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SYNTHESIS AND IN-VITRO CYTOTOXIC SCREENING OF TRIETHYLAMMONIUM {[7-HYDROXY-4,9-DINITRO-8-(PHENYLCARBAMOYL) BICYCLO[3.3.1]NONA-3,7-DIEN-2-YLIDENE (OXIDO)-λ^5-AZANYL} OXIDANIDE AGAINST EAC CELL LINES

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Summary

The title compound (TBO) with bicyclo[3.3.1]nonane skeleton was synthesized in an efficient one pot procedure employing a green methodology. The compound was structurally confirmed by UV-Visible, FT-IR, NMR and single crystal X-ray diffraction analysis. The anticancer activity of TBO was tested against Ehrlich Ascites Carcinoma [EAC] cell lines using *In-Vitro* methods such as Trypan blue dye exclusion method and MTT assay. The results revealed that TBO showed potent cytotoxicity against EAC cell lines by activating the apoptotic pathway.

Key words: Bicyclo[3.3.1]nonane, Ehrlich Ascites Carcinoma [EAC] cell lines, MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Introduction

Numerous biologically active natural products contain bicyclic systems.[1,2] These bicyclic frame works possess wide range of biological activities which include antitumor[3], antibacterial[4], antiviral[5], antiprotozoal[6], antispasmolytic[7], analgesic and anti-inflammatory[8] activities. Among the bicyclic systems, bicyclo[3.3.1] nonane systems deserve special attention, as they are key precursors in a synthetic strategy toward taxol skeleton,[9,10] a current clinically used anticancer drug of natural origin. To the best of our knowledge the anticancer activity of TBO has not been investigated, hence we attempted to synthesize and evaluate the *In-Vitro* cytotoxic effect of TBO against EAC cell lines.

Materials and Methods

Synthesis and analytical studies were carried out using laboratory and analytical grade reagents.

The bicyclic adduct TBO was synthesized using green synthetic methodology[11] via a one pot synthesis.[12] Acetoacetanilide (0.01 mol) and 1,3,5-trinitrobenzene (0.01 mol) dissolved in absolute ethanol were mechanically stirred for 2 hrs at ambient temperature in the presence of excess triethylamine. The intensely colored solution obtained after stirring was kept at 30-40°C until bright orange crystals separated out. To the above reaction mixture, copious amount of anhydrous ether is added and stirred further in an ultrasonic bath for 30 minutes. The resulting crystals were powdered well, washed with ethanol-ether solution and recrystallised from absolute alcohol. Mol. Formula $C_{22}H_{29}N_5O_8$, yield 90% , m.pt. 177°C

TBO was subjected to various spectral analysis. UV-Visible spectrum was recorded on a Lambda-25 UV-Visible spectrophotometer and emission spectrum was obtained on a Jasco Spectrofluorimeter using DMSO solvent. The FT-IR spectrum was recorded using a Perkin-Elmer spectrum RXI Infrared spectrometer. The ^1H, ^{13}C and 2D NMR (COSY) spectra were obtained from Bruker RX-300 spectrometer with DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal reference. Single crystal X-ray diffraction analysis was carried out using single crystal X-ray diffractometer CAD4/MACH3 with Mo-Kα radiation (λ= 0.71073A°) at 20°C.

λ_{max} (absorbance) = 505 nm, λ_{max} (emission) = 556 nm. FT-IR υ (cm^{-1}) 1630 (C=Ostr), 1545 (-NO_2 asymm), 1400 (-NO_2symm). ^{1}HNMR DMSO-d6 δ (ppm) = 1.25-1.30 (t, 9H, CH_3, J=7.2 Hz), 3.07- 3.14 (q, 6H, -CH_2, J=7.2 Hz, 7.5 Hz), 4.3 (m, bridgehead CH), 4.7 (m, bridgehead CH), 5.1 (m, bridiging $HCNO_2$), 7.1-7.6 (m, 5H, Ar-H), 8.4 (s, CH propenide proton), 10.96 (s, NH), 14.95(s, OH).^{13}CNMR DMSO-d6 δ (ppm) = 13.57(s, 3C, CH_3), 51.24 (s, 3C, CH2), 35.60 (s, CH_2), 36.99 (s, bridgehead CH), 40.14 (s, bridgehead CH), 85.18 (s, bridging $HCNO_2$), 104.93 (s, =C-CO), 125.28 (s, propenide carbon), 125.54 (s, 2C, ortho carbons of phenyl ring), 128.73 (s, para carbon of phenyl ring), 133.32(s, 2C, meta carbons of phenyl ring), 132.48 (s, C=NO_2), 136.91 (s, C-NH), 143.01 (s, C-NO_2), 174.75 (s, -C=O), 175.12 (s, =C-OH).

Cells

EAC cell lines obtained from a recognized centre was maintained and used for experiments.

In-Vitro cytotoxicity

Short-term cytotoxicity was assessed by incubating 1 $X10^6$ EAC cells in 1 ml phosphate buffer saline at 37°C for 3 hrs in CO_2 atmosphere with varying concentrations of TBO. The viability of the cells was determined by the trypan blue exclusion method[13].

MTT Assay

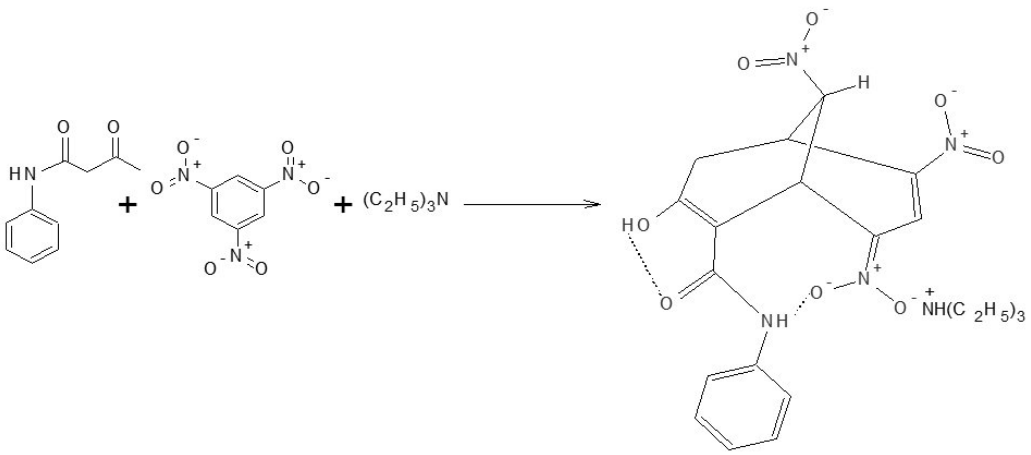
EAC were cultured in 96 well plates with growth medium RPMI 1640 and 10% FCS. Increasing concentrations of TBO were added to the cells and incubated at 37°C for 14 hrs in CO_2 incubator with 5% CO_2. The media was replaced with a fresh growth medium along with 20µl of 3-(4,5-dimethylthiazol-2-

yl)2,5diphenyltetrazolium bromide (MTT). Again it was incubated for 4 hrs at 37°C. After incubation purple precipitate was clearly visible under the microscope. Then the growth medium was removed and 200ml of 0.1% 0.1N acidic isopropyl alcohol was added to the cells to dissolve the MTT- Formazan crystals. Then the covered plates were kept in the dark at 18-24°C overnight. The samples colour were read at 570 nm. Experiments were repeated thrice. The average was calculated, and compared with the control test samples. The percentage growth inhibition was calculated using the following formula.[14]

$$\% \text{ Growth Inhibition} = \frac{\text{Control OD - Treated OD}}{\text{Control OD}} \times 100$$

Results and Discussion

The TBO adduct prepared from acetoacetanilide, 1,3,5-trinitrobenzene and triethylamine possess three nitro groups, an amide linkage, a rigid bicyclic skeleton similar to the naturally occurring taxol molecule and a diketo structure hydrogen bonded through enolic proton (Scheme1). All these characteristics are supported by FT-IR, NMR and single crystal XRD studies.



Scheme 1: Synthesis of TBO

The coloured bicyclic adduct TBO gives a single maximum absorption at 505 nm. Further, the molecule possess fluorescence property as evinced by the stokes shift (51 nm) of this absorption maximum from 505 nm to 556 nm in the emission spectrum. In the carbonyl region of the FT-IR spectra, the complex has no appreciable absorption above 1630 cm^{-1} although in acetoacetanilide a peak is observed at 1720 cm^{-1}. This can be explained by considering the enolisation of the diketo moeity. The absorptions at 1500-1445 cm^{-1} are due to anionic NO_2 asymmetric and symmetric stretching modes.[12,15] The bridgehead and bridging $HCNO_2$ protons appear as multiplets in ^1H NMR spectrum around ~4-5 ppm. The five protons are observed around 7.0-7.6 ppm in which the two ortho protons appear as a doublet at 7.6 ppm. The two meta protons give distinct signals at 7.4 ppm and 7.3 ppm respectively. The peak at 7.1 ppm corresponds to the para proton of the phenyl ring. The propenide proton appears at 8.4 ppm as observed by earlier workers.[12,15-19] The amide N-H proton which is deshielded due to hydrogen bonding exhibits peak at 10.9 ppm. The appearance of peak at 14.9 ppm in the bicyclic adduct indicate that there is an enolic hydrogen which is hydrogen bonded to the carbonyl attached to the N-H group.[12] These findings were supported by ^{13}C NMR spectrum in which the enolic diketo structure exhibit peaks at 174 ppm (-C=O) and 175 ppm (=C-OH). The close proximity of the two signals indicate the similarity of the two carbonyl groups, even though one carbonyl in the bicyclic skeleton is present in enolic form (C-OH) and the other being present as an amide carbonyl (CONH).

Inorder to reveal the configuration of the bridging ($HCNO_2$) proton which occurs as multiplet around 5 ppm, a COSY spectral analysis is performed. This multiplet may arise due to spin-spin coupling with the methylene protons present on one side of the bicyclic ring besides the bridgehead protons. The absence of cross peaks in the COSY

spectrum revealed that the bridging proton must be present on the side of the nitro propenide moiety as in Scheme 1. The presence of four double bonds in TBO are identified by single crystal X-ray diffraction analysis (C=O 1.250 A°, C=C-OH 1.320 A°, C=C-NO_2 1.356 A°, C=NO_2 1.360 A°). Further, the presence of distorted chair envelope conformation in TBO may be inferred from bond angles (bridgehead =C-CH-CNO_2 112.5°, bridgehead H_2C-CH-CNO_2 113.7°, bridging H-C-NO_2 109.5°) as reported by earlier workers.[20,21]

Cytotoxic Effect of TBO on EAC cells (Trypan Blue method)

The results of the present study revealed that TBO exhibited significant increment in non-viable cells in trypan blue cell viability assay at all test concentrations. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue whereas dead cells do not. Hence, dead cells are shown as a distinctive blue colour under light microscope.[22] At low concentration (50 µg/ml) TBO showed 21.29% cytotoxicity where as at high concentration (1000 µg/ml) showed 69.43% cytotoxicity (Table 1).

Table 1: Cytotoxic Effect of TBO on EAC cells (Trypan Blue method)

Concentration of TBO (µg/ml)	Viable cells	Viable cells (%)	Death cells	Death cells (%)
Control	130	92.19	11	7.81
50	85	78.71	23	21.29
100	96	73.28	35	26.72
250	71	63.96	40	36.04
500	68	46.50	64	51.15
1000	37	30.57	84	69.43

Cytotoxic effect of TBO on EAC cell lines (MTTAssay)

Cytotoxic activity of the TBO was also assessed through MTT assay. 24 hrs treatment with the TBO showed inhibition of EAC cells. TBO showed 100% cytotoxicity with IC_{50} value of 151.71 µg/ml (Figure 1). The reduction of tetrazolium salt is now widely accepted[23] as a reliable way to examine cell proliferation. The yellow tetrazolium MTT is reduced by metabolic active cells by the action of mitochondrial dehydrogenase enzymes. The death of the cells caused by the test drug under study might be due to the loss of mitochondria which is one of the hallmarks of the apoptosis pathway.

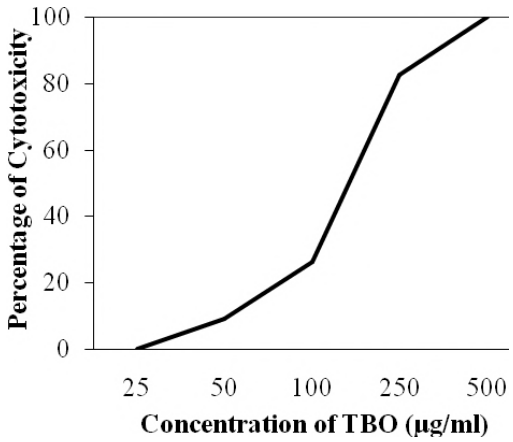


Figure 1. Determination of cytotoxicity by MTT Assay in EAC cell lines

In the present work, TBO was synthesized by a green method and the presence of bicyclo[3.3.1]nonane skeleton with enolised diketo structure in TBO was confirmed by spectral studies. The *In-Vitro* studies conducted clearly depicted that the synthesized TBO possess potent cytotoxicity against EAC cell lines by activating apoptotic pathway.

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