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Benzoin Thiosemicarbazone Inhibits Growth and Triggers Apoptosis in Earlich Ascites Carcinoma Cells through an Intrinsic Pathway

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Abstract Anticancer drug development is now an emerging field of research all over the world. In this study we attempt to synthesize a novel, simple, inexpensive and safe chemical agents Benzoin thiosemicarbazone (BTSC) and studied against Ehrlich Ascites Carcinoma (EAC) cells bearing Swiss Albino mice by monitoring in vivo tumor cell growth inhibition, survival time of tumor bearing swiss albino mice. MTT colorimetric assay was done to assess the in vitro effect of the test compound. The intrinsic apoptotic pathway induced by BTSC was evidenced by p53 or tumor protein, B-cell lymphoma 2 (BCL-2), B-cell lymphoma extra-large (BCL-xL), BCL-2 associated X protein (BAX), cleavage of caspase-9 and caspase-3 and poly-ADP ribose polymerase (PARP-1). Reactive oxygen species (ROS) generation after BTSC treatment was determined by 2´, 7´- dicholorodihydrofluorescein diacetate (DCFH-DA) staining. The compound was found to possess pronounced anticancer effect. Maximum cell growth inhibition, enhancement of life span was found 73.53% and 52.17% at the dose of 8 mg/kg (*i.p*) respectively. The induction of apoptosis by BTSC occurred through an ROS-dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway, and was regulated by the BCL-2 protein family. The compound exerted a potent antitumor effect toward Ehrlich ascites carcinoma cells through the induction of apoptosis via an intrinsic pathway. Thus, this study provides evidence to carry out further researches in a way to formulate novel anticancer drugs.

Keywords: *EAC cells, benzoin thiosemicarbazone, intrinsic pathway, ROS, caspase inhibitor*

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1. Introduction

Schiff bases are condensation products of aldehydes and ketones with primary amines and were first reported by Hugo Schiff in 1864. These are the compounds containing imine or azomethine (-HC=N-) functional group and are found to be a versatile pharmacophore for design and development of various bioactive lead compounds. In recent times schiff bases and schiff base metal complexes have drawn the attention of many researchers in medicinal and pharmaceutical fields due to a broad spectrum of biological activities like anticancer [1,2], antimicrobial [3], anti-tubercular, anti-inflammatory [4] and analgesic [5], antiviral, pesticidal [6] etc.. Several reports have been published as the successful use of sulphur containing schiff bases and schiff base complexes with thiosemicarbazones have showed better results [7-10]. However, further investigation is necessary for biological studies in order to understand more about their antitumor mechanism.

Apoptosis is a process of programmed cell death regulated by cellular signaling pathways and characterized morphologically by cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation and global mRNA decay. Apoptosis can be initiated through one of two pathways, one is intrinsic pathway and other is the extrinsic pathway [11]. In intrinsic (mitochondrial) pathway the tumor protein p53 and Bcl-2

becomes active which is layered on the surface of the mitochondria, detect damage and activate a class of proteins called Bax. This protein punch holes in the mitochondrial membrane, causing cytochrome c to leak out [12]. This cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), which is free floating in cells cytoplasm. Using energy from the ATPs in mitochondria, the Apaf-1 and cytochrome c binds to and activate caspase-9, another free floating protein. The caspase-9 then cleaves the caspase-3 and activate caspase cascade which then start a chain reaction of protein denaturation and eventually apoptosis.

The another pathway is extrinsic (death receptor) pathway initiated by death ligand and binds to cell surface receptors and activate caspase-8, which then activate downstream caspase-3 directly which play essential role in programmed cell death [13]. These two pathways are executed mainly by caspases a family of endoproteases that provide critical links in cell regulatory networks controlling inflammation and cell death with caspase-8 and -9 engaging in the extrinsic and intrinsic pathways, respectively [14,15]. In addition, reactive oxygen species (ROS), a series of oxygen metabolism byproducts, have a role in cell signaling, including; apoptosis; gene expression; and the activation of cell signaling cascades [16].

In this present paper we have reported anticancer activities of benzoin thiosemicarbazone against EAC cells in swiss albino mice and the mechanism of apoptosis.

2. Materials and Methods

2.1. Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

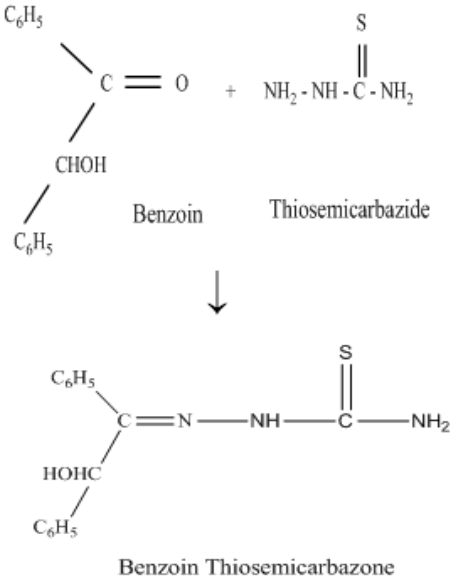
2.2. Experimental Animal

Adult male Swiss albino mice, 5–7 weeks old (25 ± 4 g body weight), were collected from animal resource branch of the International Centre for Diarrheal Disease Research (ICDDR'B), Mohakhali, Dhaka, Bangladesh. Animals were housed in iron cages containing sterile paddy husk and saw dust as bedding material under hygienic conditions with a maximum of six animals in a cage. They were maintained under controlled conditions (12:12 h light–dark with temperature 22 ± 5°C). Standard mouse diet (recommended and prepared by International Centre for Diarrheal Disease Research, Bangladesh Mohakhali, Dhaka) and water were given in adequate.

Ethic approval was obtained from institutional animal, medical ethics, bio-safety and biosecurity committee for experimentations on animal, human, microbes, and living natural sources from Institute of Biological Sciences, University of Rajshahi, Bangladesh (225/320-IAMEBBC/IBSc).

2.3. Synthesis of the Compounds

Synthesis of Benzoin thiosemicarbazone: The compounds were synthesized according to the method as described in the literature [17,18]. For benzoin thiosemicarbazone (BTSC), benzoin and thiosemicarbazide (1:1 molar ratio) were mixed together and refluxed for a period of 3-4 hours and then distilled to half of the total volume. The solution was then allowed to stand overnight till a yellowish white crystalline product separated out. The crystals were washed with ethanol and recrystallized and finally dried in an oven at 50°C and stored in a desiccator.



2.4. Characterization of the BTSC

The synthesized compound was characterized by taking melting point, elemental analysis and IR spectra.

Table 1. Characterization of BTSC

Yield percentage and physical characteristics of BTSC					
Test compound		Yield %	Melting point °C	Physical Form	Solubility
BTSC		60	150-153	White crystalline solid	Ethanol, Methanol, DMSO and acetone
Compound	Elemental analytical data found (calculated) in %				
	C	H	H	O	S
BTSC	59.78 (59.98)	5.31 (5.37)	18.50 (18.65)	5.30 (5.33)	10.07 (10.67)
IR spectral data of BTSC					
Compound	$\nu(NH_2)$	ν(N-H)	ν(C=N)	ν(C=S)	ν(NH-C=S)
BTSC	-------	3379 w	1682 s	1263 w	977 s

[s= strong, w= weak, m= medium].

2.5. Cell Lines

Ehrlich ascites carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as mammary gland cancer cell in ascites in Swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6 cells/mouse. For in vitro study, EAC cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium having glucose, 2 mM L-glutamine in presence of 10% fetal calf serum, and 1% (v/v) penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.6. *In vitro* Cell growth inhibition

In vitro effect of BTSC against EAC cells growth inhibition were assayed by MTT colorimetric technique. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to its insoluble formazan, which has a purple color. For this experiment, 4×10^6 EAC cells in 200 µL RPMI-1640 media were plated in the 96-well flat bottom culture plate in presence of six different concentrations (5 µg, 10 µg, 20 µg, 40 µg, 80 µg and 120 µg/mL) of BTSC and incubated for 24h at 37°C in CO_2 incubator. EAC cells treated with dimethyl sulfoxide (DMSO), was used as control. The assays were performed in triplicates to avoid experimental errors. After the incubation period, aliquot were removed carefully, and 180 µL of phosphate buffered saline (PBS) and 20 µL of MTT were added to each well and further incubated for 8h at 37°C. The aliquot was removed again and 200 µL of acidic isopropanol was added and incubated again at 37°C for 1h. Subsequently, absorbance was taken at 570 nm in microtiter plate reader (Optical Microplate Reader, Mikura Ltd., Horsham, UK). The following equation was used to calculate the cell proliferation inhibition ratio:

$$Proliferation\ inhibition\ ratio\ (\%) = \frac{(A-B)\times100}{A}$$

Where A is the OD 570 nm of the cellular homogenate (control) without experimental compounds and B is the OD 570 nm of the cellular homogenate with experimental compounds.

2.7. *In Vivo* Cell Growth Inhibition

In vivo tumor cell growth inhibition was carried out by the method as described earlier [19]. For this study five groups of mice (six in each group) were used. All the mice were inoculated with 2×10^6 EAC cells intraperitoneally. Treatment was started after 24 hours of tumor inoculation and continued for 6 days. Groups 1 to 3 were treated by BTSC at the doses of 2 mg/kg (*i.p.*) 4 mg/kg (*i.p.*) and 8mg/kg (*i.p.*) respectively per day per mouse. Group 4 received standard drug Bleomycin (0.3 mg/kg, *i.p.*). Treatment with only normal saline (0.98%) was considered as untreated control (group 5). The mice of all the groups were sacrificed on the 6th day after transplantation and tumor cells were collected by repeated intraperitoneal wash with 0.98% saline. Viable tumor cells per mouse of the treated groups were compared with those of control. The cell growth inhibition was calculated by using the following formula:

$$\%\ Cell\ growth\ inhibition = (1-T_{w/}C_w)\times100$$

where
T_w = Mean of number of tumor cells of the treated group of mice
C_w = Mean of number of tumor cells of the control group of mice.

2.8. Average Tumor Weight and Survival Time

The antitumor effects of BTSC was assessed by measuring average tumor weight, mean survival time (MST) and percentage increase of life span (% ILS) [20]. These parameters were measured under similar experimental conditions as stated in the previous experiment (cell growth inhibition). Treatment was continued for 10 days. Tumor growths were monitored daily by measuring weight change. MST of each group (6 in each) was monitored by recording the survival time. MST and % ILS were calculated by using the following equations.

$$MST = \frac{survival\ time\ (days)\ of\ each\ mouse\ in\ a\ group}{Total\ number\ of\ mice}$$

Percent increase of life span,

$$\%\ ILS = \frac{MST\ of\ treated\ group}{MST\ of\ control\ group}\times100.$$

2.9. Cell Morphologic Change and Nuclear Damage

Morphological observation of cells in absence and presence of BTSC were studied [21] using a fluorescence microscope (Olympus iX71, Seoul, Korea). EAC cells were collected from the treated and untreated EAC cells bearing Swiss albino mice and washed twice with phosphate buffer saline (PBS). Then cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) at 37 °C for 20 min and washed twice again with PBS.

2.10. DNA Fragmentation Assay

DNA fragmentation assay by agarose gel electrophoresis was determined by the method described previously [22]. EAC cells collected from mice treated with experimental compound BTSC at the dose of 8mg/kg/mice (five consecutive days) and from control mice. The cells were washed with PBS and suspended again in PBS. The total DNA was isolated by using a DNA extraction kit (Promega, Madison, WI, USA) and analyzed by electrophoresis on 1.5% agarose gel containing 0.1µg/mL ethidium bromide and visualized under Ultraviolet illuminator.

2.11. Effect of Caspase Inhibitors on EAC Cells

In order to confirm the involvement of caspases [23] in the experimental compounds induced cell death, the

untreated EAC cells were collected on day six of tumor inoculation and washed with PBS. Then these cells were incubated in CO_2 incubator (RPMI-1064 media) with Z-DEVD-FMK (caspase-3 inhibition, 2µmol/mL) and Z-IETD-FMK (caspase-8 inhibitor, 2µmol/mL) for 2h. Then the cells were treated with BTSC and incubated for another 24 hours at 37°C in CO_2 incubator. Finally, the cells were counted using hemocytometer and determined the effects of BTSC on cell growth inhibition in presence or absence of specific inhibitor.

2.12. Measurement of Reactive Oxygen Species (ROS) Generation

The ROS level of the cells were examined using 2´, 7´-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, USA). The cells were collected from control mice and treated mice on day six of tumor inoculation and washed with PBS at 1200rpm for 2minutes. Then the cells were incubated with DCFH-DA 10µL (50µM final concentration) at 37°C for 30minutes in the dark. Again the cells were washed twice with PBS and maintained in 1mL culture medium. ROS generation was assessed using a fluorescence microscope at excitation and emission wavelengths of 485nm and 530nm, respectively [24].

2.13. Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using TRIzol method from mice receiving BTSC at the dose of 8mg/kg/day and control EAC bearing mice on day six of tumor implantation. Total RNA was used as a template for reverse transcription using the following protocol: each 20µL reaction contained 125µM deoxynucleotide (dNTP), 100 pmol random hexamer, 100 pmol oligo dT_{18} primer, 50 units of MuLV reverse transcriptase (New England Bio lab, Ipawich, MA, USA), diethylpyrocarbonate (DEPC) treated water and 3µg total RNA. Briefly, RNA and oligo dT_{18} primer where incubated at 70°C for 15 minutes then immediately placed on ice, after which the other components were added and incubated at 42°C for 1h and then at 70°C for 15 minutes. Expression of one housekeeping gene (GAPDH) and eight growth regulatory genes, namely, Bcl-2, Bax, p53, Bcl-xL, PARP 1 and Caspase-3,-8,-9 were examined using these cDNA as template for PCR. Reaction mixture were prepared containing 1X of Taq polymerase, 25pmol each of forward and reverse primer, 2.5mM of each dNTP and 0.25U of platinum Tag polymerase (Tiangen, Beijing, China), 2µL cDNA and nuclease-free water to a total volume of 25µL. Primer sequences are listed in Table 2. BioRad (Hercules CA, USA) gradient thermal cycler was used for amplifications. All the PCR products were analyzed in 1.5% agarose gel and Gene Ruler 1kb DNA ladder (Fermentus, Pittsburgh, PA, USA) was used as marker. The cycling condition for initial PCR activation step of 3 min at 95°C, followed by 35 cycles of 95°C/1 min. For p53, Bcl-2 and Caspase-3, -8, -9 genes 55°C/1 min, 72°C/1 min and a final extension of 72°C/1 min. In the case of Bax, GAPDH and Bcl-xL, the annealing temperature was 54°C.

2.14. Statistical Analysis

The experimental results have been expressed as the mean ± S.E.M. Data have been calculated by one was ANOVA followed by Dunnett “t” test for the determination of statistical significance using SPSS software of 20 version. The difference was considered to be statistically significant when $p < 0.05$.

Table 2. Primer sequences and PCR protocol used for the RT-PCR assay

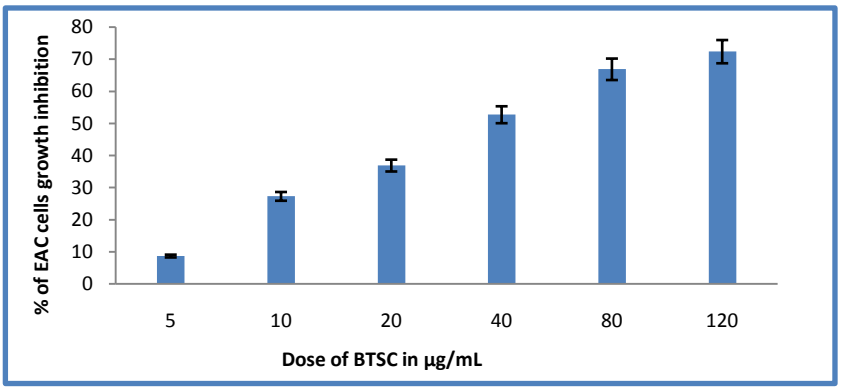
Sl. No.	Target genes	Primers	Amplification (kb)
1	Bcl-2 F Bcl-2 R	5´-GTGGAGGAGCTCTTCAGGGA-3´ 5´-AGGCACCCAGGGTGATGCAA-3´	0.200
2	BAX F BAX R	5´-CGCCCACCAGCTCTGAGCAGA-3´ 5´-GCCACGTGGGCGTCCCAAAGT-3´	0.500
3	P^{53} F P^{53} R	5´-GCGTCTTAGAGACAGTTGACT-3´ 5´-GGATAGGTCGGCGGTTCATGC-3´	0.550
4	Bcl-X F Bcl-X R	5´-TTGGACAATGGACTGGTTGA-3´ 5´-GTAGAGTGGATGGTCAGTG-3´	0.700
5	Caspase-3 F Caspase-3 R	5´-TTAATAAAGGTATCCATGGAGAACACT-3´ 5´-TTAGTGATAAAAATAGAGTTCTTTTGT-3´	0.300
6	Caspase-8 F Caspase-8 R	5´-CTGCTGGGGATGGCCACTGTG-3´ 5´-TCGCCTCGAGGACATCGCTCTC-3´	0.450
7	Caspase-9 F Caspase-9 R	5´-ATGGACGAAGCGGATCGG-3´ 5´-CCCTGGCCTTATGATGTT-3´	0.400
8	PARP-1 F PARP-1 R	5´-AGGCCCTAAAGGCTCAGAAT-3´ 5´-CTAGGTTTCTGTGTCTTGAC-3´	0.270
9	GAPDH F GAPDH R	5´- GTGGAAGGACTCATGACCACAG-3´ 5´- CTGGTGCTCAGTGTAGCCCAG-3´	0.350

3. Results

3.1. *In vitro* EAC Cell Growth Inhibition with BTSC

In vitro effect of BTSC on EAC cells growth inhibition have been investigated by MTT assay. The BTSC induced EAC cells death is a dose dependent manner (Figure 1). The BTSC showed 72.40% cancer cell growth inhibition at the concentration of 120 µg/mL. The effect decreased with the reduction in concentration of the experimental compound and it reached to 8.7% at 5 µg/mL.

In vivo tumor cell growth inhibition was observed with BTSC at the doses of (2 mg/kg *i.p.*, 4 mg/kg *i.p.*, 8 mg/kg *i.p.*) per mouse per day. Maximum cell growth inhibition (73.53%) was found at the dose of 8 mg/kg (*i.p*) whereas *Bleomycin* shows 88.2% cell growth inhibition at the dose of 0.3 mg/kg *(i.p)* (Table 3).



Data are expressed as the mean of results in 3 repeated experiments. Error bar represent standard error of mean.

Figure 1. *In vitro* effect of BTSC on EAC cell growth inhibition

Table 3. Effect of the BTSC and *Bleomycin* (antitumor drug) on cell growth inhibition *in vivo*

Treatment	Dose, mg/kg (i.p.)	No. of EAC cells in mice on day 6 after tumor cell inoculation × 10^7	% Cell growth inhibition
Control (untreated EAC cells bearing mice)	-	2.180±0.082	-
EAC+ Bleomycin	0.3	0.257±0.010***	88.2
EAC+BTSC	2	0.1050±0.023***	51.83
	4	0.858±0.009***	60.61
	8	0.577±0.004***	73.53

Mice were inoculated 2×10^6 EAC cells/mouse (*i.p.*) on days 0. Treatment was started after 24 hours of tumor cell transplantation. Number of mice in each experiment were six (n=6); the results were shown as mean ± S.E.M (Standard error of mean). Treatment was continued for 6 consecutive days. Where significant values are *p<0.05, **p<0.01, and ***p<0.001 when compared with control.

Table 4. Effect of BTSC and *Bleomycin* on survival time and increase of life span of EAC cell bearing mice

Treatment	Dose, mg/kg (*i.p.*)	Mean survival time mean ± S.E.M. (days)	% Increase of life span
Control (untreated EAC cells bearing mice)	-	23±0.98	-
EAC + Bleomycin	0.3	43±0.86***	86.95
EAC+BTSC	2	28±0.79*	21.74
	4	32±0.88*	39.13
	8	35±1.09**	52.17

Data are expressed as the mean of results in 6 mice ± S.E.M. Treatment was continued for 10 consecutive days. Where significant values are *p<0.05, ** p<0.01 and *** p<0.001 when compared with control.

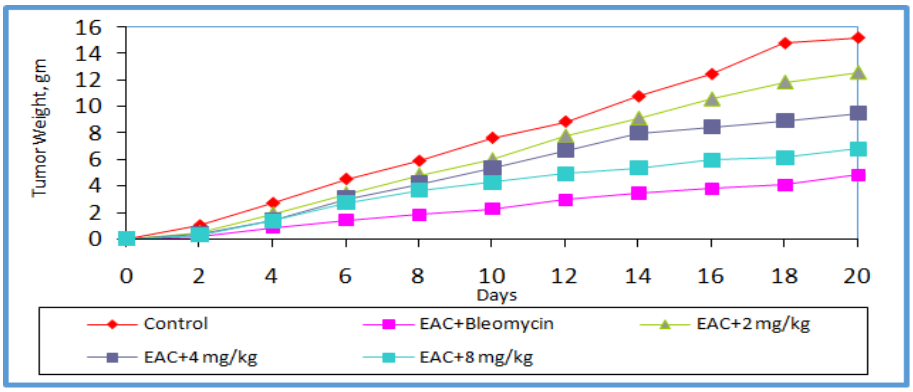


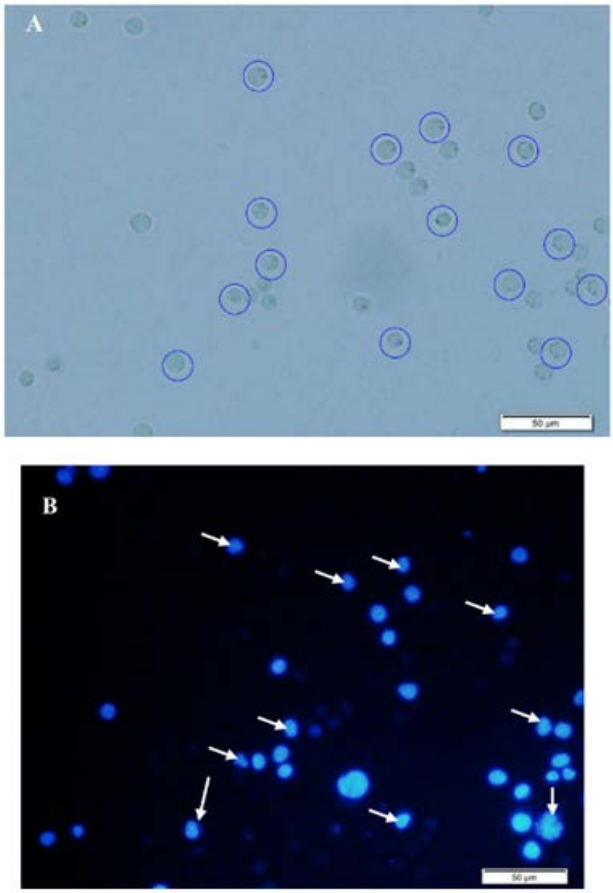
Figure 2. Effect of BTSC on average tumor weigh

The mean survival time (MST) of the untreated tumor bearing mice was 23 days. With the treatment of BTSC, the value was found to be increased. About 52.17% enhancement of life span was found at 8 mg/kg (*i.p*) with BTSC (Table 4).

The treatment with BTSC also reduced the rate of tumor growth. At day 20, BTSC at the dose of 8 mg/kg *i.p* reduced the tumor weight by 33.31% as compared to that of control mice (Figure 2).

3.2. Morphological Changes and Nuclear Damage Induced by BTSC

Morphological changes of EAC cells in absence and presence of BTSC at the dose of 8 mg/kg/day, were studied using a fluorescence microscope (Olympus iX71, Seoul, Korea) and confirmed by 4', 6-diamidino-2-phenylindole (DAPI) staining. Round and homogeneously stained nuclei were observed in the control group (Figure 3A) and the cells of treated group exhibited manifest fragmented DNA in nuclei (Figure 3B). Apoptotic morphologic alterations (e.g. fragmentation, cell membrane blebbing and nuclear condensation) were also observed clearly by optical microscopy and these results suggested that experimental compounds could induce apoptosis of EAC cells.



Comments: Cells were collected from untreated EAC bearing mice A (optical) and BTSC treated EAC bearing mice B (fluorescence). Arrows indicate apoptotic features (condensed chromatin and nuclear fragmentation).

Figure 3. Effect of BTSC [8 mg/kg/day (*i.p.*)] on morphological changes of EAC cells

3.3. DNA Fragmentation Assay

The activation of the endogenous Ca^{2+}/Mg^{2+} dependent endonuclease is the most distinctive biochemical hallmark of apoptosis. This activated endonuclease mediated the cleavage of internucleosomes and generates oligonucleotide fragments of about 180-200 base pairs length or their polymers. A DNA distinct band was obtained in agarose gel electrophoresis of DNA preparation from the control group. On the other hand, experimental compound BTSC treated EAC cells shown smear-like DNA degradation which is characteristic feature of apoptosis induction control group. Result was shown in Figure 4.

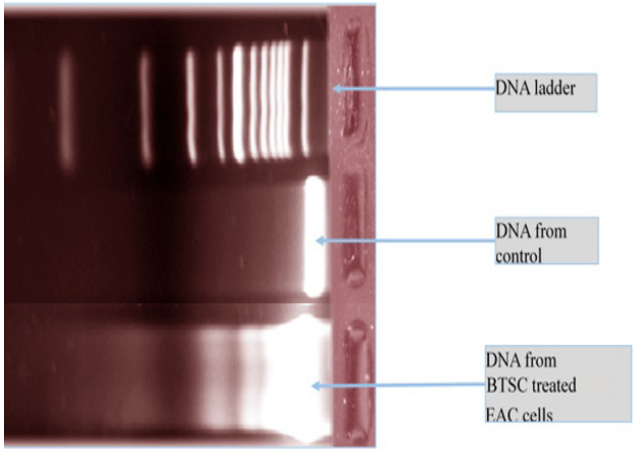


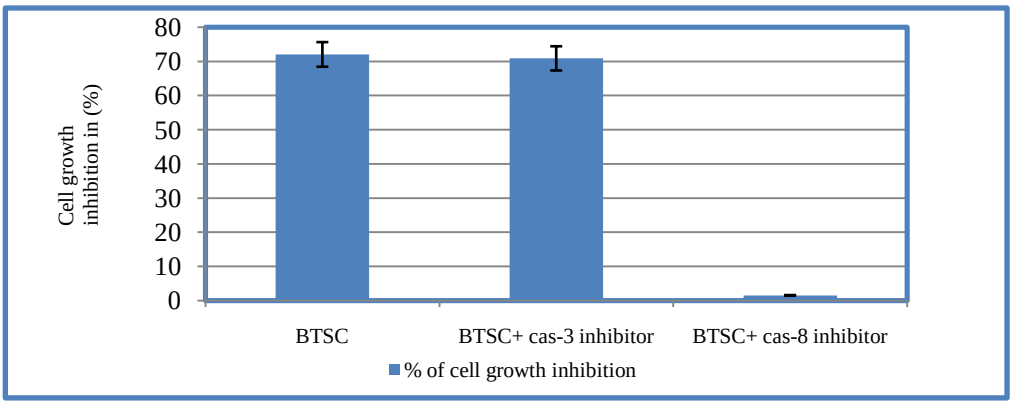
Figure 4. DNA fragmentation assay in BTSC-treated mice

3.4. Effect of Caspases on BTSC-induced Cytotoxicity in EAC Cells

Caspase inhibitors Z-DEVD-FMK (caspase-3 inhibitor) and Z-IETD-FMK (caspase-8 inhibitor) were used to detect the involvement of specific caspases in the apoptotic cell death of EAC cells induced by the treatment of experimental compound BTSC. In the presence of BTSC growth inhibition of EAC cells were 78.89% and it decreased to 3.88 and 75.01% for in presence of Z-DEVD-FMK (caspase-3 inhibitor) and Z-IETD-FMK (caspase-8 inhibitor) in the culture medium (Figure 5).

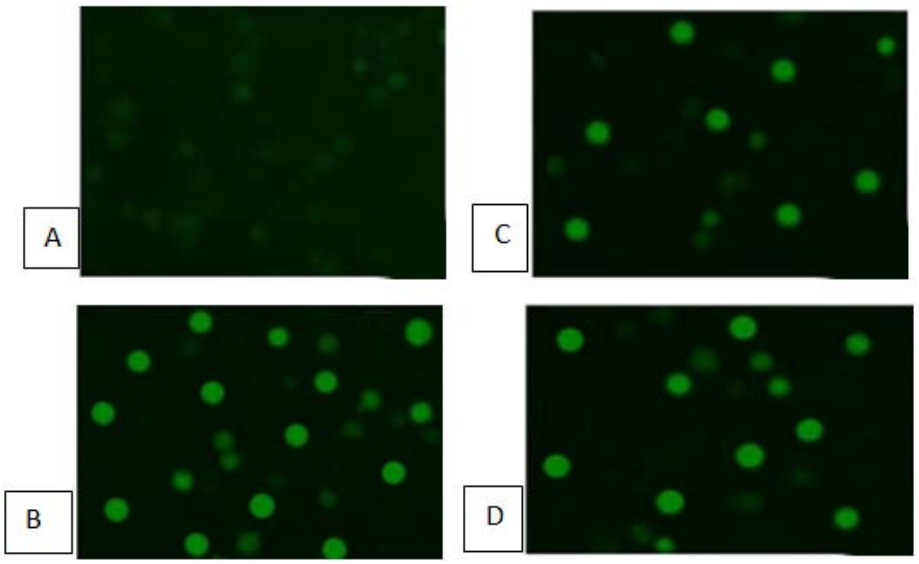
3.5. Determination of Intracellular Reactive Oxygen System in EAC Cells

2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) is a common fluorescent probe that can be used for detecting several ROS in biological media. The DCFH-DA first hydrolyzed to DCFH by intracellular esterase, which is then oxidized by reactive species and originates a fluorescent compound 2′, 7′-dichlorofluorescein (DCF), whose fluorescence intensity is proportional to the levels of ROS. Here, DCFH-DA was used to assess the effect of BTSC on ROS generation in EAC cells after 24h treatment. As shown in Figure 6, the untreated control cells displayed little green fluorescence, while increasing concentrations of the experimental compound resulted in much stronger signals, with 8 mg/kg (*i. p.*) the compound producing the strongest intensity.



Numbers of mice in each experiment were 6. The results are shown in mean±SEM. Significant value is, **p<0.01.

Figure 5. Effect of caspases on BTSC-induced cytotoxicity in EAC cells



Effects of $Ni(BTSC)_2$ on the generation of ROS in EAC cells after 24 h treatment. Observation of cells by fluorescence microscopy (200× magnification) after incubation with DCFH-DA. (A) Control cells, (B) 2 mg/kg (*i.p*), (C) 4 mg/kg (*i.p.*) and (D) 8 mg/kg (*i.p.*).

Figure 6. Effect of BTSC on intracellular ROS generation

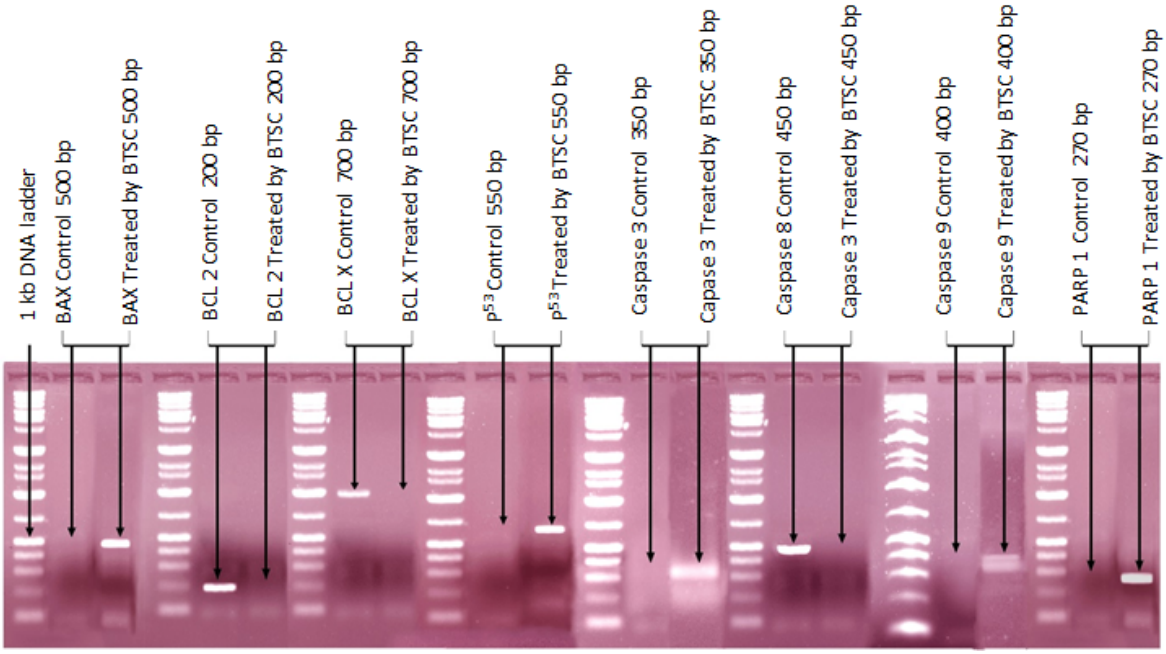


Figure 7. Expressions of different cancer-related genes in BTSC-treated EAC cells

3.6. Altered Expressions of Cancer-related Genes in BTSC Treated EAC Cells

Reverse transcription PCR was used to study the mRNA expression levels of several tumor-related anti-apoptotic genes (Bcl-2, Bcl-xL and caspase 8) and pro-apoptotic genes (p53, Bax, PARP-1, caspase-3 and caspase-9) in control and BTSC-treated EAC cells (Figure 7). GAPDH primers were used for an amplification reaction to confirm the suitability of the purified RNAs and the samples were found to be suitable for RT-PCR. The control cells showed high expressions of Bcl-2, Bcl-xL and caspase 8 genes, whereas EAC cells treated with BTSC showed reduced Bcl-2, Bcl-xL and caspase 8 mRNA expressions. In addition, the p53, Bax, PARP-1, caspase-3 and caspase-9 genes showed increased expressions in treated EAC cells. On the other hand, no expression of these genes was in control mice.

4. Discussion

The results presented in the above section showed that the schiff base BTSC inhibited EAC cells proliferation *in vitro* in a dose dependent manner (Figure 2). *In vivo* effect of the compound is quite comparable to those of *bleomycin* at dose 0.3 mg/kg (*i.p.*). At the dose of 8 mg/kg (*i.p.*) the BTSC showed 77.15% cell growth inhibition, whereas *bleomycin* showed 88.20% cell growth inhibition.

In the present study BTSC induced apoptosis was confirmed by the observation of the changes in nuclear morphology and cell shape as compared to that of the control EAC cells. Given that the activity of the caspase-3 and caspase-8 were blocked significantly by the z-DEDV-fmk and z-IETD-fmk inhibitors respectively. From our result it is clear that BTSC showed enhanced caspases-3 and caspases-9 activities, while caspase-8 was not affected, suggesting that apoptosis occurred mainly through the mitochondrial pathway.

Similarly, this study showed induction of apoptosis in BTSC-treated EAC cells are regulated by numerous growth related genes. Among them, Bcl-2 (B cell lymphoma gene 2) family gene was believed to be the first one attributed in the apoptotic process [25]. Among the apoptosis-related genes, Bax, Bid, and Bak act as pro-apoptotic, and other members Bcl-2, Bcl-xL, and Bcl-W act as anti-apoptotic class [26]. In the present study, we found that Bcl-2 and Bcl-xL expressions were observed in control mice, and they were absent in mice treated with BTSC, whereas pro-apoptotic genes such as Bax, P53, and PARP-1 expressions showed opposite results. We found overexpression of these genes in BTSC-treated mice in comparison with that of control mice (Figure 7). Increased expression of these genes could lead to cytochrome c release from mitochondria, which binds to apoptotic protease activating factor-1 (Apaf-1) and ATP. Which then binds to procaspase-9 to create a protein complex known as an apoptosome. The apoptosome then activates caspase-3 and caspase-9, leading to the cleavage of PARP-1 [27]. Altered expression of these proapoptotic and antiapoptotic genes in EAC cell upon BTSC treatment further confirmed the induction of programmed cell death of cancer cell.

It has been believed that in cancer progression and prevention ROS acts as a double-edged sword. Moderate levels of ROS maintain essential mechanisms of cancer cell survival, such as proliferation and angiogenesis [28], while high levels of ROS lead to the destructive effects on cancer cells through pathways such as apoptosis and autophagy [29,30]. In this study, it is revealed that treatment with BTSC for 24 h increased ROS production in EAC cells in a dose-dependent manner. Since the stability of the mitochondria is closely related to ROS balance, it can be said that apoptosis occurred in this study mainly through the mitochondrial pathway.

5. Conclusion

This compound may therefore be considered primarily as potent anticancer agent and the possible mechanism for the anticancer effect is ROS dependent mitochondria-mediated intrinsic pathway rather than an extrinsic pathway and was regulated by the Bcl-2 protein family. In order to ascertain these compounds as novel potential anticancer drugs, it is necessary to carry out further experiments against other cancer cell lines with higher test animals and with advanced techniques. However these findings definitely give positive support to carry out further researches in a way to formulate novel anticancer drugs.

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