17-Oxo-17a-Aza-D-Homo-5-Androsten-3β-Yl Esters: Cytotoxic To Liver and Neuroblastoma Cancer Cell Lines

Neelima Dhingra[1], T.R.Bhardwaj[2]
[1,2]University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh-160014 (India)

***Corresponding author:**
E-mail: neelimad08@gmail.com

ABSTRACT

Introduction and positioning of the heteroatom in the parental steroid skeleton has significant effect on the activity profile of these compounds. A substantial amount of biologically relevant azasteroids have been reported as antifungals, antilipemic, neuromuscular blocking agents, local anaesthetics, antimicrobials, and GABA receptor antagonist. Recent work from our laboratory has described the synthesis and evaluation of 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl ester derivatives as potential 5-alpha reductase inhibitors. The current study was undertaken to further investigate the cytotoxicity of the newly synthesized derivative using some more human cancer cell lines. Seven human cancer cell lines have been exposed to 1X 10^{-5}m (single dose) of compounds (1-8), Finasteride, 5-fluorouracil, mitocycin 6 and, adriamycin were used as reference drugs for comparison. The cell growth was measured after 24h, using ELISA reader, after staining with sulforhodamine B dye (SRB), which binds to basic amino acid residues in the trichloroacetic acid (TCA) fixed cells. All the experiments were carried out in quadruplicate. The newly synthesized azasteroids derivatives displayed significant cytotoxicity against liver and neuroblastoma cell lines as compared to standard reference drugs. This together with, earlier reported antiproliferative activity against prostate cancer cell lines (DU-145) and 5-alpha reductase inhibitory activity, indicates that 17a-azasteroids seems to possess potential eliciting in vitro cytotoxicity. Results obtained from these studies shall be used as guidelines for further development of novel compounds to be used chemotherapeutic agents.

Keywords: *Cytotoxicity; Human cancer cell lines; 17a-aza-D-Homosteroids; sulforhodamine B dye*

INTRODUCTION

Neoplasm is described by abnormal increase in the number of cells, which may result not only from increased cell proliferation but also from decreased level in programmed cell death (apoptosis) [1]. Cells die in response to development signals, and the process is characterized by number of biochemical changes. Any influence between the physiological process of cell proliferation and cell death may lead to change in organ size with the subsequent development of abnormalities in particular organ [2]. So it is reasonable to assume that cytotoxic agents act by killing the cells or by preventing the cell proliferation, thus are useful for the treatment of disease that involve abnormal or uncontrolled cell proliferation.

The steroid system, selected by the evolutionary process to perform some of the most fundamental biological functions, has not only inspired the biochemists and endocrinologists, but has also become the basis of the most phenomenal developments in medicinal chemistry. Naturally occurring steroids have been modified at different positions to get active molecule which show less or few undesirable side-effect. Replacement of one or more atoms in steroids with different groups also affects its chemical properties which lead to change in biological activity [3]. The chemistry and pharmacology of 4-aza [4], 6-aza [5], 7-aza-des-*A*-steroids [6], 19-Nor-10-aza [7], 15,16 di-aza [8,9], 17aza, 17a-aza [10-14] and 4,17-diaza [15] steroids as gamma amino butyric acid (GABA) receptor antagonistic, antifungal, antineoplastic, mutagenic, anti-inflammatory [10-14], antilipemic [16], neuromuscular blocking agents [17], local anesthetics and antimicrobials [6], has been described by number of investigators Fig.1 [18,19].

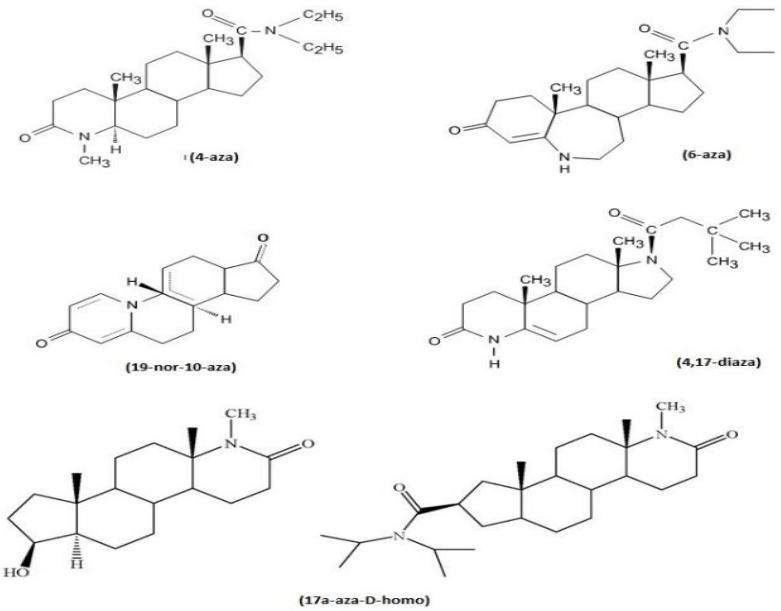

Figure 1: Structure of some reported potent azasteroids

Recent published studies from our laboratory described the synthesis of 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl ester steroids Fig.2 and their evaluation as antiproliferative agents and 5-alpha reductase inhibitors. This group of steroids has displayed significant antiproliferative activity as compared to reference drug finasteride, against human prostate cancer cell line (DU-145) with IC_{50} values being in the range of 5.2-33.1μM and significant increase in the serum androgen level of testosterone has been found as compared to control [20,21].The observation that the location of the aza group in the parent steroid skeleton bring about notable changes in the pharmacological activity and our experience from our previous studies further prompted us to investigate the cytotoxicity of the these newly synthesized derivative using some more human cancer cell lines.

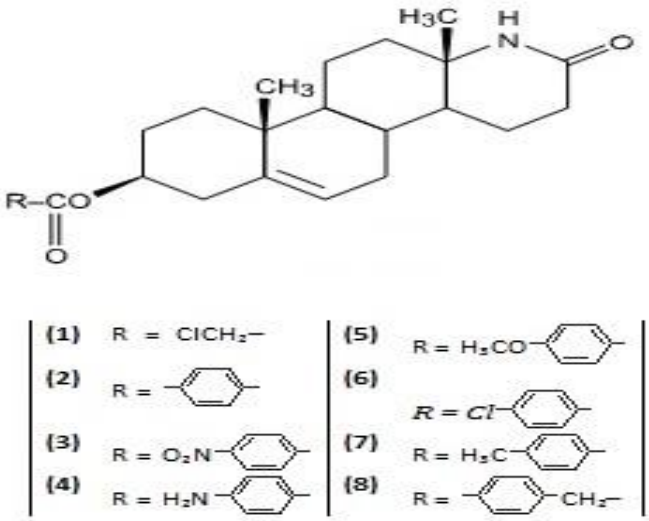

Figure 2 Structure of synthesized and evaluated 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl ester [20,21]

METHODS

A Chemicals and biochemical:
Reagent grade chemicals were used without purification. Complete growth medium (RPMI-1640), fetal bovine serum, tricholoroacetic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, and dimethyl sulfoxide were purchased from Sigma Aldrich. Finasteride was obtained as a gift sample from Cipla, Bombay (India) and was of analytical grade (assay 99.9%).

B cell culture:
The cell line panel consisted of 7 human cancer cell line Liver (Hep-2), Neuroblastoma (IMR-32) and colon (Colo-205, HCT-15, 502713 HT-29 and SW-620). Cells were grown in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator.

C Preparation of test materials:
A stock solution of 20 mg/ ml of each extract was prepared. DMSO was used for dissolving 5% alcoholic extracts, 50% aqueous–DMSO for 50% alcoholic extracts and distilled water for aqueous extracts. The stock solution were serially diluted to obtain working test solution with complete growth medium (RPMI-1640 medium with 2mM glutamine, 100µg/l streptomycin, pH 7.4, sterilized by filtration and supplemented with 10% fetal bovine serum and 100 units / ml penicillin before use) containing 50 µg/ ml of gentamycin to obtain working test solutions of 200 µg/ ml. The working test solution were not filtered/ sterilized but microbial contamination was controlled by addition of gentamycin in complete growth medium used for dilution of stock solution to prepare working test solutions

D Cytotoxicity assay (Sulforhodamine B SRB assay):
In vitro cytotoxicity against seven human cancer cell lines was determined using 96-well tissue culture plates [22, 23]. The cells were grown in tissue culture flasks in complete growth medium at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in a carbon dioxide incubator. The cell suspension of required and cell density (1-2 lakhs /ml) depending on the mass doubling times of cell lines was prepared in complete growth medium for determination of cytotoxicity. The aliquots of 100 µl of cell suspension were added to each well on a 96 well tissue culture plates. The blank wells contained complete medium in place of cell suspension. Simultaneously, control experiment with positive controls containing known anticancer drugs agents were also carried out. The cells were incubated for 24 hrs.
The test material (100µl in each well) was added after 24 hr to well containing cell suspension and blank wells. The cells were allowed to grow in presence of test material by further incubating the plates for 48 hr. At the end of incubation period the cell growth was stopped by gently layering trichloroacetic acid (50%TCA, 50µl/well) on top of the medium in all the wells. The plates were incubated at 4°C for 1 hr. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins and air dried.
The cell growth was measured by staining with sulforhodamine B dye. Sulforhodamine B dye (SRB, 0.4% in 1% acetic acid, 100µl/well) was added to each well and plates were allowed to stand at room temperature for 30 minutes. The plates were washed with 1% acetic acid four times and then dried. Tris-HCl buffer (0.01M, pH 10.5, 100µl/ml) was added to each well to solublise the dye. The plates were shaken gently for 10 minutes on a shaker and the optical density was recorded on ELISA reader at 540 nm. The cell growth was determined by subtracting mean optical density (OD) value of respective blank from the mean OD value of experimental set. Percent growth in presence of test material was calculated by considering the growth in absence of test material as 100% and in turn percent growth inhibition in presence of test material was calculated. All the experiments were carried out in quadruplicate.
Cell viability (%) = OD of treated cells / OD of control cells ×100

RESULTS AND DISCUSSION
Earlier reports concerning the chemical synthesis and the biological evaluation of 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl ester derivatives has revealed the importance of lactam for increased antiproliferative activity and suitable structure for the development of

new 5-alpha reductase inhibitors. Present study was undertaken to explore the cytotoxic potential of newly synthesized steroidal derivatives , and compounds 1-8 were submitted to the Indian Institute of Integrative Medicine, Jammu (formerly Regional Research laboratory) for *in vitro* preclinical antitumor screening programme using sulforhodamine B dye (SRB assay) against 7 human cancer cell lines derived from liver cancer (Hep-2), neuroblastoma (IMR-32) and colon cancer cell lines (Colo-205, HCT-15, 502713, HT-29, SW-620,) [22,23]. Compounds have been assayed at1 x 10^{-5}M (single dose), finasteride, 5-fluorouracil, mitomycin 6 and adriamycin were used as reference drugs for comparison. Results summarized in Table 1, are expressed as % growth inhibition of synthetic steroidal derivatives on different cancer cell lines. Compounds that inhibited the growth of any one of cell line to 40% or more were considered to be active cytotoxic agents. The conclusions summarized in Fig.3 and 4 Table 2 are percentage growth inhibition ± SEM of four replicate measurements against liver and neuroblastoma cell lines and are based on significance p<0.05.

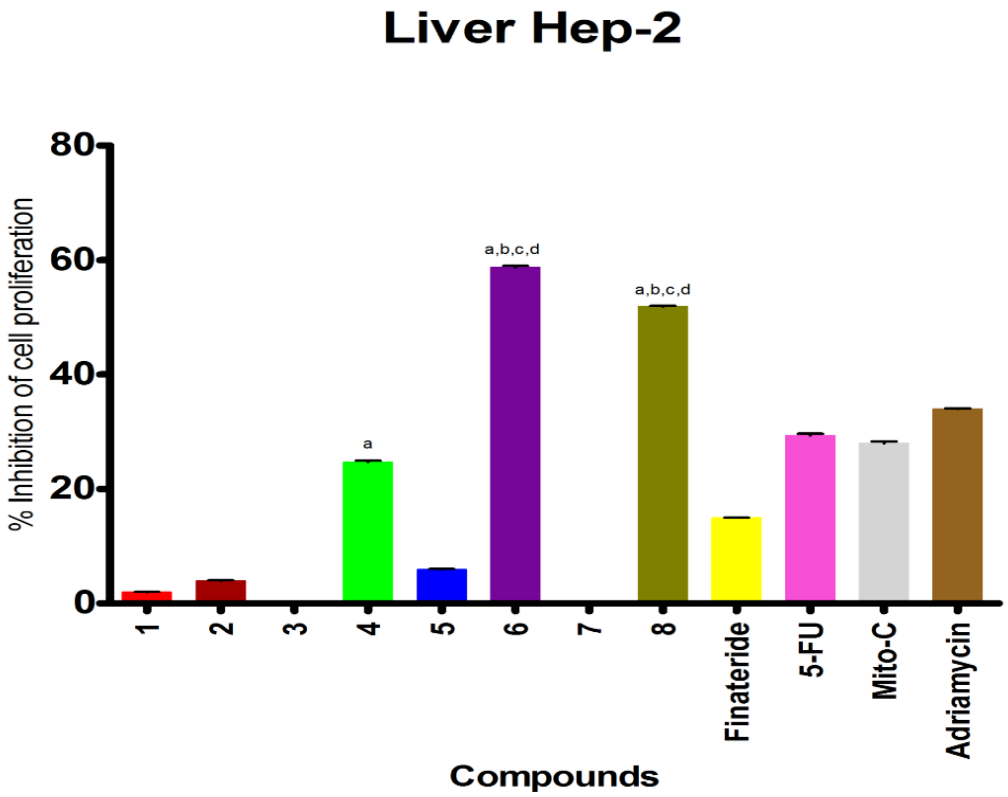

Fig.3 Data expressed as the mean ± SEM against liver cell lines (Hep-2), [a]P<0.05 as compared to Finasteride, [b]P<0.05 as compared to 5-FU, [c]P<0.05 as compared To Mito-C, [d]P<0.05 as compared to Adriamycin.

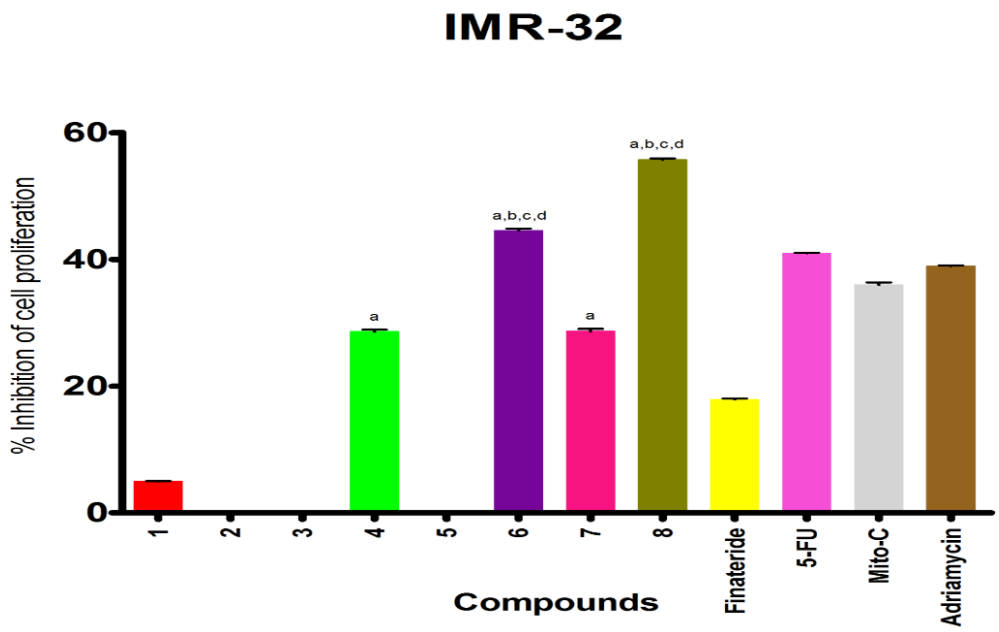

Fig.4 Data expressed as the mean ± SEM against Neuroblastoma cell line (IMR-32), [a]P<0.05 as compared to Finasteride, [b]P<0.05 as compared to 5-FU, [c]P<0.05 as compared to Mito-C, [d]P<0.05 as compared to Adriamycin.

Table 1: Percent Growth inhibition at 1x10^{-5} m concentration in the 7 cell lines

Compound	Liver	Neuro blastoma	Colon				
	Hep-2	IMR-32	Colo-205	HCT-15	502713	HT-29	SW-620
1	2	5	32	12	22	2	26
2	4	0	26	8	10	3	16
3	0	0	17	11	14	13	13
4	25	29	22	8	3	0	29
5	6	0	27	17	19	0	27
6	59	45	17	12	25	15	32
7	0	29	2	9	25	11	0
8	52	56	11	4	25	13	17
Finasteride	15	18	23	13	0	0	6
5-FU 1X10^{-5}M	30	41	38	61	66	51	11
Mito C 1x10^{-5}M	28	36	0	19	43	Ni	Ni
Adriamycin 1x10^{-5}M	34	39	13	29	51	Ni	Ni

Not significant inhibition (Ni)

Table 2: % growth inhibition expressed as the mean ± SEM against liver and neuroblastoma cell lines (Hep-2) and (IMR-32)

Compound	% growth inhibition (mean±SEM)[a,b,cd]	
	Liver (Hep-2)	Neuroblastoma (IMR-32)
1	1.959±0.0325	4.971±0.066
2	3.945±0.0924	0
3	0	0
4	24.713±0.218[a]	28.588±0.304[a]
5	5.913±0.096	0
6	58.722±0.249[a,b,cd]	44.555±0.300[a,b,cd]
7	0	28.648±0.358[a]
8	51.884±0.116[a,b,cd]	55.706±0.246[a,b,cd]
Finasteride	14.977±0.245	17.918±0.120
5-FU	29.333±0.300	40.954±0.072
Mitomycin	27.984±0.350	35.982±0.382
Adriamycin	33.994±0.100	38.936±0.112

[a]P<0.05 as compared to Finasteride, [b]P<0.05 as compared to 5-FU, [c]P<0.05 as compared to Mito-C,[d]P<0.05 as compared to Adriamycin.

Among the 17a-aza-D-homo-17-one steroids 1-8, a significant activity was found for the 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl 4-chlorobenzoate (6) and 17-Oxo-17a-aza-D-homo-5-androsten-3b-yl phenylacetate (8). In particular, compound 6 and 8 exhibited significant cell line-selective cytotoxicity against the Liver (Hep-2) and neuroblastoma (IMR-32) cancer cells, 59%, 45% and 52%, 56%, respectively (Table 1). Sensitivity to cancerous cell lines varied according to cell types and the colon cancer cell lines appears to be the comparatively resistant to the tested compounds as no effective growth inhibition has been found in comparison to reference drugs. Further the inhibition properties of newly synthesized azasteroids toward liver cancer cell lines seem to be related to the electron withdrawing group at the *para* position the aromatic ring system, as compounds 4 and 7 with (NH_2-) and 7 (CH_3-) being less active with 25 and 0 %, growth inhibition respectively for Hep-2 cell. However, this hypothesis is not consistent with the results for 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl 4-nitrobenzoate (3), and 17-Oxo-17a-aza-D-homo-5-androsten-3β-yl 4-methoxybenzoate (5), which are totally inactive with 0% inhibition, thus revealing that electron withdrawing moiety does not greatly influence anticellular activity. Introduction of spacer like –CH2–separating the phenyl ring and carboxylic carbon furnished the compounds 8, with strong activity against neuroblastoma (IMR-32). Based on the present and our earlier published studies [20, 21], azasteroids seems to be the most likely candidates eliciting *in vitro* cytotoxicity effect, however, the exact mechanism of action these compounds remain to be elucidated.

CONCLUSION

Recently published paper indicated the synthesis, efficacy (antiproliferative activity and 5-alpha reductase inhibitory) and toxicity profile of complete group. In the present studies, the sulphoradamine B[6] assay (SRB assay) was used to examine cytotoxicity using 7 human cancer cell lines. Compounds 6 and 8 have been found to be most potent cytotoxic against liver (Hep-2) and neuroblastoma (IMR-32) cell line. This together with antiproliferative activity against prostate cancer cell lines and 5-alpha reductase inhibitory activity makes them interesting molecules, particular with the potential as

pharmacological tool and possibly as lead to antitumor agents. Furthermore experiments have to be carried out to fully assess the effectiveness and potency using different *in vitro/ in vivo* model including *insilico* studies by docking , before their consideration as clinical candidate as antitumour agents.

ACKNOWLEDGMENT

The grants received from University Grants Commission, New Delhi and Council of Scientific and Industrial Research, New Delhi is gratefully acknowledged. We are thankful to Indian Institute of Integrative Medicine (Formerly Regional Research Laboratory) Jammu for screening the compounds for cytotoxic activity. We are thankful to Mr Dewank, Archer Chem, Mumbai, for generous gift of Finasteride.

REFERENCES:

1. G.I Evan and K.H. Vousden, "Proliferation, cell cycle and apoptosis in cancer". Nature, vol. 411,pp 342-348, 2001.
2. N.N. Danial and S.J. Korsmeyer, "Cell death: critical control points". Cell, vol. 116, pp 205-219, 2004.
3. A. Shahab, A. Nami, S. Khan, M. Alam, M. Mushfiq, D.-U. Lee, and S.Park, "Synthesis, Spectral Characterization, and *In Vitro* Cytotoxicity of N-2′-Hydroxyethyl-Substituted Azacholestanes Prepared from 6-Oxocholestanes by Modified Schmidt Reaction". Journal of Spectroscopy, available online at http://dx.doi.org/10.1155/2013/128149. Vol. 2013
4. M. Brandt, and M.A. Levy, "3_-Hydroxy-_5-steroid dehydrogenase / 3-keto-_5-isomerase from bovine adrenals: mechanism of inhibition by 3-oxo-4-aza steroids and kinetic mechanism of the dehydrogenase". Biochemistry; vol.28, pp 140–148, 1989.
5. R.M. Patrick, and G.F. Francis, "Synthesis of a B-homo-6-azaandrost-4-ene-3-one as a novel steroidal 5_-reductase inhibitor", Tetrahedron Lett, vol.35 (18), pp 2823–6, 1994.
6. M. Krojer, M. Keller, and F. Bracher, "7-Aza-des-A-steroids with antimicrobial and cytotoxic activity". Sci. Pharm., vol. 81, pp 329–338, 2013.
7. A. Guarna, C. Belle, F. Machetti, E.G. Occhiato, A.H. Payne, and C. Cassiani, "19-Nor-10-azasteroids: a novel class of inhibitors for human steroid 5_-reductases 1 and 2", J. Med. Chem. vol.40, pp 1112–29, 1997.
8. R.Chesnut, N. Durham, R.,Brown E. Mawdsley, and K. Berlin, " Antibacterial activity of 15-azasteroids alone and in combination with antibiotics". Steroids , vol.27, pp 525–41, 1976..
9. R. Dolle, H. Allaudeen, L. Kruse," Design and synthesis of 14_-methyl-15-aza-Dhomosterols as novel antimycotics". J. Med. Chem. vol. 33, pp877–80, 1990.
10. D.F. Covey, M Han, A.S. Kumar, de la Cruz MAM, E.S. Meadows, and Y. Hu,"Neurosteroid analogues. Structure–activity studies of N-acylated 17a-aza-D-homosteroid analogues of the anesthetic steroids (3_,5_)-and (3_, 5_)-3-hydroxypregnan-20-one". J. Med. Chem. vol. 43(17), pp 3201–4, 2000.
11. C. Wang, S. Wang, Y. Xu, and Y.H. Hu, "Preparation of (5_,13_)-D-azasteroids as key precursors of a new family of potential GABA receptor modulators". Steroids, vol. 68, pp 677–83, 2003.
12. G. Patrick, and O. Kinsman, "Synthesis and antifungal activity of novel aza-D-homosteroids, hydroisoquinolines, pyridines and dihydropyridines" J. Med. Chem. vol 31, pp 615–24,1996..
13. C. Andrianopoulos, G. Stephanou, E. Politi, and N.A.Demopoulos, "Evaluation and characterization of micronuclei induced by the antitumour agent ASE [3_-hydroxy-13-_ amino-13,17 seco-5_-androstan-17-oic-13,17-lactam-p-bis(2-chloroethyl) amino phenyl acetate] in human lymphocyte cultures". Mutagenesis vol 15(3), pp 215–21, 2000.
14. C. Xenos, and C. Camoutis, " Synthesis of A- and D-homoazasteroidal isoxazoles". J Heterocycl. Chem. vol 36, pp1343–4, 1999.
15. J.W. Morzycki, Z Lotowski, A.Z. Wilczewska, and J.D. Stuart, "Synthesis of 4, 17-diazasteroid inhibitors of human 5_-reductase", Bioorg Med Chem. vol. 4(8), pp1209–15, 1996.
16. P. Catsoulacos, D. Politis, and G. L. Wampler, "Antitumor activity of homo-aza-steroidal esters of [p-[bis(2-chloroethyl)amino]phenyl]acetic acid and [p-[bis(2-chloroethyl)amino] phenyl]butyric acid," Cancer Chemotherapy and Pharmacology, vol. 10, no. 2, pp. 129–132, 1983.
17. P. M. Sabale, P.Prajapati, P.G. Kalal, D. B. Nagar, "Azasteroids as Promising Neuromuscular Blockers: A Review," Journal of Applied Pharmaceutical Science Vol. 2 (11), pp. 164-173, November, 2012 , Available online at http://www.japsonline.com, DOI: 10.7324/JAPS.2012.211292
18. T. Duong, R. H. Prager, and J. M. Tippett, "Central nervous system active compounds. II. The synthesis of some 4-, 5-, 6- and 7-substituted caprolactams," Australian Journal of Chemistry, vol.29, no. 12, pp. 2667–2682, 1976.
19. M. Martin-Smith and M. F. Sugrume, "Biological activity in steroids possessing nitrogen atoms: recent advances," Journal of pharmacy and pharmacology, vol. 16, pp. 569–595, 1964.

20. N.,Dhingra, T.R.Bhardwaj, N. Mehta, T. Mukhopadhyay, A.Kumar and M. Kumar, "17-Oximino-5-androsten-3b-yl esters: synthesis, antiproliferative activity, acute toxicity, and effect on serum androgen level". Med Chem Res vol 20, pp 817-825, 2011.
21. T.R.Bhardwaj, M.Kumar, N.Dhingra and N.Mehta, "Novel Steroidal Esters of 17-oximino-5-androsten-3β-ol". WO/2009/027994; PCT/IN08/0513.
22. A.Monk , D. Scudiero and P. Shekan, "Feasibility of a High-Flux Anticancer Drug Screen Using a Diverse Panel of Cultured Human Tumor Cell Lines" J. Nat. Cancer Inst. vol 83, pp 757-766, 1991.
23. P.Shekan, R.Storeng, and D.A.Scudiero, "New colorimetric cytotoxicity assay for anticancer-drug screening". J. Nat. Cancer Inst. vol 82, pp 1107-1112, 1990.