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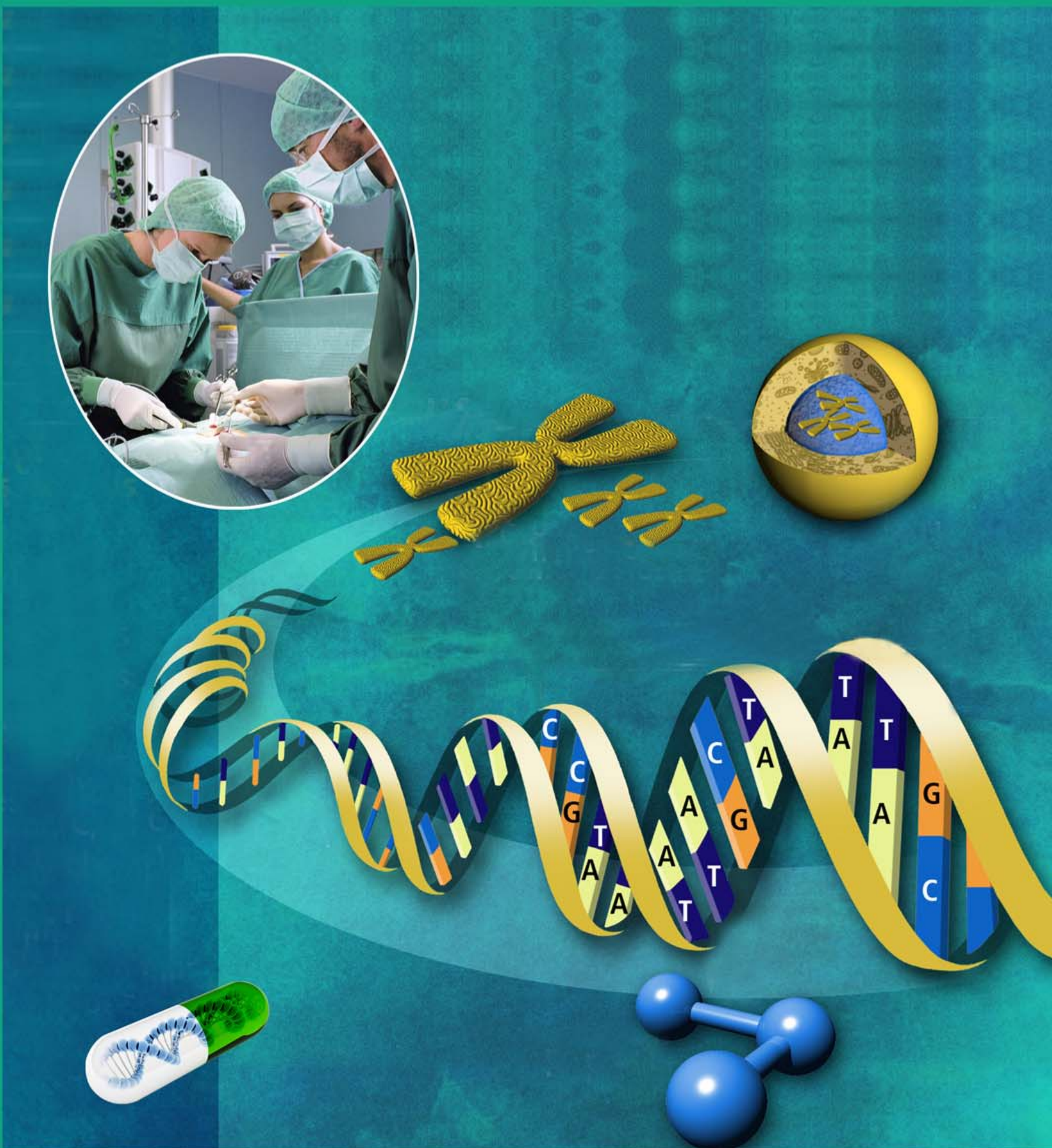

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Research Paper

EFFECT OF A SIDEROPHORE PRODUCER ON ANIMAL CELL APOPTOSIS: A POSSIBLE ROLE AS ANTI-CANCER AGENT

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Iron plays an essential role in the proliferation of aggressive tumors therefore it represents an ideal target for cancer therapy. Cell free supernatants from a siderophore producing actinobacterium previously isolated from Thailand were tested against six human cancer cell lines including malignant melanoma A 375 (ATCC no.: CRL-1619), colorectal adenocarcinoma SW620 (ATCC no.: CCL-227), gastric carcinoma Katob III (ATCC no.: HTB-103), liver hepatoblastoma HepG2 (ATCC no.: HB-8065), breast carcinoma BT474 (ATCC no.: HTB-20) and Acute T cell leukemia Jurkat (ATCC no.: CRL-2063). Following treatment of cells with the bacterial culture supernatant the cell viability of A375 cells was dramatically decreased with cell survival of less than 34 % within 48 h. The rest of the cell lines were largely unaffected. Therefore it is suggested that the actinobacterium produced a cytotoxic compound responsible for the cell death by inducing apoptotic activity. We further speculate that this compound was desferioxamine E as the bacterium is known to produce this compound under the culture conditions used.

Keywords: Siderophores, Actinobacteria, Human cell cancer lines, Apoptosis

INTRODUCTION

Cancer cells have an increased requirement for iron compared with 'healthy' cells due to their rapid division. They also posess a higher rate of iron uptake and storage (Elford *et al.*, 1970; Vaughn, 1987). Moreover the ribonucleotide reductase (actinobacterium) enzyme involved in DNA synthesis is upregulated in cancerous cells leading to increased DNA replication and cell proliferation (Buss *et al.*, 2003; Dayani *et al.*, 2004, Richardson, 1997). Iron is fundamental for the activity of ribonucleotide reductase making iron an obvious anti-cancer candidate (Thelander and Gräslund, 1983; Whitnall *et al.*, 2006).

Siderophores are low molecular weight compounds (600-1500 Daltons) that chelate ferric complexes and other ions with a high affinity. Due to the role of iron in cellular proliferation, iron chelators, such as siderophores, are agents that may be beneficial for the treatment of cancer

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(Wandersman and Delepelaire, 2004). Indeed some data demonstrated that desferrioxamines (iron-free siderophores) can significantly reduce the growth of aggresive tumours in patients suffering from neuroblastom (NB) or leukemia (Buss *et al*., 2003; Lovejoy and Richardson, 2003). Clinical studies have demonstrated that following a 4 h incubation desferrioxamine can inhibit DNA replication of NB cells (Blatt *et al.*, 1988). In addition a 72 h exposure of NB cells to 60 μM of desferrioxamine reduces the cells' viability by 80 % (Blatt and Stitely, 1987).

An actinobacterium (GenBank accession number EF585403), previously isolated from Tak province in Thailand, was tested positive for siderophore production and its main chelating agent released was identified as desferrioxamine E by HPLC analysis (Nakouti *et al*., 2012). The aim of this work was to screen the culture supernatant for anti-cancer activity using a range of cancerous cell lines and to investigate if a posssible response mechanism was due to apoptosis.

MATERIALS AND METHODS

Growth Media

Starch casein media: Casein (0.4 g), starch (1.0 g), KNO_3 (0.5 g), K_2HPO_4 (0.2 g), $MgPO_4$ (0.1 g), $CaCO_3$ (0.1 g) and bacteriological agar (20 g) were dissolved in 1 litre of distilled H_2O and sterilised by autoclaving at 121^{0}C for 15 min (solid media). Liquid media were prepared by omitting the bacteriological agar.

Culture Conditions

Spores of the strain were stored in a bacterial preservation system (Technical Service Consultants Ltd, Lancashire) at -80^{0}C and 10 μl were spread onto starch casein agar plates and incubated at 30^{0}C for a week until sporulation occurred. Fresh spores were collected aseptically (in 20 ml of sterile distilled H_2O) and an aliquot (100 μl) was introduced into a 250 ml conical flask containing 50 ml of sterile starch casein media (liquid). The cultures were supplemented with 5 g/L lysine prior to incubation on a rotary shaker (200 rpm) at 30^0 C for 4 days. The conditions were previously optimised for maximum desferrioxamine E production (Nakouti and Hobbs, 2012). Following incubation the medium was centrifuged for 20 min at 8000 g to separate the mycelia. The supernatant was filter-sterilised by using a 0.20 μm syringe filter (Corning) and stored at 4^{0}C.

Biological Assay of Anticancer Activity

The effect of the culture supernatant on the cell viability was assessed using the MTT assay (Palaga *et al*., 1996) against 6 human cancer cell lines including malignant melanoma A375 (ATCC no. CRL-1619), colorectal adenocarcinoma SW620 (ATCC no. CCL-227), gastric carcinoma Katob III (ATCC no. HTB-103), liver hepato-blastoma HepG2 (ATCC no. HB-8065), breast carcinoma BT474 (ATCC no. HTB-20) and Acute T cell leukemia Jurkat (ATCC no. CRL-2063). Cell suspensions in PRMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 100 mg/ml gentamicin were seeded into flat-bottomed 96-well plates at a density of 2-3 x 10^4 cells/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. An aliquot (50 μl) of the culture supernatant was added to the well and incubated (48 h contact time) under the same conditions.

Cell viability was calculated as $(A_{sample}-A_{blank})/(A_{control}-A_{blank})$ x 100 % while absorbance (540 nm) obtained from cells incubated with sterile PRMI-1640 medium was used as the control (positive) and cell free medium as the blank (negative). Ferrichrome (iron-free) from *Ustilago sphaerogena* (5μl of 1 mg/ml concentration) was also tested against the cell lines for anti-cancer activity. The reason behind this was that desferrioxamine E was not available in the market; therefore ferrichrome, also a hydroxamate, was purchased from Sigma-Aldrich.

Apoptosis Tests

A cell culture dish (35 x 10 mm) containing a sterile glass coverslip in RPMI-1640 medium was seeded with a cell suspention of A375 cells (final concentration of 1 x 10^6 cells/ml). The cells were allowed to attach for 24 h, followed by treatment for 48 h. They were then washed with phosphate buffer saline (PBS), fixed with 1% (v/v) glutaraldehyde in the dark for 2 h and washed with PBS. Cells which had attached to the glass coverslips, were stained with DNA dye Hoechst 33342. After being left for 5 min in the dark, apoptotic cell death was determined by observing fragmented nuclei cells under the fluorescent microscope (modified from [14]).

RESULTS AND DISCUSSION

Anticancer Activity

The effect of the strain's supernatant on cellular viability was assessed using the MTT assay. The isolate was found to produce strong cytotoxic substances specific to malignant melanoma cells (A375). Following treatment of cells with the culture supernatant (50 μl) the percentage cell viability of A375 cells was dramatically decreased with cell survival of less than 34 % (33.193 %) within 48 h (Table 1); whereas the remaining cancer cell lines exhibited similar cell viability. The experiment was an average of 12 replicates. Therefore it was suggested that the organism exhibited selective activity against the skin cancer cell line. Ferrichrome produced by *Ustilago sphaerogena* had little effect on any of the cancer cell lines (cell viability after treatement was measured to be 94 %).

Induction of Apoptosis by *Streptomyces* sp. Strain 23F

The supernatant (50 μl) of strain 23F (GenBank accession number EF585403) was tested for induction of apoptosis with malignant melanoma cells (A375). Addition of the supernatant to A375 cell culture resulted in the induction of apoptotic

Table 1: MTT Assay Data Demonstrating the Anticancer Activity of the Siderophore Producer Supernatant

Samples	1	2	3	4	5	6	7	8	9	10	11	12	Mean	Viability (%)	STD (%)
Blank	0.038	0.035	0.035	0.035	0.035	0.038	0.038	0.035	0.035	0.035	0.038	0.038	0.036	0	N/A
Control	1.178	1.226	1.293	1.190	1.198	1.227	1.210	1.293	1.193	1.224	1.194	1.290	1.226	100	N/A
Treated with Supernatant	0.421	0.444	0.454	0.422	0.440	0.424	0.422	0.443	0.423	0.423	0.441	0.421	0.431	33.19	+/–0.957
Treated with ferrichrome	1.166	1.101	1.109	1.190	1.192	1.105	1.170	1.163	1.199	1.169	1.196	1.166	1.160	94	+/–2.877

activity. Cells from the culture were stained with Hoechst dye and apoptotic nuclei were visualised under the fluorescent microscope (Figure 1). The cancer cells (A375) became smaller through shrinking and the chromatin became agglutinated resulting in nuclear condensation followed by breakdown of the nuclear membrane. The remaining 'attached' cells appeared very similar to the untreated ones. Cells treated with sterile starch casein media (negative control) did not display any DNA fragmentation and appeared similar to untreated cells (Figure 2). The results suggested that the cytotoxic compound has induced apoptosis in the A375 cell line.

Figure 1: A375 Cancer Cells Stained with Hoechst, the Apoptotic Nuclei were Visualised Under the Fluorescent Microscope (X 40 Magnification)

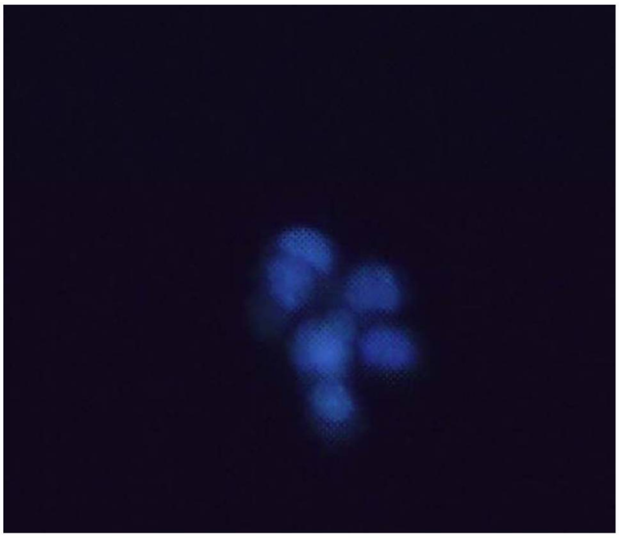
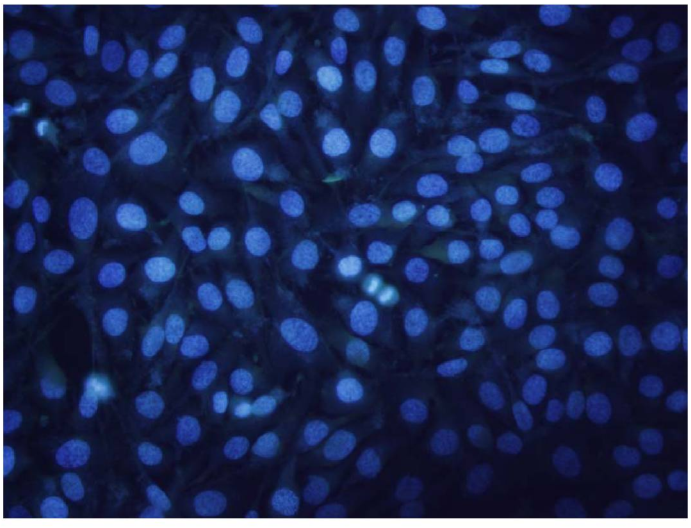


Figure 2: A375 Cells Treated with Starch Casein Media (Negative Control), Cell Division is Evident (x 40 Magnification)



CONCLUSION

The actinobacterium (GenBank accession number EF585403) released a cytotoxic substance that induced apoptosis to malignant melanoma A375 (ATCC no. CRL-1619).

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