRL 1032 (50 mg, 0.1 mmol) was reacted with DOX, HCl (50 mg, 0.086 mmol) following the method described for CCRL 1036 (see previous section) to give 39 mg (46% yield) of pinkish compound. Recrystallization from methanol (10 ml) gave pure CCRL 1033 as product. R<sub>r</sub> 0.65 using methanol/dichloromethane (1:9 v/v) as mobile phase; m.p., 177–179 °C (decomp).

IR: 3624–3437 cm<sup>-1</sup> (OH and NH), 2959–2856 cm<sup>-1</sup> (aliphatic CH<sub>2</sub>), 1721–1659 cm<sup>-1</sup> (C=O), 1618 and 1513 cm<sup>-1</sup> (aromatic ring), 1235 and 1017 cm<sup>-1</sup> (C–O). ¹³C NMR (DMSO): The ¹³C NMR spectrum showed the following resonance in ppm: 120.0 (C1-O), 110.7 (C2-O), 154.4 (C3-O), 118.9 (C4-O), 136.2 (C5-O), 38.4 (C6-O), 27.5 (C7-O), 29.8 (C8-O), 40.4 (C9-O), 134.0 (C10-O), 27.5 (C11-O), 31.9 (C12-O), 46.9 (C13-O), 49.7 (C14-O), 18.9 (C15-O), 36.5 (C16-O), 40.0 (C17-O), 18.8 (C18-O), 213.7 (C1-S), 38.7 (C2-S), 38.8 (C3-S), 39.1 (C4-S), 39.4 (C5-S), 39.7 (C6-S), 40.1 (C7-S), 40.4 (C8-S), 46.5 (C9-S), 46.8 (C10-S), 46.3 (C11-S), 46.5 (C12-S), 69.8 (C13-S), 185.6 (C14-S), 160.7 (C15-S), 119.6 (C2-X), 136.1 (C3-X), 119.9 (C4-X), 186.4 (C5-X), 155.4 (C6-X), 32.0 (C7-X), 74.8 (C8-X), 36.5 (C9-X), 69.5 (C10-X), 156.0 (C11-X), 186.4 (C12-X), 213.7 (C13-X), 66.4 (C14-X), 134.6 (C14a-X), 110.5 (C5a-X), 134.0 (C6a-X), 134.6 (C10a-X), 110.7 (C11a-X), 119.9 (C12a-X), 56.5 (CH<sub>2</sub>O-X), 100.2 (C1'-X), 29.7 (C2'-X), 46.8 (C3'-X), 67.9 (C4'-X), 66.6 (C5'-X), 16.9 (CH<sub>3</sub>5'-X). (“O” refers to estrone carbon, “X” refers to doxorubicin carbon, and “S” refers to spacer group carbon.)

Mass spectrum: m/z (relative intensity), 1008 (M<sup>+</sup>, 15%), 799 (M<sup>+</sup>-C<sub>6</sub>H<sub>5</sub>O< sub>3</sub>), 676 (M<sup>+</sup>-C<sub>2</sub>H<sub>4</sub>N<sub>2</sub>O<sub>4</sub>), 498 (M<sup>+</sup>-C<sub>7</sub>H<sub>3</sub>NO<sub>4</sub>), 289 (30%), 270 (M<sup>+</sup>-C<sub>10</sub>H<sub>2</sub>O<sub>2</sub>), 40%, 225 (M<sup>+</sup>-C<sub>3</sub>H<sub>2</sub>N<sub>2</sub>O<sub>4</sub> spacer group fragment, 80%), 154 (M<sup>+</sup>-C<sub>2</sub>H<sub>3</sub>N<sub>2</sub>O sugar moiety plus attached carbonyl group fragment, 80%), 136 (M<sup>+</sup>-C<sub>6</sub>H<sub>5</sub>NO<sub>2</sub> base peak 100%).

Elemental analysis: Calculated for C<sub>13</sub>H<sub>21</sub>N<sub>2</sub>O<sub>12</sub>, found: C, 68.05%; H, 7.32%; N, 3.17%. Requires: C, 67.85%; H, 7.14%; N, 2.77%.

**Cell lines**

The breast tumor cell line MCF-7 and MCF-7ADR (resistant to DOX and related compounds) were obtained from the National Cancer Institute (NCI), and MT-1 was derived from solid tumor by Hambly et al. (1994). The leukemia cell line K562 was obtained from European tissue culture collection, characterized by NCI and Hambly and his colleagues in the Clinical Oncology Unit, University of Bradford.

**Preparation of drug solutions**

Stock solutions of DOX and CCRL compounds were prepared in DMSO and then diluted with fresh media prior to use, so that the final concentration of DMSO was <0.1%. All compounds were diluted by a factor of 10 on addition to the cell lines to give final concentration in the range of 0.001 μM to 1 mM in logarithmic increments.

**Preparation of culture media**

RPMI 1640 liquid culture medium was purchased from Life Technologies (Paisley, UK), and to each 500 ml were added fetal calf serum (50 ml), penicillin/streptomycin 5000 IU ml<sup>-1</sup>/50 μg ml<sup>-1</sup> (5 ml), sodium pyruvate 100 mM (5 ml), and L-glutamine 200 mM (5 ml), using a sterile microbiological filter (Costar). Prepared media were kept at 4–8 °C for less than a month.

**MTT chemosensitivity assay (96-h continuous exposure method)**

Assays were carried out in round-bottomed 96-well culture plates. Cells were harvested simply from the suspension culture (K562 a leukemia cell line) or by trypsinization (MCF-7, MCF-7ADR, and MT-1) of monolayer cells during the exponential growth phase and were plated per well in 180 μl RPMI 1640 medium. Then, 20 μl of the appropriate concentration of each CCRL compound was added to give a final volume of 200 μl per well. A control was also incorporated, and also a further blank control was used that contained only RPMI 1640 medium. Plates were then incubated at 37 °C in an atmosphere of 5% CO<sub>2</sub> and 95% air. After 96 h
Results and Discussion

The five new steroidal-DOX prodrugs [CCRL 1023 (I), CCRL 1024 (II), CCRL 1033 (IV), CCRL 1035 (II) and CCRL 1036 (IV)] (Fig. 1) were prepared from estrone, DOX, and spacer groups according to modification methods described by Hartman et al. (1988). All intermediates and final compounds were characterized by typical spectroscopic methods and by satisfactory elemental microanalysis. Chemosensitivity of human breast and leukemia tumor cell lines against these compounds were determined according to semi-logarithmic graphs of % cell survival against compound concentration were produced, and IC₅₀ values were estimated.

The IC₅₀ values of tested compounds are shown in Table 1. The standard antitumor agent DOX and its analog CCRL 1023 were nonselective, giving similar IC₅₀ values in each cell line except for MCF-7ADR, as expected. However, the DOX amide analog CCRL 1023 was less cytotoxic than DOX against all cell lines tested. CCRL 1036 and CCRL 1024 were relatively inactive (IC₅₀ > 10 μM) and nonselective against all breast cancer cell lines tested. CCRL 1035 was inactive against all breast cancer cell lines in these studies (IC₅₀ > 100 μM) but showed some activity against the K562 leukemia cell line (IC₅₀ = 11.7 μM). In contrast, CCRL 1033 was selective for MCF-7 (estrogen receptor positive, ER⁺) breast tumor cells and was almost similar in potency to DOX (IC₅₀ = 0.7 μM compared to 0.5 μM for DOX), whereas it was relatively nonselective and more than 10-fold less active (IC₅₀ = 10.5 μM) against all ER-negative breast tumor cells and the K562 leukemia cell line.

These results show that DOX is nearly 10-fold more toxic than its amide analog (CCRL 1023), which confirms that the amino group of DOX is important for its biological activity, as it stabilizes the intercalation of the aglycone between adjacent base pairs of DNA (Masquelier et al., 1980). This intercalation may then cause inhibition of topoisomerase II.

The presence of a free hydroxyl group at either the 3- or 17-position of steroids is essential to maintain their affinity for ERs. The lower toxicity and lack of selectivity for ERs observed for CCRL 1024 and CCRL 1036 may be explained by an absence of free hydroxyl groups on these estrone moieties of prodrugs.

CCRL 1035, which was inactive even though it possesses a free phenolic OH at the 3-position of the estrone moiety, has a short chain spacer group (two carbons) in the 17-position that may facilitate degradation of prodrug in the culture medium as previously reported for a similar type of spacer group by Manns et al. (1993). This may be responsible for its low toxicity and lack of selectivity. However, replacing the short chain spacer group at the 17-position of estrone in CCRL 1035 with a longer alkyl group (12 carbons) results in a potent and selective prodrug (CCRL 1033) against the human ER-positive breast cancer cell line MCF-7.

Conclusions

Dox and its analog CCRL 1023 are potent but nonselective antitumor agents. Linking the alkyl spacer chain via the amine group in daunosamine sugar did not affect the selectivity but reduced cytotoxicity. Estrone amide prodrugs incorporating DOX require spacer chains greater than six carbons in length plus either a 3- or 17-hydroxyl group in the steroid moiety for ER selectivity.

CCRL 1033 (DOX amide-linked to 17-position of estrone via a 12-carbon alkyl spacer group) is a potential lead ER-affinic prodrug for the development of novel estrogenic/antiestrogenic antitumor prodrugs targeted to ER-positive breast tumors.

References


Table 1. IC₅₀ values (μM ± SD) for antitumor agents: DOX, its ester analog (CCRL 1023), and estrogenic novel prodrugs (n = 6).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MCF-7</th>
<th>MT-1</th>
<th>MCF-7ADR</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOX</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>&gt;10.0</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>CCRL 1023</td>
<td>9.0 ± 2.4</td>
<td>5.0 ± 1.2</td>
<td>22.0 ± 4.3</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>CCRL 1024</td>
<td>10.5 ± 5.2</td>
<td>10.5 ± 2.3</td>
<td>9.0 ± 4.0</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>CCRL 1033</td>
<td>0.7 ± 0.3</td>
<td>10.4 ± 2.1</td>
<td>10.5 ± 5.1</td>
<td>10.5 ± 4.4</td>
</tr>
<tr>
<td>CCRL 1035</td>
<td>&gt;100.0</td>
<td>&gt;100.0</td>
<td>&gt;100.0</td>
<td>11.7 ± 4.1</td>
</tr>
<tr>
<td>CCRL 1036</td>
<td>11.0 ± 0.1</td>
<td>12.0 ± 1.2</td>
<td>11.5 ± 2.6</td>
<td>11.0 ± 2.3</td>
</tr>
</tbody>
</table>


