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Original article

To determine the cytotoxicity of chlorambucil and one of its nitro-derivatives, conjugated to prasterone and pregnenolone, towards eight human cancer cell-lines

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ABSTRACT

Four ester prodrugs derived from the bifunctional alkylating agent chlorambucil, and one of its nitro-derivatives, 3-nitrochlorambucil conjugated to prasterone and pregnenolone, were synthesized and tested for their cytotoxic activity against eight human cell lines, using the standard MTT assay. A comparison between the esters and the controls, namely chlorambucil and 3-nitrochlorambucil would suggest that all four esters possess to varying degrees, specificity towards the breast adenocarcinoma cell line (MDA-mb468) than the other seven cells' lines tested. The overall findings are encouraging since it infers that these lipophilic esters not only have the ability to traverse specific cell membranes but also exhibit cytotoxicity towards most of the cell lines tested.

1. Introduction

Chlorambucil, also known as 4-{4-[di(2-chloroethyl)amino] phenyl}butyric acid, was first synthesised in 1953. It is an aromatic nitrogen mustard and its cytotoxicity as a bifunctional alkylating agent is due to its ability to cross-link between the bases of DNA [1]. Chlorambucil is still used as one of the front-line drugs in the treatment of chronic lymphocytic leukaemia, malignant lymphomas and advanced ovarian and breast carcinomas. Clinical use of chlorambucil is however limited by its toxic side-effects [2,3]. At physiological pH chlorambucil is predominantly ionized [4] and therefore may not rely solely on passive diffusion [5,6] in order to traverse biological membranes and reach the targeted tumour cells. It is also known to bind to plasma constituents such as albumin which reduces its ability to effectively target tumour cells [7].

The aim of this study was to determine the cytotoxicity of four steroidal esters of prasterone and pregnenolone, linked to bifunctional alkylating agents, chlorambucil and its nitro-derivative, 3-nitrochlorambucil, against three breast adenocarcinoma cell lines, namely, the estrogen-positive breast adenocarcinoma (MDA-mb468) the estrogen-negative breast adenocarcinoma (MCF-7), and the transfected breast adenocarcinoma (MDA NQ01). In addition to these cell lines, the study was also carried out to investigate the cytotoxic effects of the esters on a broader spectrum of cell types, namely, colon adenocarcinoma (Widr), brain posterior fossa medullablastoma (Daoy), lung large cell carcinoma (H460), ovarian adenocarcinoma (OVCA-3) and skin malignant melanoma (A375). The presence of the strong withdrawing nitro-group, *ortho* to the *N*,*N*-bis(2-chloroethyl) moiety [8] should reduce the reactivity of alkylating moiety by limiting the formation of the highly reactive aziridine intermediate [9]. The masking of the ionisable carboxylic moiety of chlorambucil and the corresponding nitro-derivative, via an ester bridge to a steroidal moiety, should increase the overall lipophilicity of the nitrogen mustard and possibly enhance the compounds ability to traverse cell membranes [10]. Furthermore,

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the steroidal moiety could possibly enhance the selectivity of the alkylating moiety by targeting tumour cells that have hormone receptors.

Prednimustine and bestrabucil are two clinically available hormonal alkylating agents that were specifically designed to enhance selectivity of the chlorambucil moiety. Prednimustine, derived from the combination of chlorambucil and prednisolone linked via an ester bridge, was designed to mask the carboxylic acid and to selectively target tumour cells with specific hormone receptors [11]. It was proved to be effective in the management of a number of leukaemias and lymphomas [12,13]. Bestrabucil, the benzoate of the estradiol-chlorambucil conjugate, was initially developed as a target orientated anticancer agent for the therapy of estrogen receptor-positive breast cancer [14]. Research data supporting the targeting nature of this conjugate has shown that concentration of bestrabucil and some of its derivatives, accumulate 5–10 times higher in tumour tissue of the sensitive xenografts, than in blood and muscle tissue [15].

Although prasterone and pregnenolone (Fig. 1), are known to be important steroidal intermediates in the biosynthesis of various hormones [16,17], relatively few studies have explored their potential as carriers. In addition, because there is growing interest in both prasterone and pregnenolone [18–26], it would be useful to explore the potential of these steroidal compounds linked to chlorambucil and the corresponding nitro-derivative, 3-nitrochlorambucil as potential carriers. The four esters in question (Table 1) were chlorambucil and the 3-nitrochlorambucil esters of prasterone (**C1** and **C2**), and chlorambucil and the 3-nitrochlorambucil esters of pregnenolone (**C3** and **C4**).

2. Chemistry

The esters were prepared by treating chlorambucil separately with prasterone and pregnenolone in the presence of DCC and DMAP at room temperature in dichloromethane [27] (Scheme 1). The nitro-derivatives were prepared in a similar manner after obtaining 3-nitrochlorambucil (**C6**) by treating chlorambucil at 0 °C with nitronium tetrafluoroborate in acetonitrile [8]. The four esters were tested *in vitro* for their cytotoxic effects against eight human cell lines using a growth inhibition assay. Chlorambucil (**C5**) and 3-nitrochlorambucil (**C6**) were used as controls in the investigation.

Prior to measure the cytotoxic effect of the esters on the eight human cancer cell lines, measurements involving the determination of the partition coefficients, the hydrolyzing properties and the alkylating activities of these four esters together with chlorambucil and 3-nitrochlorambucil were carried out. The measured partition coefficients for the four esters were found to be greater than 8.00 and were compared with values obtained by the application of a computer aided program. The estimated partition coefficients for the four esters ranged from 8.54 ± 0.46 to 9.18 ± 0.42, confirming the highly lipophilic nature of the esters. The partition coefficients of chlorambucil and 3-nitrochlorambucil were found to be

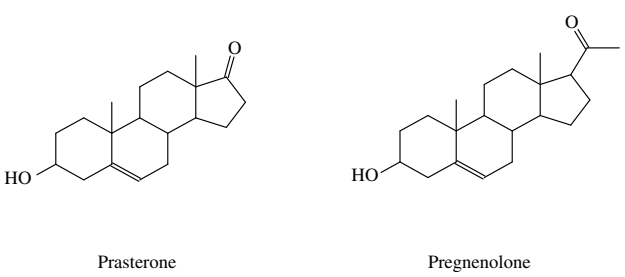
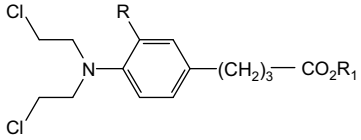


Fig. 1. Structures of prasterone and pregnenolone.

Table 1
Steroid esters of 4-{4-[di(2-chloroethyl)amino]phenyl}butanoic acid (chlorambucil).



Compound	R_1	R	Formula
C1	Prasterone	H	$C_{33}H_{45}Cl_2NO_3$
C2	Prasterone	NO_2	$C_{33}H_{44}Cl_2N_2O_5$
C3	Pregnenolone	H	$C_{35}H_{49}Cl_2NO_3$
C4	Pregnenolone	NO_2	$C_{35}H_{48}Cl_2N_2O_5$
C5	H	H	$C_{14}H_{19}Cl_2NO_2$
C6	H	NO_2	$C_{14}H_{18}Cl_2N_2O_4$

C1 is chlorambucil ester of prasterone; **C2** is 3-nitrochlorambucil ester of prasterone; **C3** is chlorambucil ester of pregnenolone; **C4** is 3-nitrochlorambucil ester of pregnenolone; **C5** is chlorambucil; **C6** is 3-nitrochlorambucil.

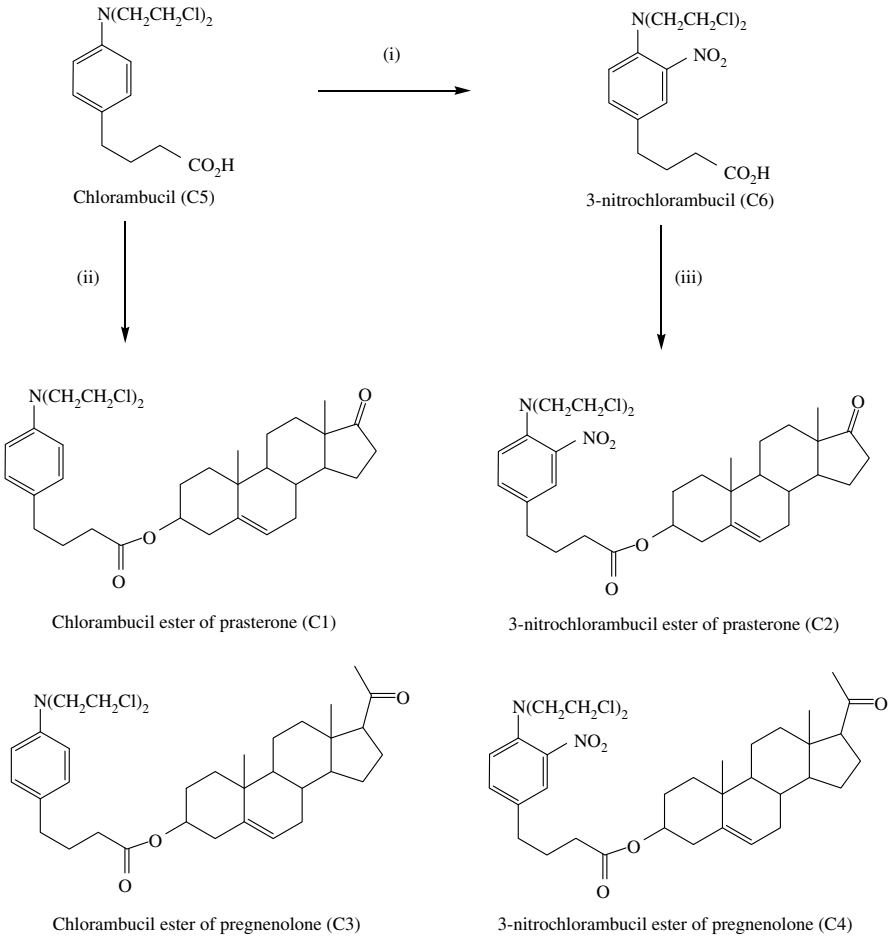
3.80 ± 0.30 and 3.70 ± 0.35, respectively, and were in close agreement with the estimated values (Table 2). The measured partition coefficient of chlorambucil was also found to be in close agreement to previous studies [10] The determined alkylating activity of 3-nitrochlorambucil ester of prasterone (**C2**) and 3-nitrochlorambucil ester of pregnenolone (**C4**) was significantly lower, presumably due to the electron withdrawing effect of the nitro-group attached *ortho* to the *N*,*N*-bis (2-chloroethyl) moiety reducing the activity of the alkylating moiety. In addition, the determined alkylating activity of the esters was much lower than that of the alkylating activity of chlorambucil and 3-nitrochlorambucil.

A similar phenomenon was observed in earlier studies [10]. It was found that on increasing the complexity of chlorambucil esters by the addition of carbons, a sharp decrease in their alkylating activity resulted. Interestingly, there was a rough correlation between the alkylating activity and the cytotoxicity of the compounds with a number of the cell lines tested.

3. Pharmacology and pharmacokinetics

A HPLC method was developed to monitor the hydrolysis of the esters and to confirm the esters possible role as prodrugs. Although the present work was centered on chlorambucil and 3-nitrochlorambucil esters of both prasterone and pregnenolone, during the developmental phase of the HPLC method, two additional esters were also analyzed, namely 3,6-dinitrochlorambucil esters of prasterone and pregnenolone. The retention times of these esters together with the esters of interest are shown in Table 3.

The method was initially developed to monitor, separately, the appearance of either chlorambucil, 3-nitrochlorambucil or 3,6-dinitrochlorambucil, however, it was found that observing the loss in peak area of the ester over time was a more accurate and reliable procedure for monitoring hydrolysis. The validation of the HPLC method also included the simultaneous separation of all the esters (see Fig. 2) in addition to the separate analysis of each of the esters. Fig. 3 shows an example of a chromatogram of 3-nitrochlorambucil ester of prasterone (**C2**). Conveniently, the hydrolysis is readily monitored by the decrease in peak area corresponding to the ester over time. On storing both the prasterone and pregnenolone derivatives of the nitrochlorambucil at 37 °C in phosphate buffer saline (pH 7.4) over a period of forty five days in sealed sample bottles resulted in 46% and 48% degradations, respectively, of these esters. This was also supported by infrared spectroscopy involving the decrease in the carbonyl ester with time. Interestingly, it has been reported that the half-life of chlorambucil in PBS is approximately 60 min, with the formation of the dihydroxy derivative 4-{4-[di(2-hydroxyethyl)amino] phenyl}butyric acid. Fortunately,



Scheme 1. Synthesis of **C1**–**C4** and **C6**. Reagents and conditions: (i) NO_2BF_4/CH_3CN (1.2 equiv.) at 0 °C under argon. (ii) Chlorambucil/CH_2Cl_2/DCC/DMAP in the presence of prasterone or pregnenolone at room temperature. (iii) 3-Nitrochlorambucil/CH2Cl2/DCC/DMAP in the presence of prasterone or pregnenolone at room temperature.

this degradation is not observed in either blood or plasma [28]. It was found that on incubating separately, all four esters (**C1**–**C4**) at 37 °C in the presence of porcine liver esterase, the rate of hydrolysis ranged from 24 to 37% and 83 to 94%, over a 2 h and a 24 h periods, respectively. Grieg et al. reported the hydrolysis of various chlorambucil ester derivatives using non-specific plasma esterases measured *in vivo*, using rat plasma, resulted in rapid cleavage ranging from less than 10 s to 5 min, depending on the size of the ester [10]. However, Grieg et al. also determined the rate of hydrolysis of chlorambucil tertiary butyl ester in freshly prepared rat liver and blood, and found that this ester was relatively stable with half-lives of approximately 2 h and 7 h, respectively [29]. This phenomenon was attributed to the steric hindrance of the tertiary butyl group. Interestingly, the esters involved in this study have similar stereochemistry's to the chlorambucil tertiary butyl esters, and after incubating them in fresh human plasma over a period 24 h, the percent of the prasterone and pregnenolone derivatives that had hydrolysed ranged from 51 to 55 and 37 to 39%, respectively.

The chlorambucil ester of prasterone (**C1**) showed activity against all the cell-lines with the exception of (Widr). The highest activity for this ester was against (MDA-mb468) with an IC_{50} of

Table 2
Log *P* partition coefficient, and *in vitro* alkylating activity of chlorambucil and chlorambucil derivatives.

Compound	Determined partition coefficient[a]	Estimated partition coefficient[b]	Alkylating activity (% of chlorambucil)[c]
C1	>8.00	8.64 ± 0.44	70
C2	>8.00	8.57 ± 0.46	8
C3	>8.00	9.25 ± 0.41	18
C4	>8.00	9.18 ± 0.42	4
C5	3.80 ± 0.30	3.70 ± 0.35	100
C6	3.70 ± 0.35	3.63 ± 0.37	38

C1 is chlorambucil ester of prasterone; **C2** is 3-nitrochlorambucil ester of prasterone; **C3** is chlorambucil ester of pregnenolone; **C4** is 3-nitrochlorambucil ester of pregnenolone; **C5** is chlorambucil; **C6** is 3-nitrochlorambucil.
[a] Determined values using the method described by Leo et al. [45].
[b] Estimated values obtained from a ACD/Log *P* software package.
[c] Measured *in vitro* at 37 °C at concentrations ranging between 0.1 and 1.0 mM. Chlorambucil is assigned 100% alkylation.

Table 3
The assignment of the peaks in the chromatogram of all the esters.

Assignment of peaks	Compound	Retention time (min)
A	3,6-Dinitrochlorambucil ester of prasterone[a]	5.74
B	3-Nitrochlorambucil ester of prasterone	7.17
C	3,6-Dinitrochlorambucil ester of pregnenolone[a]	8.64
D	Chlorambucil ester of prasterone	10.08
E	3-Nitrochlorambucil ester of pregnenolone	11.39
F	Chlorambucil ester of pregnenolone	16.88

The hydrolysis of esters **C1**–**C4** was monitored separately using porcine liver esterase and fresh human plasma.
[a] These esters were used in another HPLC study and were not analyzed for their cytotoxic properties. In addition, neither of these esters were subjected to hydrolysis using porcine liver esterase or fresh human plasma.

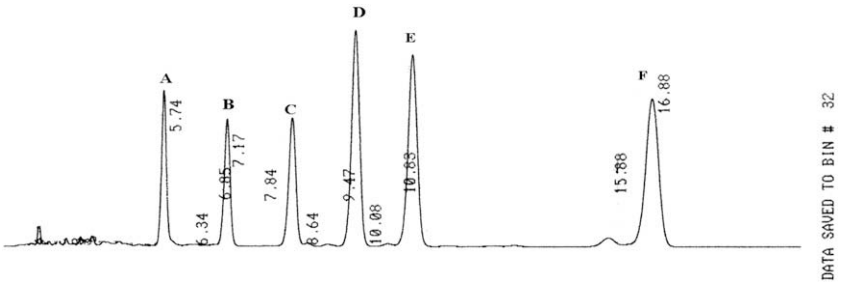


Fig. 2. A chromatogram of the simultaneous separation of all the esters indicated in Table 3. RP-HPLC conditions: mobile phase was acetic acid (50 ml of a 2% solution) made upto a litre with acetonitrile, pH = 5.2 at a flow rate of 1.2 ml/min. A C18 Symmetry Waters column (250 mm × 4.6 mm ID) fitted with a guard column (3 cm × 4.6 mm ID) was used and the wavelength was set at 254 nm.

5.48 μM. The 3-nitrochlorambucil ester of prasterone (**C2**) was only cytotoxic against (MDA-mb468) and the transfected (MDA-NQO1) cell line, with IC_{50}'s of 7.25 and 10.60 μM, respectively. The chlorambucil ester of pregnenolone (**C3**) was cytotoxic to varying degrees against all of the cell-lines, the least being the MCF-7 cell line which expresses estrogen and progesterone receptors [30], however, it was most active against MDA-mb468 cell line with an IC_{50} of 10.26 μM. The 3-nitrochlorambucil ester of pregnenolone (**C4**) was cytotoxic against (MDA-mb468), the transfected (MDA-NQO1) and the (MCF-7) cell-lines, with IC_{50}'s of 19.34, 32.56 and 68.30 μM, respectively. Both chlorambucil and 3-nitrochlorambucil were used as controls in the study involving the eight cell-lines, and afforded IC_{50}'s ranging from 0.65 to 7.13 μM and 1.16 to 13.04 μM, respectively. The effect of the nitro-group was clearly evident when comparing the cytotoxicity of chlorambucil (**C5**) and 3-nitrochlorambucil (**C6**), where the reduced activity of 3-nitrochlorambucil was attributed to the effect of the nitro-group *ortho* to *N*,*N*-bis(2-chloroethyl) moiety and closely correlated with the *in vitro* determination of the alkylating activity using *p*-nitrobenzyl pyridine. As a control measure, the cytotoxicity of both prasterone and pregnenolone was also carried out on the cell lines and was found to be non-cytotoxic at concentrations below 100 μM. The IC_{50} data can be found in Table 4.

4. Results and discussion

A number of interesting findings were obtained from investigating the activity of the compounds against the tested cell lines. The most significant finding involved the breast adenocarcinoma (MDA-mb468) cell line, where all the compounds exhibited cytotoxic activity ranging from 0.65 μM to 19.34 μM. The reason for using the transfected breast adenocarcinoma (MDA-NQO1) cell line was to assess the possible bioreductive potential of the nitro-compounds, since the added flavoenzyme NAD(P)H: quinone oxidoreductase (DT-diaphorase), via an oxygen independent pathway [31], catalyzes two-electron reduction of quinones and nitro-compounds to hydroquinones and nitroso compounds, respectively [32]. In addition, there is an over expression of DT-diaphorase throughout many tumor tissues [33,34] and its activity and gene expression have been found to be up regulated in comparison to tissue levels in lung, colon, liver and breast tumors [35,36]. DT-diaphorase is therefore an attractive target for the development of bioreductively activated chemotherapeutic agents [37]. Furthermore, DT-diaphorase is also capable of detoxifying certain potentially carcinogenic xenobiotics [38]. However, the determined cytotoxicity of the tested compounds was found to be between 1.5 and 2 fold greater in the (MDA-mb468) compared to the (MDA-NQO1) cell line. These results did not therefore provide conclusive evidence that the esters (**C2** and **C4**) were reduced to the corresponding nitroso or hydroxylamine derivatives but may suggest that the transfected cell line exhibits greater resistance by possibly detoxifying the compounds via the DT-diaphorase dependent pathway, whereas, the (MDA-mb468) cell line, possessing no functional diaphorase, is less resistant. The esters were tested for their cytotoxicity against the breast adenocarcinoma (MCF-7) cell line in order to confirm whether they displayed any specificity against estrogen dependent cell lines. However, mild cytotoxicity was only observed with esters (**C1**, **C3** and **C4**), affording IC_{50}'s of 72.5, 90 and 68.3 μM, respectively. The determined IC_{50}'s for chlorambucil and nitrochlorambucil were 4.5 and 23 μM, respectively and would therefore imply that esters (**C1**, **C3** and **C4**) do not exhibit any significant specificity for the estrogen dependent cell line.

A comparison between the IC_{50} data obtained for prasterone and pregnenolone esters, would suggest that prasterone esters are in general, more cytotoxic and the data roughly correlates with that

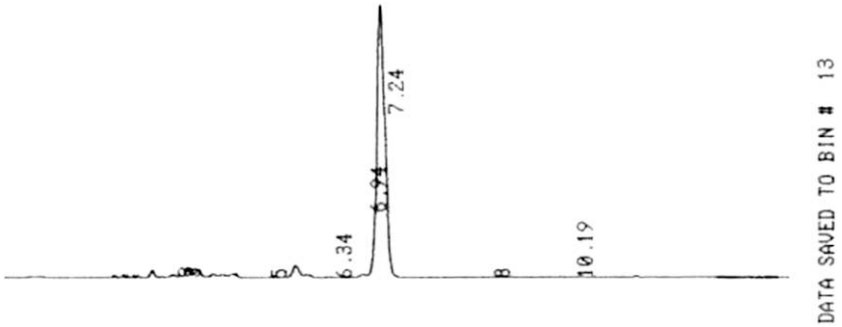


Fig. 3. An example of a chromatogram of one of the esters, namely, 3-nitrochlorambucil ester of prasterone mentioned in Table 3 with a retention time of 7.17 min. RP-HPLC conditions: mobile phase was acetic acid (50 ml of a 2% solution) made upto a litre with acetonitrile, pH = 5.2 at a flow rate of 1.2 ml/min. A C18 Symmetry Waters column (250 mm × 4.6 mm ID) fitted with a guard column (3 cm × 4.6 mm ID) was used and the wavelength was set at 254 nm.

Table 4
The IC_{50} values for chlorambucil and the chlorambucil derivatives on eight human cell-lines.

Cell line IC_{50} μM	Widr	Daoy	MDA468	MDA NQ01	MCF-7	H460	OVCA-3	A375
C1	>100	19.61 (±6.9)	5.48 (±5.6)	11.83 (±1.4)	72.5 (±2.0)	11.85 (±1.5)	82.50 (±1.2)	61.42 (±8.9)
C2	>100	>100	7.25 (±3.1)	10.60 (±5.7)	>100	>100	>100	>100
C3	86.30 (±2.0)	41.90 (±1.3)	10.26 (±6.7)	20.53 (±2.1)	90 (±1.5)	36.16 (±1.4)	88.80 (±3.7)	74.21 (±7.2)
C4	>100	>100	19.34 (±3.4)	32.56 (±4.3)	68.3 (±2.5)	>100	>100	>100
C5	7.13 (±5.1)	1.36 (±1.5)	0.65 (±3.0)	0.79 (±2.9)	4.5 (±1.3)	2.19 (±6.6)	8.92 (±2.7)	2.58 (±6.2)
C6	13.04 (±3.9)	5.30 (±3.7)	1.16 (±6.3)	3.75 (±4.0)	23 (±3.1)	9.53 (±1.1)	15.56 (±2.1)	9.46 (±7.1)

All values are given in μM, however, the values shown in brackets are the standard deviations for the % growth inhibition.
C1 is chlorambucil ester of prasterone; **C2** is 3-nitrochlorambucil ester of prasterone; **C3** is chlorambucil ester of pregnenolone; **C4** is 3-nitrochlorambucil ester of pregnenolone; **C5** is chlorambucil; **C6** is 3-nitrochlorambucil.

obtained for their alkylating activity. Furthermore, due to the presence of the nitro-group in esters (**C2** and **C4**), the determined alkylating activity was significantly reduced compared to esters (**C1** and **C3**) which roughly correlated with the activity obtained for these esters in all but two of the cell lines tested. Generally, when a strong electron withdrawing group such as a nitro-group is attached *ortho* to the *N*,*N*-bis (2-chloroethyl) moiety, the potential to alkylate is reduced. This phenomena was observed when comparing compounds **C1** with **C2**, **C3** with **C4**, **C5** with **C6**, in addition to **C1** with **C4** and **C2** with **C3**. However, there is no correlation, as one would expect, between the magnitude of alkylation and the degree of cytotoxicity in the cell lines tested.

5. Conclusion

The findings are encouraging because the four esters possess, to varying degrees, some specificity towards the breast adenocarcinoma cell line (MDA-mb468), with chlorambucil esters of prasterone (**C1** and **C2**) displaying higher activity. However the chlorambucil ester of prasterone (**C1**) and the nitrochlorambucil ester of pregnenolone (**C3**) also exhibited relatively significant cytotoxic activity towards brain posterior fossa, medullablastoma (Daoy) and lung large cell carcinoma (H460) cell lines. An additional advantage of linking chlorambucil and its nitro-derivative to prasterone and pregnenolone is that the resulting esters exhibit increased lipophilic properties, which should thus increase their ability to traverse blood-tissue barriers. However, this must be balanced by their ability to be cleaved at some stage in order to avoid possible accumulation within the cell membrane and thus be prevented from entering the cytosol. From the results, one can conclude that the esters did not show improved cytotoxicity towards the cell lines tested, compared to the parent drugs, in fact, it was not the intention to develop compounds with greater activity but compounds that possess cytotoxicity similar to or close to the parent drugs, in the hope of being more selective to cancerous cells, and less damaging to normal cells and tissues. A comparison between the activity of the esters and the parent drugs, does however, require some degree of caution, since it is inconclusive as to whether the difference in activity was due to intrinsic properties of the esters or whether it was attributed to the partial or complete bioconversion of the esters into the active parent drugs. It would certainly be of interest to determine the precise mode of action of these esters in order to confirm whether or not they truly function as prodrugs. The esters may also be subject to efflux by P-glycoproteins or by ABC transporters, thus limiting their bioavailability [39–42]. It would be of interest to investigate the cytotoxicities of these esters in cells that over-express P-glycoproteins, initially targeting the breast adenocarcinoma cell line (MCF-7) using matched controls and an MCF-7 cell line that over-expresses *P*-glycoprotein. Studies are now underway in order to answer some of these questions.

6. Experimental section

6.1. Materials

The IR spectra for the compounds were recorded as either a chloroform solution or as an oil using a Genesis FTIR spectrometer. ^{1}H NMR were recorded in deuterated chloroform using a Bruker drx500 (500 MHz), a amx360 (360 MHz), and a Avance dpx300 (300 MHz) instrument. Tetramethylsilane was used as internal standard, and *J* values are given in Hz. ^{13}C NMR spectra were recorded using the listed NMR spectrometers at 125.77, 90.55 and 75.47 MHz, respectively. Mass spectra were recorded on a VG ZAB 2SE high resolution mass spectrometer, with Opus V3.1 and DEC 3000 Alpha Station and microanalysis were recorded on a LEEMANS C E440 Elemental Analyzer. Reactions were monitored, whenever possible by TLC on silica gel plates (G_{254}) and column chromatography was performed using C60 silica gel (35–70 μm). Samples were centrifuged using a Sanyo MSE Microcentaur and esters were incubated (hydrolysed) in a Memmert UM200 incubator.

Three HPLC systems were used at various stages of the investigation – GBC LC 1110 HPLC pump connected with a 20 μl loop, a GBC LC 1210 UV/vis detector and a Hewlett Packard HP3395 integrator. The second system involved the use of a Varian Vista Series 5000 LC pump connected to a 20 μl loop, a Waters 486 UV/vis detector and a Hewlett Packard HP3395 integrator. The third system involved the use of a Gilson 305 pump, connected to a 20 μl loop, a Dionex UVD 340S diode array detector controlled by a Dell Optiplex GX1 computer equipped with Chromeleon (version 6.10). The unit was connected to a Hewlett Packard DeskJet 890C printer.

Chemicals: dichloromethane, tertiary butanol, *N*,*N*-dicyclohexylcarbodiimide, 4-dimethylaminopyridine, nitronium tetrafluoroborate, sodium acetate, chlorambucil, isopropanol, *n*-octanol, HPLC grade acetonitrile, sodium chloride, potassium dihyhrogen phosphate, disodium hydrogen phosphate potassium chloride, porcine liver esterase, suspended in 3.2 M ammonium sulphate. (340 μl equivalent to 16.5 mg of protein), absolute ethanol and water were all purchased from Sigma. Ethyl acetate, C60 silica gel, light petroleum ether (40–60), ethanol (96%), deuterated chloroform and acetone (analar) were purchased from Merck. Pregnenolone and prasterone were purchased from Acros Organics.

4-(4-Nitrobenzyl) pyridine and 3-amino-1-propanol were purchased from Lancaster Research Chemicals. The media used for the tissue culture was RPMI 1460 supplemented with 2 mM L-glutamine and 10% foetal bovine serum. The RPMI and L-glutamine were purchased from Sigma while the foetal bovine serum was purchased from PAA Laboratories.

6.2. Methods

6.2.1. Cell lines used in the investigation

The ATCC human cell lines used in the study were cultured at The Patersons Institute for Cancer Research, Manchester, UK and

The Cancer Research Council Unit, Bradford, UK. The cell lines used were: colon adenocarcinoma (Widr); brain posterior fossa medullablastoma (Daoy); breast adenocarcinoma (MDA-mb468); breast adenocarcinoma cell line (MDA-NQO1) transfected with flavoenzyme NAD(P)H: quinone oxidoreductase (DT-diaphorase); breast adenocarcinoma (MCF-7); lung large cell carcinoma (H460); ovarian adenocarcinoma (OVCA-3) and skin malignant melanoma (A375).

*6.2.2. General procedures for the synthesis of compounds **C1**, **C2**, **C3**, **C4** and **C6***

*6.2.2.1. Synthesis of 4-{4-[di(2-chloroethyl)amino]-3-nitrophenyl} butanoic acid (**C6**).* A solution of nitronium tetrafluoroborate (2.0 g, 15.64 mmol) in acetonitrile (60 ml) was stirred at 0 °C under argon, and after 15 min chlorambucil (4.0 g, 13.16 mmol) in acetonitrile (60 ml) was added dropwise over a period of 10 min. After being stirred for an additional 40 min at 0 °C, the reaction mixture was stirred at room temperature for 1 h and then poured into an excess of water (150 ml). The organic material was extracted with dichloromethane (3 × 60 ml), washed with water (3 × 60 ml) and dried over anhydrous sodium sulphate. The sodium sulphate was removed by filtration and the dichloromethane was removed by evaporation under reduced pressure to afford the crude product which was chromatographed on a column of silica gel with ethyl acetate-light petroleum (2:1) as eluent to give the 3-nitrochlorambucil as a dark orange oil (2.51 g, 55%). Found: C, 47.9; H, 5.40; N, 8.20; Cl, 20.20%. $C_{14}H_{18}Cl_2N_2O_4$ requires C, 48.15; H, 5.20; N, 8.00; Cl, 20.30%; ν_{max} ($CHCl_3$)/cm^{-1} 1720 (C═O) and 2800–3500 br (OH); δ_H (300 MHz; $CDCl_3$) 1.98 (2H, m, CH_2CH$_2$CO$_2$H), 2.42 (2H, t, *J* 6.9, CH$_2$CH_2CO$_2$H), 2.70 (2H, t, *J* 7.5, ArCH_2), 3.50 [8H, m, N(CH_2CH_2Cl)$_2$], 7.30 (1H, d, *J* 9.0, *meta* to NO_2, $C_6H_3NO_2$), 7.35 (1H, dd, *J* 8.2 and 2.0, *para* to NO_2, $C_6H_3NO_2$), and 7.54 (1H, d, *J* 2.0, *ortho* to $NO_2C_6H_3NO_2$); δ_C ($CDCl_3$) 25.8 (*C*H$_2$CH$_2$CO$_2$H), 32.9 and 33.9 (*C*H$_2$CH$_2$CH$_2$), 41.5 (2 × *C*H$_2$Cl), 55.9 (2 × H$_2$*C*N), 124.8 [*C*H, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 126.8 (*C*H, *ortho* to NO_2), 133.3 (*C*H, *para* to NO_2), 138.2 (*C*–CH$_2$), 140.6 (*C*–NO_2), 146,4 [*C*–N(CH$_2$CH$_2$ Cl)$_2$], 177.0 (*C*O$_2$H); *m*/*z* (EI) 348.

*6.2.2.2. Synthesis of (3β)-3-hydroxyandrost-5-ene-17-one, 4-{4-[di(2-chloroethyl)amino] phenyl}butyrate (**C1**).* General procedure was used for preparing all four esters.

A solution of chlorambucil (1.0 g, 3.29 mmol) in dichloromethane (40 ml) was stirred at room temperature for 10 min, after which time, prasterone (1.09 g, 3.78 mmol) in dichloromethane (10 ml) was added dropwise over 5 min. A solution of DCC (780 mg, 3.78 mmol) in dichloromethane (20 ml) was added after which time DMAP (1.2 mg, 9.8 μmol) was added to catalyze the reaction. The reaction mixture was sealed and stirred for 20 h at room temperature. The resulting suspension was treated with acetonitrile (30 ml) in order to enhance the precipitation of the by-products. The precipitate was filtered and the solvents were evaporated under reduced pressure to afford the crude product. The crude product was redissolved in ethyl acetate (50 ml), leaving behind undissolved by-product which was removed by filtration. The ethyl acetate was removed by evaporation under reduced pressure to give an oil which was chromatographed on a column of silica gel using ethyl acetate-light petroleum (1:2) as eluent to afford the ester as an off-white oil (1.19 g, 63%). Found: C, 68.53; H, 7.41; N, 2.40; Cl, 12.19%. $C_{33}H_{45}Cl_2NO_3$ requires C, 68.97; H, 7.89; N, 2.43; Cl, 12.33%; ν_{max} ($CHCl_3$)/cm^{-1} 1731 cm^{-1} (C═O); δ_H (300 MHz; $CDCl_3$) 0.88 (3H, s, CH_3 on C18), 1.05 (3H, s, CH_3 on C19), 2.30 (2H, t, CH$_2$CH_2CO$_2$), 2.36 (1H, m, C*H*, on C8), 2.41 (2H, m, CH_2, on C16), 2.56 (2H, m, ArCH_2), 3.60–3.72 (8H, m, 2 × CH_2Cl and 2 × CH_2CH$_2$Cl), 4.61 (1H, m, C*H*, on C3), 5.40 (1H, d, C*H*, on C6), 6.65 [2H, d, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 7.08 [2H, d, *meta* to N(CH$_2$CH$_2$Cl)$_2$]; δ_C ($CDCl_3$) 13.5 (C18), 19.5 (C19), 20.5 (C11), 22.2 (C15), 26.4 (*C*H$_2$CH$_2$CO$_2$), 27.2 (C2), 29.6 (C12), 31.5 (C7)[1], 31.8 (C8)[1], 34.1 (ArCH$_2$ and CH$_2$CH$_2$CO$_2$), 36.0 (C16), 36.8 (C10), 37.0 (C1), 38.1 (C4), 40.3 (2 × *C*H$_2$Cl), 47.4 (C13), 50.0 (C9), 51.6 (C14), 53.8 (2 × *C*H$_2$CH$_2$Cl), 73.5 (C3), 112.4 [2 × *C*H, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 121.8 (C6), 129.7 [2 × *C*H, *meta* to N(CH$_2$CH$_2$Cl)$_2$], 131.0 (*C*–CH$_2$), 139.8 (C5), 43.8 (*C*–N), 172.2 (O–*C*═O), 220.9 (C═O); *m*/*z* (EI) 575.

*6.2.2.3. (3β)-3-Hydroxyandrost-5-ene-17-one, 4-{4-[di(2-chloroethyl)amino]-3-nitrophenyl}butyrate (**C2**).* The final step involved the removal of ethyl acetate by evaporation under reduced pressure to give an oil which was chromatographed on a column of silica gel using ethyl acetate-light petroleum (1:2) as eluent to afford the ester as a light orange oil (690 mg, 70%). Found: C, 63.82; H, 7.22; N, 4.47; Cl, 11.28%. $C_{33}H_{44}Cl_2N_2O_5$ requires C, 63.97; H, 7.16; N, 4.52; Cl, 11.44%; ν_{max} ($CHCl_3$)/cm^{-1} 1731 cm^{-1} (C═O); δ_H (300 MHz; $CDCl_3$) 0.88 (3H, s, CH_3, on C18), 1.05 (3H, s, CH_3, on C19), 2.30 (2H, t, CH_2, CH$_2$CH_2CO$_2$), 2.36 (1H, m, C*H*, on C8), 2.41 (2H, m, CH_2, on C16), 2.66 (2H, m, ArCH_2), 3.40–3.59 (8H, m, 2 × CH_2Cl and 2 × CH_2CH$_2$Cl), 4.60 (1H, m, C*H*, on C3), 5.40 (1H, d, C*H*, on C6), 7.30 [1H, d, C*H*, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 7.35 [1H, dd, C*H*, *meta* to N(CH$_2$CH$_2$Cl)$_2$], 7.54 (1H, d, C*H*, *ortho* to NO_2, $C_6H_3NO_2$); δ_C ($CDCl_3$) 13.5 (C18), 19.5 (C19), 20.3 (C11), 21.8 (C15), 26.1 (*C*H$_2$CH$_2$CO$_2$), 27.7 (C2), 31.0 (C12), 31.3 (C7)[1], 31.4 (C8)[1], 33.7 (*C*H$_2$CO$_2$), 33.9 (ArCH$_2$), 35.8 (C16), 36.7 (C10), 36.9 (C1), 38.0 (C4),41.4 (2 × *C*H$_2$Cl), 47.4 (C13), 50.0 (C9), 51.6 (C14), 55.9 (2 × *C*H$_2$CH$_2$Cl), 73.8 (C3), 121.9 (C6), 124.8 [*C*H, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 126.7 (*C*H, *ortho* to NO_2), 133.3 (*C*H, *para* to NO_2), 138.5 (*C*–CH$_2$), 139.8 (C5),140.5 (*C*–NO_2), 146.3 (*C*–N), 172.4 (O–*C*═O), 221.0 (C═O); *m*/*z* (EI) 620.

*6.2.2.4. (3β)-3-Hydroxypregn-5-ene-20-one, 4-{4-[di(2-chloroethyl) amino] phenyl}butyrate (**C3**).* The final step involved the removal of ethyl acetate under reduced pressure to afford an oil which was chromatographed on a column of silica gel with ethyl acetate–light petroleum (1:3) as eluent to give the ester as an off-white oil (1.18 g, 60%). Found: C, 69.36; H, 8.20; N, 2.33; Cl, 11.58%. $C_{35}H_{49}Cl_2NO_3$ requires C, 69.75; H, 8.19; N, 2.32; Cl, 11.76%; ν_{max} ($CHCl_3$)/cm^{-1} 1730 cm^{-1} (O–C═O) and 1704 cm^{-1} (CH$_3$–C═O); δ_H (300 MHz; $CDCl_3$) 0.63 (3H, s, CH_3, on C18), 1.01 (3H, s, CH_3, on C19), 2.13 (3H, s, CH_3, on C21), 2.28 (1H, m, C*H*, on C8), 2.30 (2H, t, CH$_2$CH_2CO$_2$), 2.50 (1H, m, C*H*, on C17), 2.54 (2H, t, ArCH_2), 3.60–3.72 (8H, m, 2 × CH_2Cl and 2 × CH_2CH$_2$Cl), 4.60 (1H, m, C*H*, on C3), 5.37 (1H, d, C*H*, on C6), 6.64 [2H, d, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 7.07 [2H, d, *meta* to N(CH$_2$CH$_2$Cl)$_2$]; δ_C ($CDCl_3$) 13.2 (C18), 19.3 (C19), 21.2 (C11), 22.8 (C16), 24.5 (C15), 26.1 (*C*H$_2$CH$_2$CO$_2$), 27.7 (C2), 31.5 (C7)[1], 31.7 (C21), 31.8 (C8)[1], 33.8 (*C*H$_2$CO$_2$), 34.0 (ArCH$_2$), 36.6 (C10), 36.9 (C1), 38.0 (C4), 38.6 (C12), 40.3 (2 × *C*H$_2$Cl), 43.9 (C13), 49.8 (C9), 53.7 (2 × *C*H$_2$CH$_2$Cl), 56.7 (C14), 63.6 (C17), 73.6 (C3), 112.4 [2 × *C*H, *ortho* to N(CH$_2$CH$_2$Cl)$_2$], 122.2 (C6), 129.7 [2 × *C*H, *meta* to N(CH$_2$CH$_2$Cl)$_2$], 131 (*C*–CH$_2$), 139.6 (C5), 143.9 (*C*–N), 172.9 (O–*C*═O), 209.6 (CH$_3$–C═O); *m*/*z* (EI) 603.

*6.2.2.5. (3β)-3-Hydroxypregn-5-ene-20-one,4-{4-[di(2-chloroethyl)-amino]-3-nitro-phenyl}butyrate (**C4**).* After the removal of the ethyl acetate under reduced pressure, the resulting oil was chromatographed on a column of silica gel using ethyl acetate-light petroleum (1:3) as eluent to afford the ester as a light orange oil (780 mg, 66%). (Found: C, 64.55; H, 7.36; N, 4.32; Cl, 10.86%. $C_{35}H_{48}Cl_2N_2O_5$ requires C, 64.90; H, 7.47; N, 4.32; Cl, 10.94%); ν_{max} ($CHCl_3$)/cm^{-1} 1730 cm^{-1} (O–C═O) and 1703 cm^{-1} (CH$_3$–C═O); δ_H (300 MHz; $CDCl_3$) 0.63 (3H, s, CH_3, on C18), 1.01 (3H, s, CH_3, on C19), 2.13 (3H, s, CH_3, on C21), 2.28 (1H, m, C*H*, on C8), 2.30 (2H, t, CH$_2$CH_2CO$_2$), 2.50

[1] Possible reversed assignment.

(1H, m, C*H*, on C17), 2.61 (2H, t, ArCH_2), 3.39–3.50 (8H, m, 2 × CH_2Cl and 2 × CH_2CH_2Cl), 4.60 (1H, m, C*H*, on C3), 5.35 (1H, d, C*H*, on C6), 7.30 [1H, d, C*H*, *ortho* to N($CH_2CH_2Cl)_2$], 7.35 [1H, dd, C*H*, *meta* to N($CH_2CH_2Cl)_2$], 7.54 (1H, d, C*H*, *ortho* to NO_2, $C_6H_3NO_2$); δ_C ($CDCl_3$) 13.2 (C18), 19.3 (C19), 20.9 (C11), 22.8 (C16), 24,4 (C15), 26.1 ($CH_2CH_2CO_2$), 27.7 (C2), 31.5 (C7), 31.7 (C21), 31.8 (C), 3.7 (CH_2CO_2), 34.0(ArCH_2), 36.6 (C10), 36.9 (C), 38.0 (C4), 38.(C12), 41.5 (2 × CH_2Cl), 43.9 (C13), 49.8(C9), 55.9 (2 × CH_2CH_2Cl), 56.7 (C14), 63.6 (C17), 73.9 (C3), 122.4 (C6), 124 [C*H*, *ortho* to N($C_2CH_2Cl)_2$], 126.7 (C*H*, *ortho* NO_2), 133.3 (C*H*, *para* to NO_2), 139.5 (C5), 140.(*C*–NO_2), 146.3 (*C*–N), 172.4 (O–*C*═O), 209.6 (CH_3–*C*═O); *m*/*z* (EI) 648.

6.3. Alkylating activity [29,43,44]

The alkylating activity of each compound was determined at concentrations ranging from 0.1 mM to 1.0 mM, dissolved in 50% (v/v) acetone/ethanol solution. The compounds (0.2 ml) were added separately to screwed capped test tubes containing 1 ml of 0.2 M acetate buffer (pH 5.6). Each tube was treated with 0.5 ml of 5% (w/v) solution *p*-nitrobenzyl pyridine in acetone and the mixture was incubated for 4 h at 37 °C. Each tube was then treated with 3.0 ml of 25% (v/v) 3-amino-1-propanol in tertiary butyl alcohol and the coloration of the reaction product was measured using a visible spectrophotometer at 560 nm. The alkylating activity of the five compounds was compared with that of an equimolar chlorambucil solution.

6.3.1. *Log* P *values*

The six compounds of known weight (10 mg) were added separately to 50 ml volumetric flasks and dissolved in *n*-octanol and then made up to the mark [29]. A portion (4 ml) of each of the solutions was transferred separately to screwed capped centrifuge tubes and an equal volume of water was added to each tube. The tubes were capped and shaken on a rotator for 1 h at 20 °C, after which they were centrifuged for 10 min at 3600 g and the *n*-octanol layer was carefully separated from the water layer. Portions of the original solutions were diluted to obtain a concentration of 0.8 mg%. The shaken solutions were also diluted in a similar manner and comparisons were made using UV spectroscopy between 200 and 400 nm. The log *P* values were then calculated according to Leo et al. [45].

6.3.2. In vitro *cytotoxicity assay [46]*

The standard assay for cytotoxicity employed a 96 well format using 400–1000 cells per well in 100 μl. Each drug dose was represented by three wells. Media (100 μl) containing doubling dilutions of 2× drug were added to triplicate sets. Control wells received 100 μl of media alone. The plates were then incubated in a humidified atmosphere for five days at 37 °C, with 5% carbon dioxide. After incubation 50 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (3 mg/ml) was added to each well and the plate returned to the incubator for 3 h. The process is known as metabolic reduction and requires an active mitochondrial function to reduce the salt. The media and excess MTT were then aspirated from each well and the formazan crystals solubilized in 200 μl of DMSO. The plates were then read using a multiscan microplate reader (Titretech) at 540 nm with subtraction at 620 nm to allow for turbidity. The resultant output was processed using an excel spreadsheet. The percentage of growth inhibition absorbance of the drug treated wells/mean absorbance of the control well × 100 and the IC_{50} (concentration of compound required to inhibit 50% growth) were calculated.

6.3.3. *General procedure used to determine the hydrolysis of the esters using porcine liver esterase [47]*

The stock solution of the ester was prepared by dissolving the ester (50 mg) in absolute ethanol (10 ml) and transferring it to a 25 ml volumetric flask and making upto the mark with absolute ethanol. The phosphate buffer was prepared by dissolving sodium chloride (2 g), potassium dihydrogen phosphate (50 mg), disodium hydrogen phosphate (250 mg) and potassium chloride (50 mg) in distilled deionised water and making up to the 250 ml mark in a volumetric flask (pH 7.4). After incubating the esterase enzyme (20 μl) in buffer (2 ml) for approximately 15 min at 37 °C, the ester (200 μl) was added. The zero reaction involved incubating the ester in buffer at 37 °C for 15 min prior to being analyzed by HPLC. A sample of the reaction mixture (200 μl) was then removed and transferred to microfuge tubes at set time intervals and treated with acetonitrile (200 μl). The mixture was then vortexed for 30 s and then centrifuged for 10 min at 11,600 g. A portion of the supernatant (200 μl) was transferred to a microfuge tube and mobile phase (200 μl) was added and mixed. A standard solution of the ester was prepared by transferring 5 ml of the stock solution to a 50 ml volumetric flask, making upto the mark with the mobile phase, and then transferring 3 ml of this solution to a 25 ml volumetric flask and making upto the mark with mobile phase and finally, 5 ml of this solution was transferred to a 10 ml volumetric flask and made upto the mark to afford a final concentration of the standard solution of 1.20 mg%. Both the standard and sample solutions were analyzed using a validated HPLC method (unpublished data), derived from a modified version of a published method [48]. The esters were also stored in phosphate buffer saline at 37 °C and monitored for their stability using the HPLC method, supported by infrared spectroscopy, over a period of forty five days. The chromatographic conditions: RP-HPLC, the mobile phase consisted of acetic acid (50 ml of a 2% solution) made upto a litre with acetonitrile, pH 5.2 and the flow rate was set at 1.2 ml/min. A C18 Symmetry Waters column (250 mm × 4.6 ID) fitted with a guard column (3 cm × 4.6 mm ID) was used and the wavelength was set at 254 nm.

6.3.4. *General procedure used to determine the hydrolysis of the esters using human plasma*

A stock solution of the ester was prepared by dissolving the ester (50 mg) in absolute ethanol (10 ml) and transferring it to a 50 ml volumetric flask and making upto the mark with the same solvent. The ester (50 μl) was transferred to a microfuge tube and treated with mobile phase (950 μl), mixed using a vortex mixer and 500 μl of the solution was transferred to another microfuge tube and made upto 1 ml with mobile phase and vortexed. Finally, 500 μl of this solution was transferred to a another microfuge tube, made upto 1 ml with mobile phase, and mixed in the usual manner, to afford a 1.25 mg% concentration of standard solution.

Freshly prepared plasma (850 μl) was transferred to a test tube, treated with phosphate buffer saline (100 μl) and stock solution of the ester (50 μl), sealed with parafilm, vortexed for 2 min and then placed into an incubator set at 37 °C. The incubated sample was treated in the following manner prior to analysis. 500 μl of the mixture was transferred to a microfuge tube and treated with 500 μl of mobile phase, mixed for 2 min using a vortex mixer and the resulting precipitate was removed by centrifugation at 11,600 g for 20 min. 500 μl of the supernatant was removed and transferred to a microfuge tube and made upto 1 ml with mobile phase.

The content of the tube was then centrifuged at 11,600 g for a further 10 min.

A comparison between the peak areas of the standard and the prepared samples were then carried out by the HPLC method in order to estimate the percent of ester hydrolyzed with time.

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