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Synthesis of Mitoxantrone Analogues and their in-vitro Cytotoxicity

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Abstract: Mitoxantrone (Mx) is a powerful neoplastic agent but its cardiotoxicity has become a concern. Since we developed an improved method for the preparation of 4, 5-diaminochrysazin which is a precursor for the synthesis of mitoxantrone, we attempted making several analogues having 3 carbon to 7 carbon atom side chains starting from beta alanino, cyclopropyl, cyclopentyl, cyclohexyl and benzyl groups. All these compounds showed a comparable cytotoxicity in-vitro when tested against MCF-7 breast cancer and Hela cervical cancer cell lines. Among these, 1, 4-bis (cyclohexylamino) 5, 8-dihydroxyanthraquinone-9, 10-dione appeared promising for further in-vivo studies.
Keywords: Mitoxantrone analogues, 4, 5-diaminochrysazin, in-vitro toxicity, MCF-7 breast cancer cells, Hela lung cancer cells.

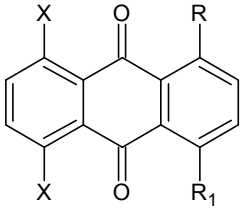
1. Introduction

N-Alkyl derivatives of anthracene-9, 10- diones exemplified by drugs ametantrone(**1**) and mitoxantrone(**2**)[1-3] are potent anti-cancer agents with an efficacy and therapeutic index exceeding adriamycin, methotrexate or 5-fluorouracil[4-9]. However, while the additional hydroxyl groups present at 5-and 8-positions of mitoxantrone lead to tenfold increase in its antineoplastic activity over ametantrone, this beneficial enhancement is unfortunately countered by a tenfold increase in its cardiotoxicity. Mitoxantrone is a drug of choice for the treatment of worsening relapsing-remitting multiple sclerosis (MS), secondary progressive MS.[7,8] However, these clinical applications are limited due to the accumulative and irreversible cardiotoxicity[6]. Recently, mitoxantrone was found to induce a progressive increase in mitochondrial mass in the cancer cells but not in the cardiac cells[9].

This suggests the opportunities to look for novel anthraquinones with reduced cardiotoxicity. The planar tricyclic structure of anthraquinone is essential for interacting with DNA base pairs. Perhaps by introducing different side chains at 1- and 4- positions in anthraquinone skeleton with a variety of substituents, which may form additional interactions with the double-strand DNA (ds-DNA) or DNA-topoisomerase II (TOP2) cleavable complex to increase their binding affinities and selectivities. In the literature, various anthraquinone-peptide conjugates [10-13] were reported to demonstrate remarkable DNA binding and exhibit cytostatic or cytotoxic activities.

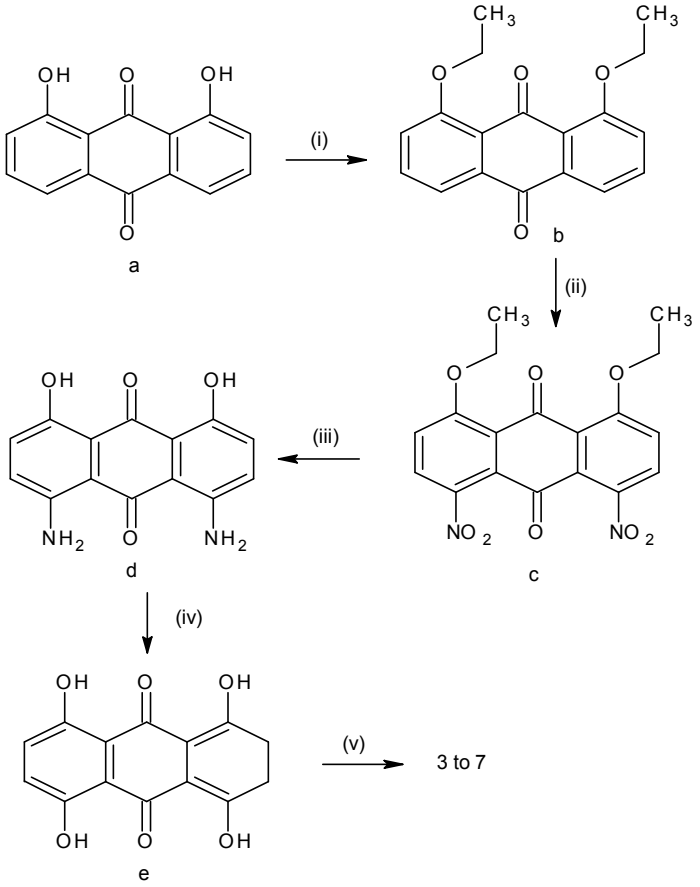
In this paper, we report the synthesis, in vitro cytotoxicity of a few analogues of mitoxantrone having 3 carbon side chains to 7 carbon atoms starting from beta alanino, cyclopropyl, cyclopentyl, cyclohexyl and benzyl groups.

2. Results and discussion



1) R&R_1=-NH $CH_2CH_2NHCH_2CH_2OH$; X=H
2) R&R_1=-NH $CH_2CH_2NHCH_2CH_2OH$; X=OH
3) R= -NH $CH_2C_6H_5$, R_1 and X= OH
4) R, R_1= -NH-C_6H_{11} and X= OH
5) R, R_1= -NH-C_5H_9 and X=OH
6) R, R_1= -NH-$(CH_2)_2$-CO_2H and X= OH
7) R, R_1= -NH-C_3H_5 and X= OH

Synthesis of 1, 8-dihydroxy-4, 5-diaminoanthraquinone (**d**) involves three steps starting from chrysazin (**a**) viz., ethylation, nitration followed by reaction with hydroiodic acid (HI)[14]. Treatment of **d** with sodium hydrosulfite in alkaline solution leads[15] to leuco-1,4,5,8-tetrahydroxyanthraquinone (**e**), this on condensation with appropriate amine in an inert gas atmosphere followed by oxidation of the resulting intermediate with oxygen gave 1,4-diamino substituted anthraquinones (Figure 1). All compounds were fully characterized .



(i) K_2CO_3/DMF/Diethylsulphate; (ii) $H_2SO_4/HNO_3/H_3BO_3$;
(ii) HI; (iv) $Na_2S_2O_4$/NaOH; (v) Amine/Ethanol

Figure 1: Synthetic sequence of mitoxantrone analogues by IR, NMR and Mass spectrometry.

Table 1: Cytotoxic activity of compounds 3-7 on HeLa cell line (cervical epithelial adenocarcinoma cell line) and MCF-7 (Breast carcinoma cell line).

Compound	IC50 (nM) ± SD] * HeLa	IC50 (nM) ± SD] * MCF-7
Mitoxantrone	59.36 ± 5.24	101.16±18.49
3	73.82 ± 5.23	107.93±3.9
4	73.7 ± 0.89	105.78±5.18
5	75.66 ±13.61	112.65±5.69
6	79.51±7.28	115.2±10.53
7	76.88±3.49	104.48±7.86

* Values are average of three independent experiments ± standard deviation, conducted in triplicate for each concentration.

Compounds **3** to **7** were subjected to in vitro testing using breast carcinoma MCF-7 & cervical cancer Hela cell lines for their cytotoxicity (Table 1) in comparison with mitoxantrone. It is observed that all compounds **3** to **7** exhibited very good inhibitory activity in both cell lines in comparison with mitoxantrone. While IC_{50} values in MCF-7 breast cancer cell line for all compounds ranged between 100 and 120 nM, but in Hela cervical cancer cell line still lower concentrations ranging 70 to 80 nM were effective. 1,4-biscyclohexylamino-5,8-dihydroxyanthraquinone (**4**) exhibited relatively better inhibitory activity while bis beta-alanino-5,8-dihydroxyanthraquinone (**6**) showed relatively less inhibitory activity against both cell lines. Relatively better inhibitory activity exhibited by compound **4** may be due to flexible nature of cyclohexyl moiety which undergoes different conformations under different conditions and also due to lipophilicity conferred to the molecule by the cyclohexyl group and which may increase the affinity for the cell membrane [16]. Since these molecules showed very good in-vitro activity we plan to carry out in-vivo testing in future to find out the cardiotoxicity.

3. Conclusion

In conclusion, we have synthesized compounds **3** to **7** for the first time by an elegant process starting from chrysazin and studied their cytotoxic activities. Cyclohexylamino substituted anthraquinone molecule showed cytotoxic activity on par with mitoxantrone which has found to possess increased cardiotoxicity.

4. Experimental

4.1 *General methods/ instruments*

Chemicals and reagents of laboratory grade were obtained from local dealers and were used without further purification. IR spectra were recorded on Nicolet avatar 320 Ft-IR spectrometer, ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ / DMSO-d_6 at 200MHz on Bruker A G spectrometer. Chemical shifts are reported in ∂ units down field from tetramethylsilane as internal standard. Mass spectra were recorded using GCMS-QP2010S (direct probe) and on Q-TOF micro^{TM} AMPS MAX10/6A system. Melting points are uncorrected and were determined with a melting point apparatus (Acro Steels Pvt. Ltd.).

4.2. *Preparation of 4, 5-diaminochrysazin (d);*

This compound has been prepared by following the method described in reference [14]

4.2.1 Preparation of leuco-1, 4, 5, 8-tetrahydroxyanthraquinone (e);

To an aqueous sodium hydroxide solution (10%, 1L) containing n-butanol (50ml) was added 4, 5-diaminochrysazin (**d**) (38.3g, 142mmol) with stirring the resulting dark-blue suspension was deaerated by stirring for 15min while a stream of N_2 was bubbled through it. Sodium hydrosulfite (22.5g, 123mmol) was gradually added with stirring, while the reaction mixture was heated and maintained at 60^0c for 30 min. after being cooled to room temperature, the reaction mixture was neutralized with HCl (4N) and allowed to stand. The resulting precipitate was collected by filtration, washed with water and dried in vaccuo at 50^0c to give leuco-1,4,5,8-tetrahydroxyanthraquinone(**e**) 35g, 90% as a brown flake, mp 230-235^{0}c (decomposition); ^{1}H NMR (DMSO-d_6): ∂ 3.0(s, 4H), 7.15(s,2H), 9.8(s,OH), GC-MS(DI): 274(M^+).

4.2.2. 1-benzylamino-4, 5,8-trihydroxyanthra quinine - 9, 10-dione (3);

To a stirred solution of leuco-1,4,5,8-tetrahydroxyanthraquinone(**e**) (1g, 3.64mmol) in 100 ml ethanol was added benzylamine (5g, 46.7mmol) and heated to 85^0C for 15 h under nitrogen atmosphere and then stirred at room temperature for another 10 h.

Filtered the reaction mixture and washed with methylene dichloride to get a bluish brown solid (0.3g, yield=23%); mp 194^{0}c (decomposition); IR (KBr); 3260,3050,2900, 2850,1600,1569, 1496, 1454, 1392, 1300,1257, 1172, 1087, 968, 790, 744, 698, 551, 466 cm^{-1} ; ^{1}H NMR($CDCl_3$); ∂ 4.64(d, 2H), 7.16 to 7.37(m,9H), 10.34(b, 1H), 12.39(s,1H), 13.01(s,1H), 13.32(s,1H); ^{13}C NMR($CDCl_3$, 50MHz); ∂ 46.9, 124.2, 126.3, 126.8, 127.6, 128.9, 129.0, 156.8, TOF-MS (m/z): Calcd for $C_{21}H_{15}NO_5$ (M+H)=362.1028, found=362.2043.

4.2.3. 1, 4-bis (cyclohexylamino)-5, 8-dihydroxy anthraquinone-9, 10-dione (4);

Using compound **e** (1g, 3.64mmol) and cyclohexylamine (5g, 50mmol) as starting materials the title compound **4** was prepared as described under compound **3**. It was obtained as a bluish brown solid (1.0g, yield=63%); mp 220^{0}c (decomposition).; IR (KBr); 3400, 2927, 2854, 1566, 1446, 1392, 1149, 1080, 956, 825, 729, 675, 551, 482, 428 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200MHz); ∂ 1.25- 1.46 (m,20H), 3.75 (br, 2H), 7.1(s,2H), 7.21(s, 2H), 10.70(b, 2H), 13.59(s, 2H); ^{13}C NMR($CDCl_3$, 50MHz); ∂ 24.3, 25.4, 33.2, 50.9, 108.6, 115.5, 124.3, 129.2, 145.7, 155.2, 184.6; TOF-MS (m/z): Calcd for $C_{26}H_{30}N_2O_4$ (M+)=434.2206, found=434.1571.

4.2.4. 1,4-bis (cyclopentylamino)-5, 8-dihydroxy anthraquinone-9, 10-dione (5);

Using compound **e** (1g, 3.64mmol) and cyclopentylamine (5g, 58.8mmol) as starting materials the title compound **5** was obtained as a blue-Broun solid (0.3g, yield=20%); mp 240^{0}c (decomposition); IR (KBr); 3400, 2954, 1604, 1558,1500, 1450, 1396, 1353, 1164, 1080, 972, 825, 663, 547, 478 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200MHz); ∂ 1.64-2.1(m,16H), 4.10(m,2H), 7.10(s, 2H), 7.23(s, 2H), 10.75(br, 2H), 13.55(s, 2H); ^{13}C NMR ($CDCl_3$, 50MHz); ∂ 24.5, 34.5, 54.4, 109.2, 115.9, 124.9, 125.0, 146.5, 155.7, 185.2; TOF-MS (m/z): Calcd for $C_{24}H_{26}N_2O_4$ (M+)=406.1893, found=406.2104.

4.2.5. 1, 4-bis (beta-alanino-5, 8-dihydroxy anthrax quinone-9, 10-dione (6);

Using compound **e** (1g, 3.64mmol) and beta-alanine (5g, 56.1mmol) as starting materials the title compound **6** was obtained as a blue-Brown solid (0.3g, yield=20%); mp >280^{0}c ; IR (KBr); 3400, 2900, 2657, 1743, 1693, 1608, 1562, 1454, 1350, 1172, 1076, 972, 825, 628, 547,470 cm^{-1}; ^{1}H NMR (DMSO-d_6, 200MHz); ∂ 2.65(br, 4H), 3.69(m, 4H), 7.13(s, 2H), 7.53(s, 2H), 10.57(br, 2H), 13.46(s, 2H); ^{13}C NMR (DMSO-d_6, 50MHz); ∂ 34.6, 38.7, 107.7, 115.7, 124.7, 125.5, 147.0, 154.9, 173.1, 183.7; TOF-MS (m/z): Calcd for $C_{20}H_{18}N_2O_8$ (M+) =414.1063, found=414.2769.

4.2.6. 1, 4-bis (cyclopropylamino)-5, 8-dihydroxy anthraxquinone-9, 10-dione (7);

Using compound **6** (1g, 3.64mmol) and cyclopropylamine (5g, 87.8mmol) as starting materials the title compound **7** was obtained as a blue-Brown solid (0.3g, yield=20%); mp >280^{0}c ; IR (KBr);3400, 2900, 2800, 1608, 1569, 1454, 1404, 1342, 1299, 1211, 1164, 1022, 960, 806, 628, 474 cm^{-1}; ^{1}H NMR ($CDCl_3$, 200MHz); ∂ 0.73(m, 4H), 0.94 (m,4H), 2.60(m, 2H), 7.13(s, 2H), 7.72(s, 2H), 10.20(br, 2H), 13.42(s, 2H); ^{13}C NMR ($CDCl_3$, 50MHz); ∂ 7.9, 24.3, 29.6, 108.2, 115.0, 124.8, 125.2, 147.5, 155.5, 186.2; TOF-MS (m/z): Calcd for $C_{20}H_{18}N_2O_4$ (M+Na)=373.1164, found=373.1162.

4.3 Biological activity

4.3.1 In vitro growth inhibition assay

The cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma- Aldrich Inc., USA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., USA) in a CO_2 incubator. The cytotoxicity of the compounds was measured by MTT assay[17]. The cells were plated in a 96-well plate at the density of 10,000 cells per well (HeLa) and 10,000 cells per well (MCF-7). After 24 hours, the cells were treated with different concentrations of analogues of mitoxantrone (25 nM to 400 nM). The cells were further incubated for 24 hours. The cytotoxicity was measured by adding 5mg/ml of MTT (Sigma- Aldrich Inc., USA) to each well and incubated for another three hours. The purple formazan crystals were dissolved by adding 100µl of DMSO to each well. The absorbance was read at 570 nm in a spectrophotometer [Spectra Max 340]. The cell death was calculated as follows:

Cell death =100- [(test absorbance/ control absorbance) x100]

The test result is expressed as the concentration of a test compound which inhibits the cell growth by 50% (IC_{50}).

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