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ULTRASTRUCTURAL STUDIES ON THE EFFECT OF *SPIRULINA PLATENSIS* ON EXPERIMENTALLY INDUCED BLADER CANCER IN RATS.

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ABSTRACT

This work aimed to investigate the protective effect of *Spirulina platensis* on the ultrastructural changes of rat bladder cancer induced by dibutyl nitrosamine (DBN) precursors using scanning and transmission electron microscopes. Scanning electron micrographs (SEM) of the urinary bladder of *Spirulina* treated animals showed tightly adherent urothelial cells with polygonal profiles as that of the control rats. Also, the overall appearance of the urothelium in transmission electron micrographs (TEM) was much similar to like that of the control animals. The carcinogenicity of DBN precursors was clearly demonstrated in the form of long papillomas and nodular aggregates protruding into the lumen. Moreover, the results showed the occurrence of microvilli covered with glycocalyx. Deformed mitochondria, extensive rough endoplasmic reticulum, large number of ribosomes and polysomes were clearly visible throughout the cytoplasm of the urothelial cells. The nuclei contained masses of condensed chromatin especially peripheral to the nuclear envelope. The animals treated with DBN and spirulina showed long microvilli on the surface of the urothelial cells. In addition, large areas of the basal lamina lost some of their urothelial cells. The apoptotic cells were also identified by characteristic ultrastructural features suggesting that *Spirulina platensis* is a chemotherapeutic agent that cause apoptosis to tumor

cells by reduction of the number of malignant cells to a single layer.

INTRODUCTION

In recent years, there is an increasing awareness that certain naturally occurring compounds in plants and other sources, have protective effects against environmental mutagens/carcinogens and endogenous mutagens (***Premkumar et al., 2004).***

Spirulina platensis is a cyanobacterium grown in certain countries and used as food for human and animal consumption. Over the last few years, it has been experimentally proven, *in vivo* and *in vitro* studies that *Spirulina platensis* is effective in the treatment of certain allergies, anemia, cancer, hepatotoxicity, viral and cardio- vascular diseases, hyperglycemia, hyperlipidemia, immunodeficiency and inflammatory processes, in addition to some other symptoms. These activities are attributed to *Spirulina* as a whole or to some of its components including fatty acids omega-3 or omega-6, beta-carotene, alpha-tocopherol, phycocyanin, phenol compounds, and a recently isolated complex, Ca-Spirulan ***(Chamorro et al. 2002).***

Bladder cancer is one of the most common malignancies occurring worldwide. The incidence of bladder cancer varies throughout the world ***(Boring et al., 1994),*** the highest incidence rates are generally found in industrially developed countries, particularly North America and Western Europe and areas associated with endemic schistosomiasis infection, including parts of Africa and the Middle East ***(Parkin et al., 1993).***

A very large volume of literature has accumulated on the carcinogenic activity of a whole range of nitroso compounds, reviewed by ***Druckery et al.(1967)*** and Magee and ***Barnes (1967).*** So far, nitrosodibutylamine (DBN), butyl-4-hydroxybutylnitrosa-amine (BBN), N-butyl-N-(3-carboxypropyl)-nitrosamine,N-ethyl-N-(4-hydroxybutyl)-nitrosamine,nitrosomethyl-dodecylamine and N-methyl-N-nitrosourea (MNU) have been shown to be potent inducer of urinary bladder tumours in rodents.The present work aimed to study the effect of *Spirulina platensis* on bladder cancer induced by DBN precuies in rats.

MATERIALS AND METHODS

The DBN precursors solution was prepared by mixing by the 2000 ppm of Sodium nitrite ($NaNO_2$) with 1000 ppm Dibutylamine (DBA) using a magnetic stirrer for 15 minutes until the solution became turbid.

The axenic strain of *Spirulina platensis* used in this work was obtained from Laboratory of Algal Technology, Mansoura University. It was cultured on liquid Zarrouk medium (1966) under 3500 Lux, 25°C and pH 9.5 as batch cultures. It was harried in the stationary phase after 21 days, dried and mixed as 1% in animal food.

Male albino rats *Rattus rattus* weighing approximately 120±5 g were obtained from Helwaan breeding farm and were acclimated to laboratory condition for one week prior to experiment. Rats were divided into 4 groups and to treated as follows:

a) Normal control group: Animals were fed on a standard diet and given normal tap water *ad libtium* for 12 months .

b) *Spirulina* treated group: Animals were fed on a standard diet mixed with 1% *Spirulina.* and given water *ad. libtium* for 12 months.

c) Carcinogen treated group; Animal were fed on the same standard diet and given DBN precursors in the drinking water for 6 months followed by normal drinking water for another 6 months.

d) Carcinogen and *Spirulina* treated animals: Animal were- fed on standard diet mixed with 1 % *Spirulina* mixed throughout the experimental period. They were also given DBN precursors in the drinking water for 6 months followed by normal water for another 6 months.

The animals were sacrificed at the end of the experiment and immediately the urinary bladder was dissected out and fixed, for 2 hours, in 2.5% glutraldehyde buffered in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C and post fixed in 1% cold osmium tetraoxide in 0.1M sodium cacodylate at pH 7.2, for 3 hours. The specimens were dehydrated through graded ethanol and embedded in resin. Ultrathin sections were stained in uranyl acetate followed by lead

citrate as described by Reynolds (1963), and examined in Joel Electron Microscope (JAPAN) operating at 60 kv.

Some of the dehydrated specimens were dried critical point according to standard procedure (Hayat, 1978). The specimens were then glued onto aluminium stubs using silver paste and with the urothelial surface to be examined facing upwards. When the paste had dried, the specimens were coated with a layer of gold in a sputtering diode coating unit in the prescribed manner. The samples were examined in an ISI-60 scanning electron microscope operating at 30 kv using the backscattered electron imaging.

RESULTS AND DISCUSSION

Control group

Scanning EM (Fig1) showed that the superficial cells appeared as polygonal cells. Transmission electron micrograph (Fig 2) illustrating the plasma membrane limiting the urinary face of the superficial cells which usually showed an angular or scalloped contour. The angular regions were separated by short slightly elevated, crest-like regions. The plasma membrane in the angular regions measured about 12 nm in thickness and showed asymmetry in its triple-layered structure. Its outer lamina facing the lumen of the bladder was thicker than the inner lamina facing the cell cytoplasm; the asymmetry was not evident in the crest regions (Fig 2). The superficial cells were generally the largest cells of the urothelium. The organelle content of the superficial cells was generally higher than the intermediate cells lying below. A noticeable feature of the superficial cells was the presence of many fusiform vesicles especially in the peripheral cytoplasm adjoining the luminal membrane. The nuclei of these cells invariably exhibited a fibrillogranular network structure. The nuclear envelop was smooth and showed no indentations. The chromatin in the nucleus of the superficial cells was mostly in the diffuse form and was distributed more or less uniformly. Mitochondria were present in considerable numbers in superficial cells. The apical plasma membrane of this intermediate cell, in general, showed a symmetrical structure, although there was an asymmetrical unit

membrane-limited vesicle in the immediate vicinity of this membrane. Occasionally one such vesicle appeared to be in continuaty with the plasma membrane. The basal cell is smaller and less differentiated than those of the overlying layers. The basal lamina is continuous and separates the urothelium from the lamina propria (Fig. 2).

The ultrastructure of the urothelia of rat bladder has been described by various workers and showed the same appearance of the present investigation ***(Walker, 1960; Koss, 1967, 1969; Clayson, 1975, Phillips and Davies, 1980).***

Spirulina treated animals:

SEM and TEM showed the overall appearance of the urothelium much like that of the control animals (Figs. 3&4).

This is in agreement with previous studies, that showed no significant differences between control and *Spirulina* treated animals in food intake, growth rate or carbon dioxide production. All animals remained apparently healthy, and had similar organ weights. These studies suggested that *Spirulina* may be safely used as a supplemented source of proteins, vitamins and minerals in rat diets ***(Toshi et al., 1991; Tranquille et al., 1994 and Narsinga, 1996).***

DBN precursors treated animals.

By SEM, the luminal face of the bladder was in the form of long papillomas and nodular aggregates protruding into the lumen (Fig. 5).

Low power TEM (Figs. 6 - 8) showed the general appearance of the urothelium. The cell layers were composed of relatively small cells which are more or less uniform in size and morphology from the basal layer to the surface.

The intercellular spaces contained long microvilli -like processes of adjoining cell borders interdigitating with each other. One of the most striking features of the urothelium was at the lumenal surface which possessed numerous closely spaced microvilli of varying dimensions covered with a thick electron dense cell coat (glycocalyx) (Fig.6). The superficial cells contained few rounded vesicles near the surface. The mitochondria were scattered throughout the cytoplasm unlike in the normal cells where they

intended to localize at the apical region. One of the organelle which was relatively prominent in these cells was the rough endoplasmic reticulum which seemed to be more extensive than that of normal urothelium, profiles of this endomembrane system were present throughout the cytoplasm. Several small, but independent, Golgi complexes were present in the cell. The cytoplasmic matrix contained an abundance of free ribosomes and polysomes (Figs.6, 7).

Another feature unique to the papilloma of this group was the occurrence of intracytoplasmic laminae (Tunnel). These formations were appearing in the superficial cells or in the cells of the immediately adjacent lower layer (Fig.6)

The ultrastructure of the cells situated deeper in the urothelium was similar to that described above. Several profiles of rough ER and a large number of ribosomes and polysomes were clearly visible in the intermediate and basal layers of the cells (Figs.7and 8). The mitochondria of these cells were distended and appeared abnormal or deformed with incomplete cristae. Some of the mitochondria did not show any internal organization and some of these structures undergoing lysis with the formation of myeline-like (figs. 6 and 7). The basal lamina was generally uninterrupted and its ultrastructure was as in normal urothelium (Fig.8).

The nuclei of all the urothelial cells were more or less similar in their ultramorphology, and contained masses of condensed chromatin distributed throughout the nucleus and especially along the nuclear envelope. The nucleoli in all the cell layers were compact (Figs.6, 7& 8).

DBN was found to induce urinary bladder tumours in rats, (***Tsuda et al., 1987, Zakhary et al.,1994 and Mochizuki et al.,1997),*** mice ***(Bertram and Craig, 1972 , Suzuki et al., 1983 and Nishikawa et al.,2003),*** hamsters (***Althoff and Kruger, 1975 and Moore et al.,1987***) and dogs ***(Okajima et al.,1981)*** as in the present investigation.

DBN precursors and *Spirulina* treated animals:

The scanning electron micrographs in Figure (9) showed long intertwined microvilli of adjoining cells. The surface of the cells showed also the presence of profuse microvilli.

In bladders of this group, TEM showed large areas where most of the urothelium had desquamated, leaving only a single layer of cells. Even these persisting cells seemed to be loosely attached to the basal lamina. In this group, the cells showed shrinkage rather than swelling, which is a morphologic feature of apoptosis. The cells were smaller in size, the cytoplasm was dense and the organelles, although relatively normal were more tightly packed. The luminal cell surface showed perfuses number of microvilli. The nuclei were deeply invaginated and sometimes broken and the chromatin materials were dense (Figs. 10&11).

The present results indicated that *Spirulina platensis* has acurative effect against DBN-inuced bladder papilloma in rats. Moreover, ultrastructural examination of these speciments revealed an improvement in most the histopathological alterations induced by DBN.

Our work is in agreement with that of ***Pardhasaradhi et al.(2003)*** who studied the effect of *Spirulina platensis* on a rat histiocytic tumor line. They reported that *Spirulina* is a chemotherapeutic agent that causes apoptosis to tumor cells. This shown in the present investigation by the reduction of the number of malignant cells to a single layer. Moreover ***Premkumar et al.(2004)*** found that *Spirulina* given by oral route to mice inhibited the genotoxicity induced by cisplatin and urethane.

Fig. (1): Scanning electron micrograph of the luminal surface of urothelium in control rat, showing the normal appearance of polygonal superficial cells. 1.500X. **(Inst.)** High power transmission electron micrograph of the plasma membrane limiting the urinary face of the superficial cells in control rats showing the plaques (P) of asymmetric unit membrane structure, and the asymmetry was not evident in the interplaque regions (IP)(X,120.000)

Fig. (2): Transmission electron micrograph of the two upper layers of urothelium in control rat showing the asymmetrical unit membrane (AUM) that limit the urinary face of the cell. The luminal membrane has a scalloped appearance, fusiform vesicles (FV) in the peripheral cytoplasm of the superficial and intermediate cells. It also shows the presence of mitochondria (M), large nucleus (N) and the endoplasmic reticulum (ER). 25.000X. **(Inst.):**

Transmission electron micrograph of the urinary bladder in control rat showing the abundance of fusiform vesicles (FV) in the apical cytoplasm of the intermediate cell, mitochondria (M), endoplasmic reticulum (ER), large number of ribosomes (R) in the cytoplasm of the basal cell, basal laminae (BL) and lamina propria (LP) with the normal appearance. (X ,20.000)

Fig.(3): Scanning electron micrograph of the luminal surface of urothelium in *Spirulina* treated rats showing the normal appearance of tightly adherent cells with polygonal profiles, network of ridges like that in control rats 15.000X. **(Inst.):** An apical portion of the superficial cell in *Spirulina* treated rats showing the asymmetrical unit membrane (AUM) that limits the urinary face of the cell.(X, 80.000)

Fig. (4): Transmission electron micrograph of the urinary bladder in *Spirulina* treated rats showing the superficial cell (S) with its characteristic feature, fusiform vesicles (FV) in the peripheral cytoplasm adjoining the luminal membrane, the intermediate cell (I) containing considerable amount of mitochondria (M), nucleus (N) and endoplasmic reticulum (ER), the basal cell (B) with smaller nucleus (N) and more electron dense than the overlying cells, basal lamina (BL) and lamina propria (LP) (X ,20.000)

Fig. (5): Scanning electron micrograph of the luminal surface of the urinary bladder in a carcinogen treated rat showing the formation of long papillomas and nodular aggregates protruding into the lumen. (X ,150)

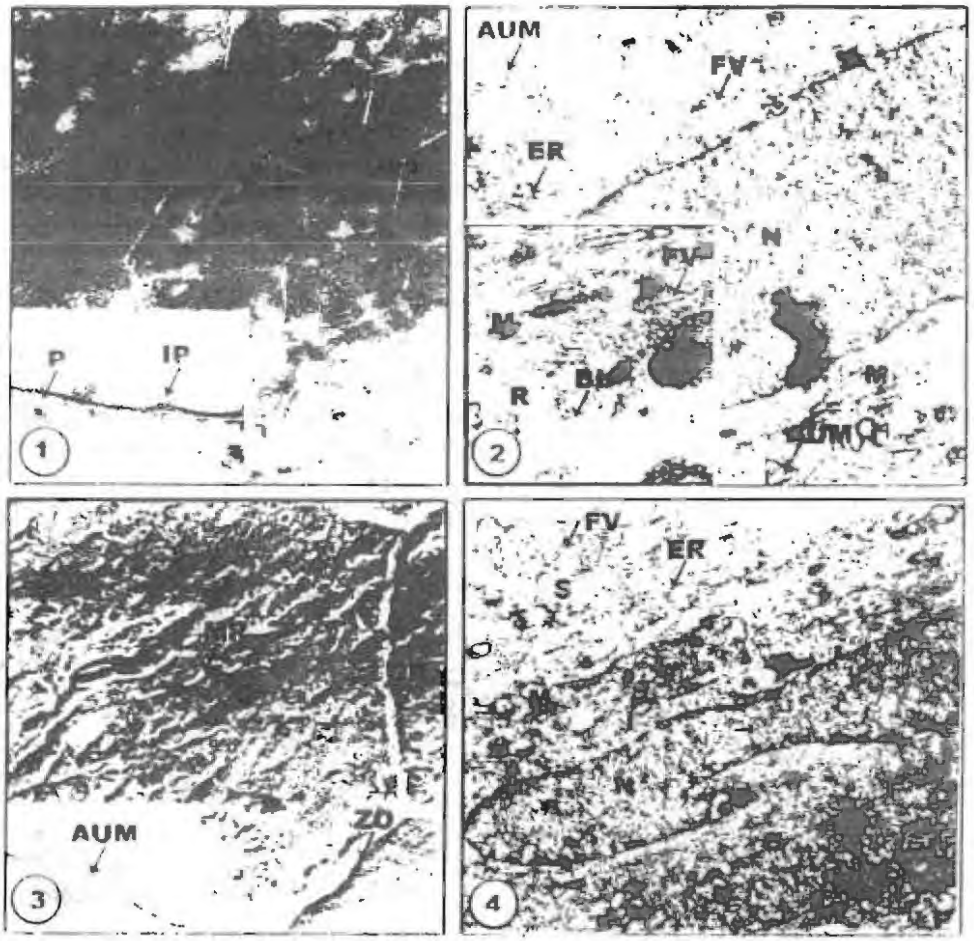
Fig. (6): Transmission electron micrograph of superficial and intermediate cells of the urothelium in carcinogen treated rats showing nucleus (N), tunnel (T) in a intermediate cell, microvilli (MV), mitochondria (M), free ribosomes (R). (X ,10.000)

Figs. (7): Transmission electron micrograph of the urinary bladder from a carcinogen-treated rat showing the intermediate cells. Note the presence of dilated rough endoplasmic reticulum (RER), Golgi apparatus (GA), free ribosomes and polysomes (R), deformed mitochondria (M) and nucleus (N). (X ,10.000)

Fig. (8): Transmission electron micrographs of the urinary bladder from a carcinogen treated rat showing the intermediate. basal cells and lamina propria. Note the presence of microfilament (MF). blood capillary (BLC) and basal lamina (BL). (X ,6.000)

Fig.(9): Scanning electron micrograph of the luminal surface of urothelium in a carcinogen + *Spirulina* treated rat showing the presence of profuse microvilli on the luminal surface of the cells and hightly raised cells borders of the small round cells. (X ,20.000)

Figs. (10 and 11): Transmission electron micrographs of the urothelia in carcinogen+ spirulina treated rats showing the presence of microvilli (MV) on the luminal surface of the cells, fragmented nucleus (N), wide intercellular space with cytoplasmic processes (*), deformed mitochondria (M), dilated endoplasmic reticulum (ER), broken basal lamina (BL) and the presence of collagen fibers (CF) in the lamina propria. (X,15.000 and X, 10.000)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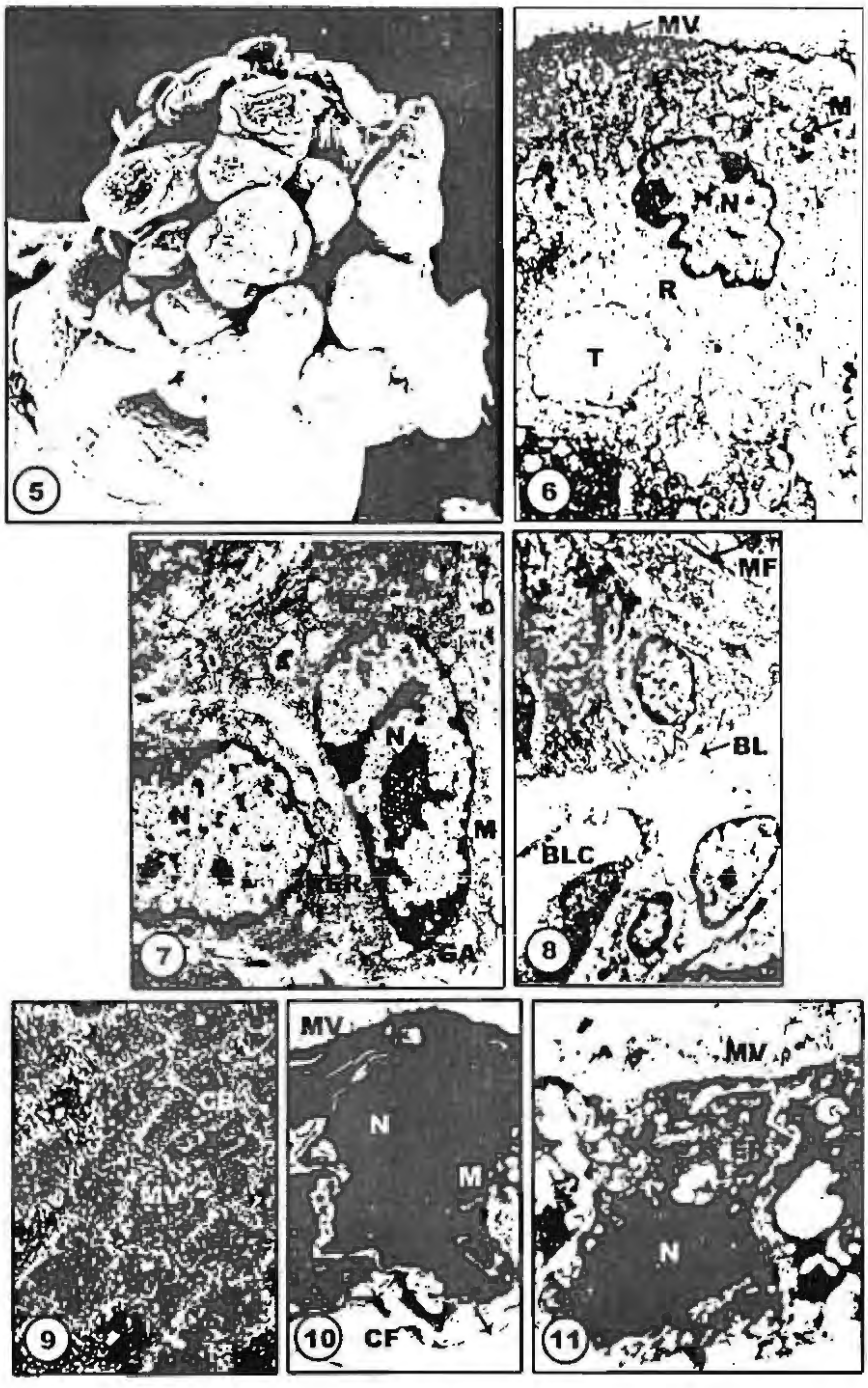
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الملخص العربي

يهدف هذا البحث الي دراسة التاثير الواقي لطحلب الاسبيرولينا على سرطان المثانة المستحدث تجريبيا بالمشتقات المسرطنة النيتروزامينية فى ذكور الجرذان باستخدام كلا من الميكروسكوبين الالكترونى الماسح و النافذ.

اوضحت نتائج الميكروسكوب الالكترونى الماسح انه لا يوجد اختلاف فى تركيب سطح الخلايا المبطنه لتجويف المثانة فى كل من المجموعة الضابطة و المجموعة المعاملة بطحلب الاسبيرولينا وأكد ذلك الميكروسكوب الالكترونى النافذ حيث لا يوجد تغيرات تركيبية مرضية فى الخلايا الطلائية المختلفة.

كان التاثير المسرطن لمشتقات النيتروزامين واضحا على التركيب الدقيق للخلايا الطلائية المبطنه للمثانة و ذلك فى تجمعها و بروزها خارج تجويف المثانة و بظهور الخميلات على سطح الخلايا و لكن بصوره غير واضحه حيث انها مغطاه بطبقة من السكريات المخاطية و كذلك وجدت خميلات في التجاويف بين الخلوية. وكذلك ظهرت بعض العضيات فى صورة مرضية وبكثرة مثل الميتوكوندريا ، الشبكة الاندوبلازمية الخشنة و الريبوسومات فى سيتوبلازم الخلايا الطلائية في مختلف الطبقات. بالاضافة الى التغير الملحوظ في شكل النواة حيث ازدادت المادة الكروماتينية المتغايرة (الكثيفة) على غشاء النواه.

و فى المجموعة المعاملة بمشتقات النيتروزامين و المعالجة بطحلب الاسبيرولينا ، اظهر الميكروسكوب الالكتروني الماسح وجود الخميلات على اسطح الخلايا و المتركزة خاصة على جدران الخلايا المبطنة للمثانة و اوضح الميكروسكوب الالكترونى النافذ التركيب الدقيق المميز للخلايا السرطانية الميتة موتا مبرمجا. اظهرت الدراسة ان لطحلب الاسبيرولينا تاثير مميت للخلايا السرطانية حيث ظهرت مما علي طبقة واحدة من الخلايا.

يتضح من نتائج هذه الدراسة أن طحلب الاسبيرولينا لها تأثير مميت على خلايا السرطان مما اقتصر وجودها على طبقة واحدة من الخلايا.