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Synthesis of androstene oxime-nitrogen mustard bioconjugates as potent antineoplastic agents

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ABSTRACT

In the present study, synthesis and antineoplastic activity of phenylacetic acid and benzoic acid nitrogen mustard conjugates of various steroidal oximes are reported for the first time. The conjugation was achieved through a more stable oxime-ester linkage and the resulting newly synthesized conjugates were evaluated *in vitro* on various human cancer cell lines for cytotoxicity. The extent of their alkylating activity was investigated by the *in vitro* colorimetric 4-(*p*-nitrobenzyl)pyridine (NBP) assay. The 17*E*-steroidal oxime-benzoic acid mustard ester 3β-acetoxy-17*E*-[*p*-(*N*,*N*-bis(2-chloroethyl)amino)]benzoyloxyimino-androst-5-ene (**8**) emerged as the most potent conjugate having significant cytotoxicity on most of the NCI 60-cell lines. Outstanding growth inhibition was observed on the IGROV1 ovarian cancer cell line with GI_{50} = 0.937 μM. In general, the D-ring derived androstene oxime-nitrogen mustard conjugates were found to possess better antineoplastic activity over a variety of cancer cells in comparison to those derived from other rings of the steroid skeleton.

1. Introduction

Conjugating alkylating agents with steroidal nuclei has been an attractive approach to fabricate cancer specific cytotoxic agents for targeting various hormone responsive tumors such as breast, prostate and endometrium. Conceptualizing that the steroidal part could play the role of the “biological platform” on which the alkylator could easily be transported through the cellular barrier to the target site, we expected several advantages such as reduced systemic toxicity, increased bioavailability, reduced dose, synergistic activity and improved specificity of cancer therapy following this approach [1]. Conjugates of nitrogen mustards with various steroidal skeletons like androstane, estrane, homo-aza-steroids and homo-aza-steroidal lactams have been synthesized to generate several potent cancer specific cytotoxic agents. This concept has led to the successful generation of a steroidal alkylating agent called estramustine phosphate sodium (**1**) (Fig. 1), which is being used in the palliative treatment of metastatic and/or progressive carcinoma of the prostate [2]. However, on further studies estarmustine was found to be devoid of any hormonal or alkylating effect, rather it binds to tubulin causing microtubule depolymerization [3,4]. This observation suggested that the steroidal alkylating agent conjugates may have their own mode of action with improved specificity towards the cancer tissue.

Studies have revealed that the steroidal part is not a mere “biological carrier” as speculated for many years in conjugates, but have their own specific cellular binding site(s) as well [5]. Hormone receptors present in the cell nucleus might be the main binding site of the steroidal part [6]. Changing the steroid skeleton or introduction of heteroatoms in the steroid skeleton have improved the inactive or already active compounds to strongly active ones. It is also observed that addition of the steroid moiety confers enhanced activity compared to the alkylating agent alone [7,8]. In some cases, toxicity remained at clinically acceptable levels and was significantly lower than the alkylating agent itself [9].

These facts encouraged us to further explore the steroidal backbone for attachment with the nitrogen mustards. Earlier, the alkylating agents have been successfully conjugated to steroid systems through a labile ester or amide linkage. It is observed that both, the position of attachment of nitrogen mustard and the basic framework of steroid nucleus, are of equal importance for the antineoplastic activity. The A- and D-rings-derived aza-steroids and homo-aza-steroidal lactams (Fig. 2) are preferred platforms for the improvement in biological activity compared to their B- and C-ring substituted counterparts [10]. The ideal nitrogen mustard moieties recognized to conjugate with these steroidal skeletons are chlorambucil, phenylacetic acid (active metabolite of chlorambucil), benzoic acid and cinnamic acid mustards (Fig. 2) [6–10]. Benzoic acid mustard and phenylacetic acid mustards conjugated to C-3 and C-17 positions of A- and D- ring modified aza-steroids and homo-aza-steroidal lactams have come out as the best templates to synthesize powerful antineoplastic agents belonging to this category [5].

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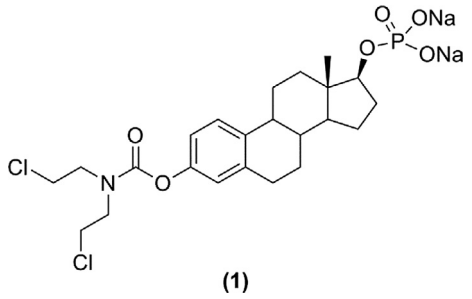

Fig. 1. Structure of estramustine phosphate sodium.

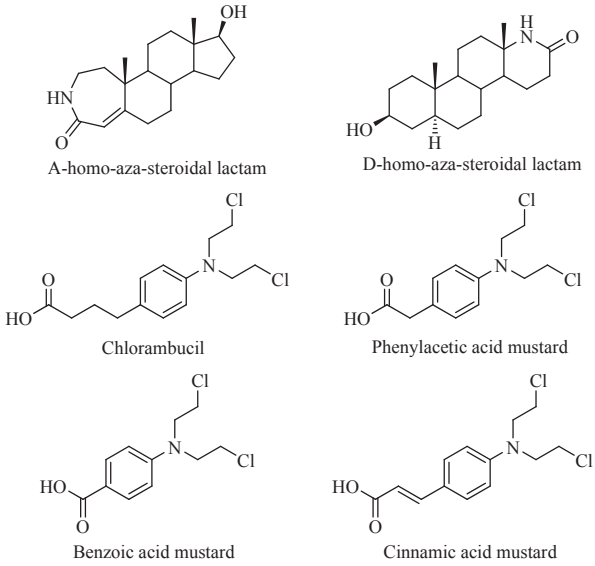

Fig. 2. Structures of the ideal steroid and mustard moieties used in the synthesis of potent antineoplastic agents.

Steroidal oximes positioned on the A- and D-rings of androstenes have structural similarity with the A- and D- ring modified aza-steroids and homo-aza-steroidal lactams. Moreover, these oximes are known to have potent cytotoxicity of their own [11,12]. Taking note of these observations, we envisioned the synthesis of esters of benzoic acid and phenylacetic acid mustards with oximes present on the A- and D-ring of androstene and a study of the effect of these modifications on the antineoplastic properties of the steroid, which we report herein.

2. Experimental section

2.1. Materials, reagents and instruments

Dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), dimethylaminopyridine (DMAP), 4-(*p*-nitrobenzyl)pyridine (NBP), deuterated-chloroform ($CDCl_3$) and triethylamine were purchased from Sigma-Aldrich Corporation, India. Sodium sulphate, sodium carbonate, silica gel-G for column chromatography (80–100 mesh size) and solvents were purchased from Merck India Pvt Ltd. Solvents were distilled and dried according to the standard procedures prior to use.

Melting points were determined on a precision digital melting point apparatus (VEEGO instruments corporation, Mumbai, India) and are uncorrected. UV readings were taken on a Perkin-Elmer LAMBDA-15 double beam spectrophotometer (Perkin-Elmer Inc., USA) supplied with matched square cuvettes of 1.0 cm light path length. Infrared spectra (wavenumbers in cm^{-1}) were recorded on Perkin-Elmer RX1 FTIR spectrophotometer (Perkin-Elmer Inc., USA) model using potassium bromide pellets. ^{1}H NMR spectra were obtained on a Bruker Avance II 400 MHz spectrometer (Bruker Corporation, Billerica, USA) using $CDCl_3$ as solvents containing tetramethylsilane as the internal standard (chemical shifts in δ, ppm). The spin multiplicities are indicated by the symbols, s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Mass spectra were obtained on an Applied Biosystems API 2000™ mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA, USA). Elemental analyses were carried out on a Perkin Elmer-2400 model CHN analyzer (Perkin-Elmer Inc., USA). The purity of the synthesized conjugates was found to be ≥95% based on the elemental analysis. Plates for thin layer chromatography (TLC) were prepared with silica gel G according to method of Stahl (E. Merck) using ethyl acetate as solvent and activated at 110 °C for 30 min. Iodine was used to develop the TLC plates.

2.2. Synthesis of steroidal oxime-nitrogen mustard conjugates

The starting nitrogen mustards *p*-(*N*,*N*-bis(2-chloroethyl)amino) benzoic acid; benzoic acid mustard (**2**) and *p*-(*N*,*N*-bis-(2-chloroethyl) amino)phenylacetic acid; phenylacetic acid mustard (**3**) were synthesized in accordance with the literature procedure [13,14]. The steroidal oximes, 3β-acetoxy-17*E*-hydroximino-androst-5-ene (**4**), 17β-acetoxy-3*E/Z*-hydroximino-androst-4-ene (**5**), 6*E*-hydroximino-androst-4-ene-3,17-dione (**7**) and 3β-hydroxy-16*E*-oximino-androst-5-en-17-one (**6**) were synthesized as reported, and purified by recrystallization from methanol to afford sufficiently pure compounds [12,15,16].

2.2.1. General method for the preparation of conjugates *8–12*

To a mixture of the steroidal oxime (0.60 mmol, 0.207 g for **4**, **5** and 0.19 g for **6**) and requisite nitrogen mustard (0.76 mmol, 0.2 g for **2** and 0.21 g for **3**) in anhydrous dichloromethane (10 mL), DCC (0.2 g, 0.97 mmol) and DMAP (0.12 g, 0.98 mmol) were sequentially added. The resulting mixture was stirred overnight. The insoluble precipitate of urea formed was filtered off. The filtrate was evaporated and acetonitrile (50 mL) was added to precipitate the residual byproduct (3 times), which was filtered and the filtrate was vacuum evaporated. The residue obtained was extracted with dichloromethane and sequentially washed with 1 N HCl, H_2O, 5% Na_2CO_3 and H_2O. The organic layer was dried over anhydrous Na_2SO_4, vacuum evaporated and purified either by recrystallization or subjecting to a column of silica gel (mesh size 80–100) using chloroform as an eluting solvent to yield the corresponding steroidal nitrogen mustard conjugates **8–12**.

2.2.1.1. 3β-Acetoxy-17E-[p-(N,N-bis(2-chloroethyl)amino)] benzoyloxyimino-androst-5-ene (8). The conjugate **8** was purified by recrystallization from a mixture of ether and hexane (50:50) to yield white needle shaped crystals (30%), m.p. 140–142 °C. FT-IRν_{max} (KBr): 2956, 1731, 1607, 1520, 1367, 1254, 1179, 1089, 833, 758. ^{1}H NMR ($CDCl_3$): δ 1.05 (s, 6H, 18 and 19-CH_3), 2.17 (s, 3H, -OCOCH_3), 3.66 (t, 4H, -N-(CH_2CH_2Cl)$_2$, J = 6.76 Hz), 3.81 (t, 4H, -N-(CH_2CH_2Cl)$_2$, J = 6.88 Hz), 4.59–4.62 (m, 1H, 3α-*H*), 5.39 (d, 1H, 6-C*H*, J = 4.96 Hz), 6.67 (d, 2H, -C*H*, J_o = 9 Hz, aromatic), 7.94 (d, 2H, -C*H*, J_o = 8.88 Hz, aromatic). ^{13}C NMR ($CDCl_3$): δ 16.84 (**C**H$_3$), 19.35 (**C**H$_3$), 20.47 (CH$_2$), 21.43 (**C**H), 23.20 (**C**H$_2$), 27.21 (**C**H$_2$), 27.70 (**C**H$_2$), 31.33 (**C**H$_3$), 33.57 (**C**H$_2$), 36.71 (**C**), 36.93 (2 X **C**H$_2$), 38.10 (**C**H$_2$), 40.10 (2 X **C**H$_2$), 45.03 (**C**), 49.99 (CH), 53.29 (2 X **C**H$_2$), 53.99 (**C**H),), 73.77 (**C**H), 110.92 (2 X Ar**C**H), 117.93 (**C**), 121.85 (**C**H), 131.76 (2 X Ar**C**H), 140.0 (Ar**C**), 149.76 (Ar**C**), 163.97 (C═O), 170.54 (C═N), 178.82 (C═O), ESI-MS *m/z*: 589.1 $[MH]^+$, 591.1 $[MH+2]^+$. Anal. calcd for $C_{32}H_{42}Cl_2N_2O_4$: C, 65.19; H, 7.18; N, 4.75. Found: C, 65.24; H, 7.23; N, 4.80.

2.2.1.2. 3β-Acetoxy-17E-[p-(N,N-bis(2-chloroethyl)amino)] phenylacetoxyimino-androst-5-ene (9). The residue obtained after workup was purified by column chromatography to yield orange colored conjugate **9** (46%), m.p. 74–76 °C. FT-IRν_{max} (KBr): 2942, 1732, 1615, 1520, 1444, 1360, 1244, 1114, 1030, 918, 803. ^{1}H NMR