**ARTICLE**

# LITHIUM CHLORIDE POTENTIATES VINORELBINE AND ACETAMINOPHEN CYTOTOXICITY IN MDAH-2774 CELL LINE IN VITRO

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**Abstract**

In this study, the effects of vinorelbine (A microtubule inhibitor), para aminophenol derived acetaminophen and LiCl (a mood stabilizing agent) on monolayer and spheroid models of MDAH-2774 human ovarian cancer cell lines were investigated using both single and combined application of the drugs. The effects of the drugs on cell proliferation, cytotoxicity and S- phase fraction of cell cycle at 24,48 and 72 hours, respectively, were evaluated in cell culture. Evaluation of each drug in monolayer and spheroid cell culture compared to control group showed that vinorelbine, lithium and acetaminophen caused significant decrease in both cell proliferation and cell cycle progression and S-Phase of DNA synthesis. However, the effect of acetaminophen in relation to the other drugs was most pronounced.

There was no significant difference between drug combination groups and single drug groups in monolayer and spheroid cell culture. The data indicate that the effects of vinorelbine, lithium chloride and acetaminophen on monolayer and spheroid models of MDAH-2774 human ovarian cancer cell line caused a decrease in cell number in the cell cycle sythesis phase and prevented cell proliferation. We observed different size and shaps autophagic vacuols and defective mitochondria in the cytoplasm of the cells treated with vinorelbine. These findings were evaluated as a sign of autophagic cell death.

**Keywords:** MDAH-2774, Acetaminophen, Lithium chloride, Vinorelbine, Cell culture, MDAH-2774 Cell Line

## 1. Introduction

Clinical and molecular findings suggest that the four major histological subtypes of ovarian carcinomas (serous, clear cell, mucinous, and endometrioid) likely represent distinct disease entities. Most malignant ovarian tumors are epithelial (carcinomas) and are thought to arise from the ovarian surface epithelium or the secondary Müllerian system, for example endometriosis [1]. The MDAH-2774 human ovarian tumor cell line

 

is deficient of p-53 and MSH-2 (mut homolog 2) proteins which prevent genomic damage by inducing apoptosis [2]. MDAH-2774 cell line was developed from cells in the ascitic fluid from a patient with endometrioid ovarian cancer and the cellular characterization was made by Friedman et al. [3].

Vinorelbine is a semisynthetic vinca alcaloid that differs from the other types in the same family such as vincristine and vinblastine, because of its relative specificity for mitotic tubule [4]. It binds to tubulins which are the subunits of mitotic microtubules, obstructs tubulin polymerization and stops cell proliferation [5,6]. In cancer cells, vinorelbine induces apoptosis [7, 8] and is used for the treatment of many types of cancers such as breast cancer, lung cancer, leukemia and lymphoma [9].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in the treatment of inflammatory diseases [10]. Acetaminophen is a members of the NSAID group and it selectively inhibits COX2 enzyme (COX) [11,12]. Over expression of COX enzymes in epithelial cells can inhibit apoptosis and increase the invasion of tumor cells [13,14]. In epithelial ovarian cancers, COX2 is over expressed and mediates angiogenesis of these tumors. The use of NSAIDs has also been linked with reduced risk from cancer-related mortality and distant metastasis. COX-2 inhibitors may be effective in the treatment of endometrial cancer via suppression of angiogenesis [15,16].

Lithium chloride is used as a mood-stabilizing agent clinically [17,18]. One of its main target is inhibition of glycogen synthase kinase 3 beta (GSK-3 β), which plays an important role in protein synthesis, cell proliferation, differentiation, movement and a variety of signaling pathways that control apoptosis [18,19,20]. Lithium treatment results in an increase in estradiol-induced proliferative and morphogenetic changes in the uterus. This action of lithium is associated with decreased expression of estrogen receptors-alpha, beta-catenin and glycogen synthase kinase-3 beta in the uterus [21]. It induces stabilization of p53 thereby ensuring protection of the cell cycle at G1/S or G2/M phases [22]. GSK-3β plays an important role in ovarian cancer proliferation implicating this kinase as a potential therapeutic target in ovarian cancer [23]. The treatment mechanism of ovarian carcinoma is still poorly understood. Therefore, the combination of conventional chemotherapy agents and GSK-3β inhibitory agents can be useful in treatment of ovarian cancer. In this study, we focused on the effect of LiCL on cytotoxicity of vinorelbine and acetaminophen in MDAH 2274 human ovarian carcinoma cell line in vitro**.**

## 2. Materials and methods

### 2.1. Cell Culture and Drugs

MDAH-2774 cells established from American Type Culture Collection (ATCC) were maintained in RPMI 1640 medium ( containing 100 U/ml penicillin and 100 µg/ml streptomycin) (Biological Industries, Haemek, Israel) supplemented with 10 % heat inactivated foetal bovine serum (FBS) (Biological Industries, Haemek, Israel). The flasks were kept in an incubator with a humidified atmosphere of 5 % $CO_2$ at 37 ºC. Cells were transferred using Ca++ and Mg++ free Hanks' basic salt solution and % 0,5 trypsin (Sigma T4799). Cell passages were carried out three times a week and semi-confluent cell cultures were used in all experiment. Final doses of vinorelbine 10 µM (Navelbine, Pierre Fabre Medicament), lithium chloride 100 µM (Sigma-Aldrich Chemie GmbH), Acetaminophen 100 µM (Bristol Myers Squibb 500 mg paracetamol) were added into cell culture in equal volumes.

 

### 2.2. Plating Efficacy

Cells were removed and single cell population was achieved by trypsinization . Cells with 98 % viability were prepared in 5 ml of RPMI-1640 and plated into a six-well plate at a concentration of 1 x $10^5$ cell/ml. All drugs were added in equal volumes of 100 µl, and after, 24, 48 and 72 h, cells were harvested with trypsin-CMF PBS (Sigma T4799).

### 2.3. Spheroid Culture

MDAH-2774 cells were cultured at 37ºC in humidified atmosphere (5% $CO_2$ in air) in RPMI-1640 medium supplemented with 10 % heat-inactivated FBS and antibiotics. Cells were passaged three times a week. Spheroid formation was achieved by seeding 1X$10^6$ cells in 5 ml of RPMI 1640-FBS 10 % in petri dishes covered with a thin layer of agar (1:1 3 % of agar and medium). Using a micropipette, spheroids (approximately 120-400 µm) were harvested by gentle repeated transfer of individual spheroids into the wells of a 24-well culture plate. Multicellular tumor spheroids (MTS) were then individually placed into the wells of a 24-well culture plate containing 1 ml of RPMI 1640-FBS 10% on a layer of 1 ml of agar in the same medium. Every three days medium was changed and replaced with fresh medium.

### 2.4. Bromodeoxyuridine-Labelling Index (BrdU-LI)

BrdU (5-bromo-2deoxyuridine (20 µM, 1:200 dilution) was added to the spheroid medium with subsequent incubation for an additional 1 h. Thereafter, spheroids were gently removed from the wells and washed in phosphate buffer saline (PBS, pH 7,4). Spheroids were fixed in 10% formaldehyde (Riedel-de Haen) in PBS for 24 h at 4 ºC and then washed in phosphate-buffered saline (PBS). Following fixation, the spheroids were dehydrated through graded ethanol (Merck 100983), cleared in toluol (Riedel-de Haen), embedded in paraffin (Kimetsan KIM-PNB/O1CP/040220) and 5 µm tissue sections were cut with microtome (Leica). Sections were dewaxed in toluol for 30 minutes. After soaking in decreasing series of ethanol, sections were washed with distilled water and PBS for 10 minutes, treated with 3 % trypsin at 37 ºC for 30 min and washed with PBS. Sections were incubated in a solution of 0.5% $H_2O_2$, then washed with PBS and incubated with primary mouse anti-BrdU antibody (Lab Vision, UK).

Next, the sections were incubated with biotinylated IgG followed by streptavidin-peroxidase conjugate (Lab Vision, UK). The sections were then washed and incubated with the AEC chromogen substrate system and counterstained with Mayer's haematoxylin.

The immunostained slides were observed under light microscopy at magnifications of x 40. BrdU-labelled cells were evaluated by the same person. BrdU positive cell types were determined by observing dark red AEC nuclear staning. Unlabelled nuclei with only blue haematoxylin staning and pale brownish nuclei were considered to be negative. Ten adjacent sections and at least 3000 cells were evaluated for each group.

### 2.5. Electron microscopic evaluations

Electron microscopic evaluations, slices obtaining form 3D spherids fixed with 2,5% glutaraldehyde in 0.1 M sodium cacodylate buffer and post fixation were made in 1% osmium tetroxide for two hours. After 1% uranlyl acetate incubation, spheroids were embeded in Epon 812. Ultra thin sections were taken from selected area and stained with Reynold's lead citrate. Sections were examined with a JEOL-JEM 1011 electron microscope ( Japan).

 

## 3. Statistical Analysis

All data are expressed by SPSS 10.0. Statistical analysis was performed by the Kruskal-Wallis analysis test. The accepted level of significance was set at $p<0.05$.

## 4. Results

### 4.1. Plating Efficacy

As shown in Fig. 1, cells in the control group showed increasing growth rate beginning with approximately $9x10^5$ cells at 24 hour and increasing to $1.6x10^6$ cells by the 72 hour. Vinorelbine at all incubations times (24,48,72 hours) decreased the cell numbers, with 75% of cell number reduction seen at the 72 hour. These decreases were statistically significant for 24, 48 and 72 hours time points ($p < 0.05$). The cell number reduction was evaluated to be dose-dependent. Lithium chloride decreased the cell number at 24 and 48 hours respectively in comparison to control with 70.6% decrease seen by the 48 hour. P values were significant ($p <0.05$). At 72hr, a non-statistically significant 65.62% cell number reduction was observed ($p > 0.05$).

A combination of vinorelbine and lithium chloride (Fig. 1) decreased cell numbers with 76.62% inhibition occurring at the 72hrs, ($p > 0.05$, $p > 0.05$, $p > 0.05$ for 24, 48 and 72 hours respectively). This result was similar to inhibition observed with vinorelbine or lithium chloride.

There was no significant effect of acetaminophen on cell proliferation at 24 hours ($p > 0.05$), A significant reduction in cell number was observed at 48 hour ($p <0.05$). At 72 hour acetaminophen group was similar to control group ($p > 0.05$).

In the vinorelbine and acetaminophen combination groups, the cell numbers decreased in comparison to control ($p <0.05$). A 70 % inhibition rate of was observed at 72 hours respectively, but it was not significant in comparison to vinorelbine.

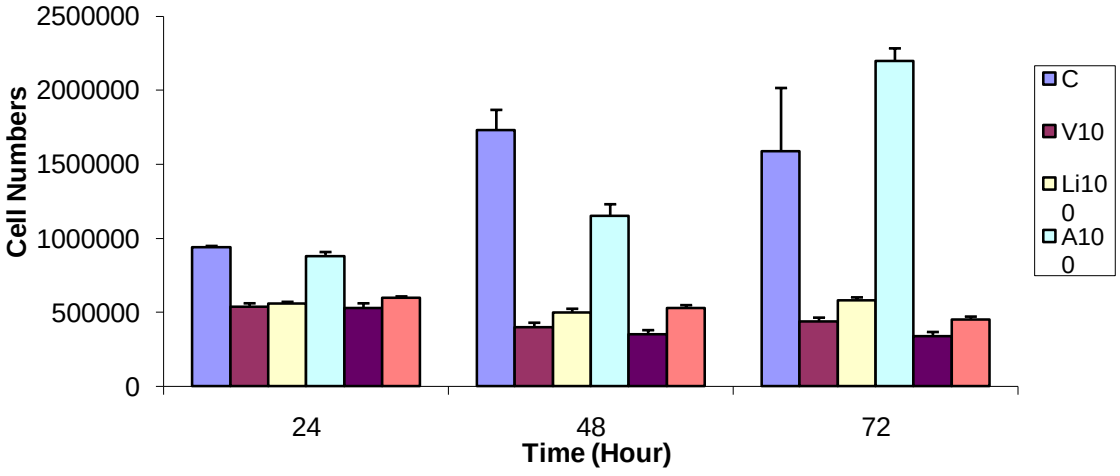


**Figure 1.** Cell proliferation results of MDAH-2774 cell line in monolayer at 24, 48 and 72 hours. Control group exerted exponentially grown. Vinorelbine, lithium chloride and their combinations inhibited proliferation at all times.

 

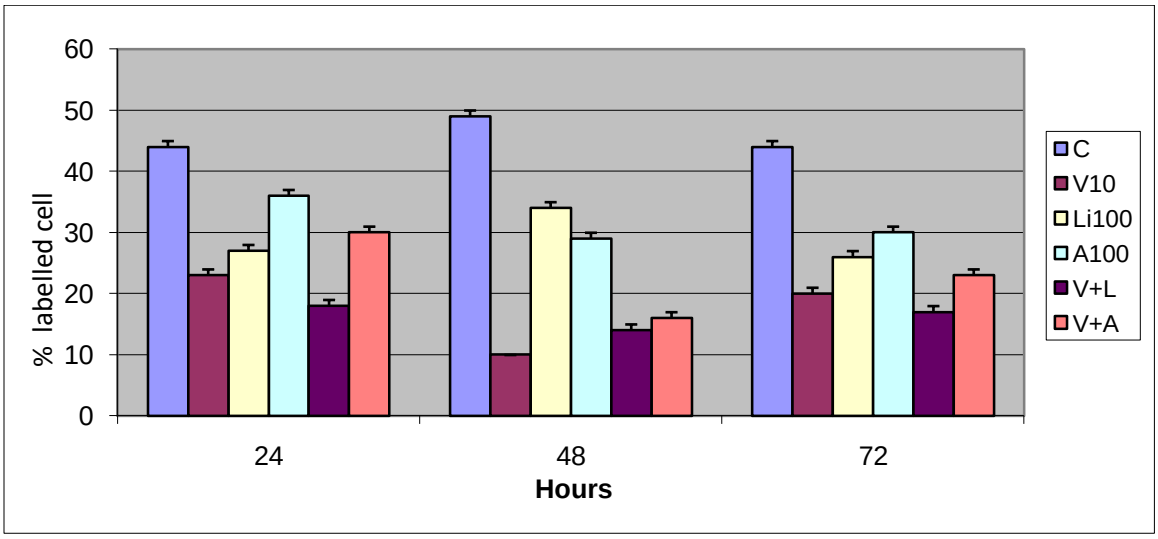


**Figure 2.** BrdU-Labelling Index in two dimensional cell culture

### 4.2. BrdU-Labelling Index (BrdU-LI) in three-dimensional cell cultures

The labelling index of the control group (Figure 4A) was determined as 18 % (Fig. 3,4,5). Vinorelbine reduced the labelling of spheroids by 7.5 % (Figure 5,6) which was statistically significant to the control ($p < 0.05$). The effect of lithium chloride was also statistically significant ($p < 0.05$) with an average detected labelling index of 6% (Fig. 3,8). In the acetaminophen group, BrdU positive cells were observed and the BrdU-Labelling index was determined as 8 % ($p < 0.05$). The labelling index of the vinorelbine and lithium chloride combination group spheroids were 5 % and this result was statistically significant compared to the control group (Figure 5) and the vinorelbine group ($p < 0.05$). The average labelling index value determined was 5 % in the vinorelbine and lithium combination groups. In the vinorelbine and acetaminophen combination group, BrdU positive cells were observed at the periphery of the spheroids and BrdU-labelling index was determined 8 %, respectively ($p < 0.05$).

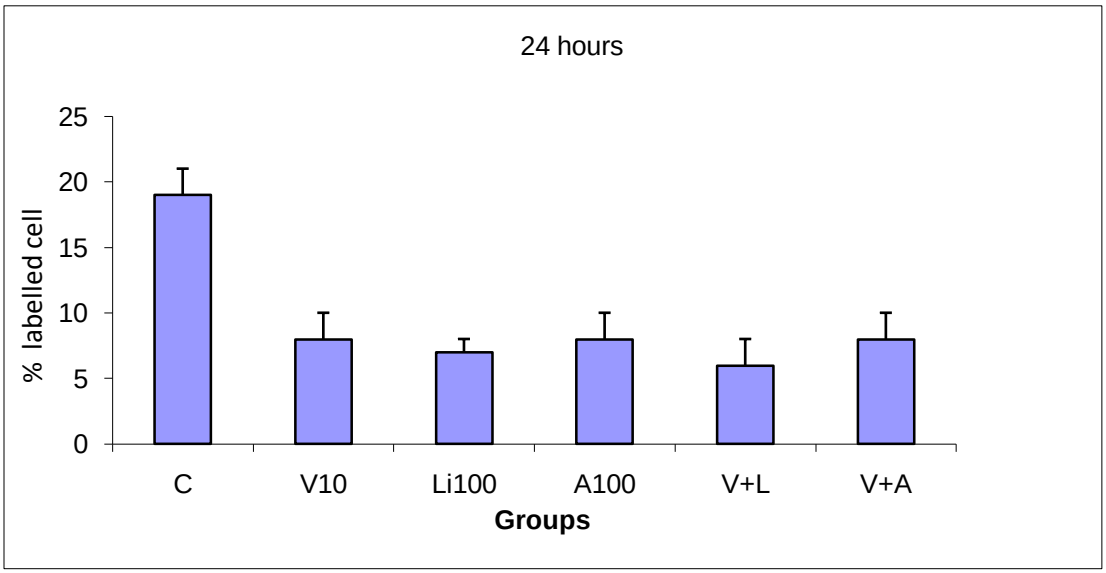


**Figure 3.** BrdU-Labelling Index in three dimensional cell spheroids

 

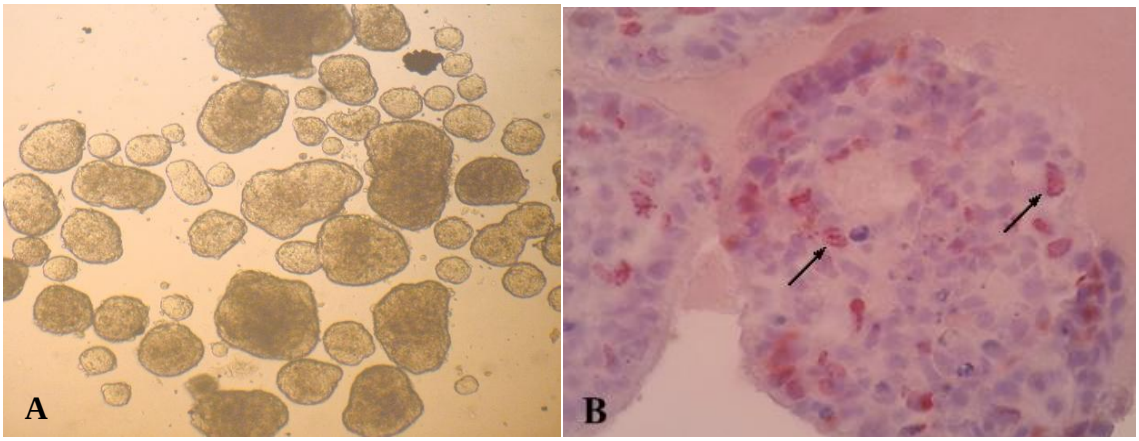


**Figure 4.** A. Control MDAH-2774 human ovarian tumor cells spheroids. The size of spheroids were calculated about 120-300 micrometer. Magnification x10. B. Control spheroids parfine section, stained with BrdU momoclonal antibody. The cells with red color nuclei were evaluated as BrDU positive cells. Magnification x40

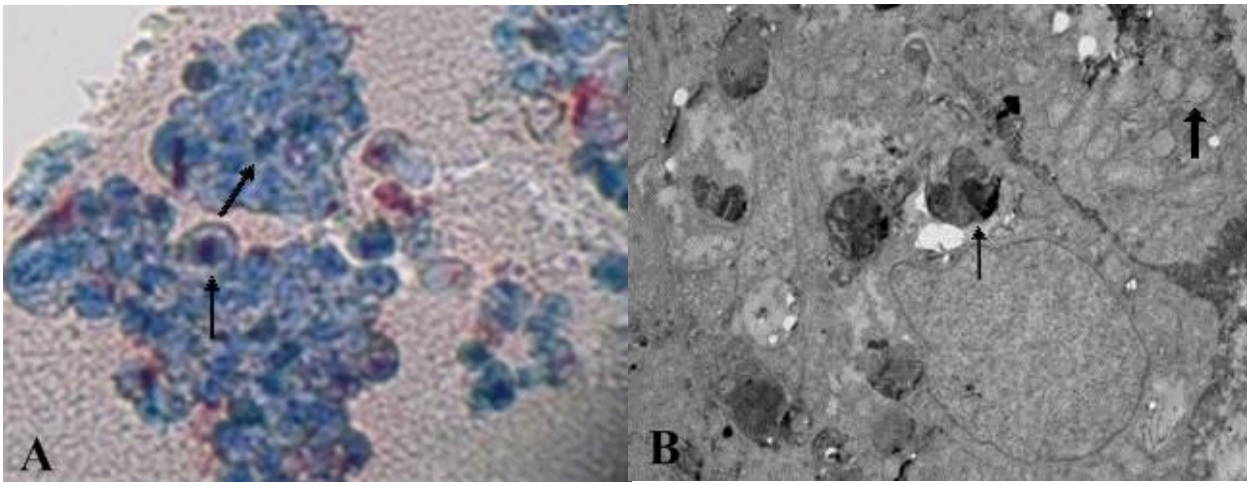


**Figure 5.** A. Vinorelbin treated and stained with BrdU spheroids. The arrow shows the unstained nuclei on both sides. Magnification x10.

B. Electron micrograph showing 10 μM vinorelbine-treated group of MDAH 2274 cells. Various size, numbers and at densities autophagic vacuoles are seen in the cytoplasm. The arrows shows several size of autophagic vacuoles on button left side ,and upper right side the dotted arrow shows defective mitochondria. N. Nucleus, Thick arrow shows mitochondria. Magnification x5000.

 

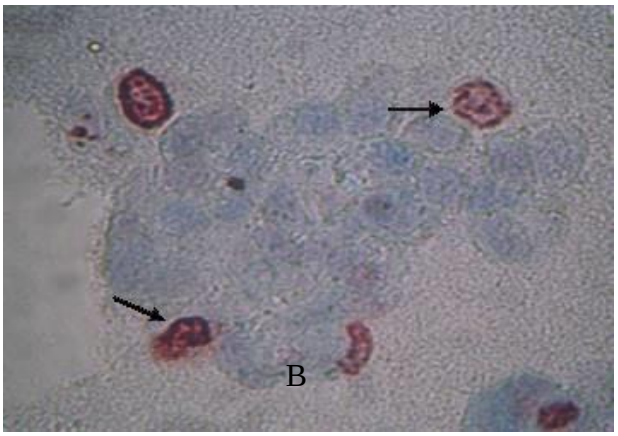


**Figure 6.** The combination of vinorelbin + acethaminophen. Arrows show red stained nucleus with monoclonal BrdU antibody. Magnification x40

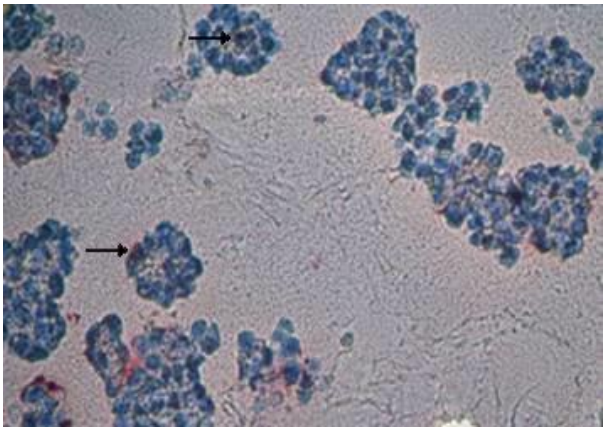

**Figure 7.** Acethaminophen treated spheroids. Magnification x10. Arrows shows red stained nucleus with monoclonal BrdU antibody.

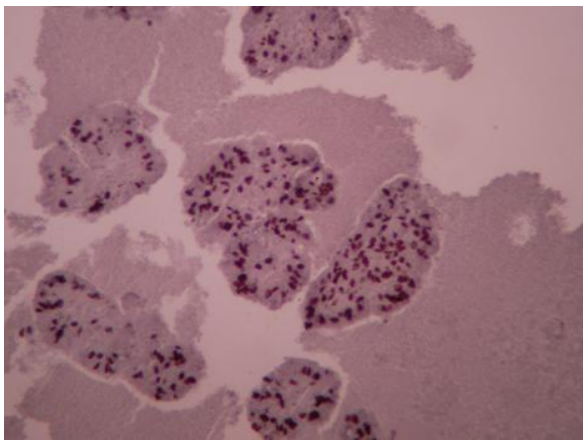

**Figure 8.** A lot of red nucleated cells stained with BrdU antibody were observed in the LiCl-treated ishikawa spheroid. Magnification x40

## 5. Discussion

Ovarian carcinoma has the highest cancer-related mortality among the gynecologigal cancers [24]. Because of the long asymptomatic nature of the disease, patients go to the doctors in the later stages of the

 

disease [23]. As a result of this, traditional chemotherapeutic agents may not be useful in such patients. We are suggesting that new or different drugs in combination with appropriate doses of chemotherapeutic agents may increase the therapeutic chance of success. In the present study, **we investigated the effects of vinorelbine, lithium chloride and acetaminophen** on cell proliferation of MDAH-2774 **cell line. We demonstrated that vinorelbine and lithium c**hloride were effective on the MDAH 2774 Cell line.

Vinorelbine is a chemotherapeutic vinca alkaloid, which is used for the treatment of non-small cell lung cancer and breast cancer and many types of cancers, including apoptosis of human lymphoma cells by reducing the amount of mitochondrial cytochrome-C [26]. In addition, vinorelbine prevents the formation of tubulin and stops the cell-cycle at metaphase by inhibiting the formation of mitotic spindle. Thus, it plays a role as an anti-mitotic agent by blocking the cell cycle at G2/M transition [27]. Vinorelbine significantly reduced the cell numbers at all incubating times and decreased the BrdU labelling Index (Figure 2). When BrdU labelling index data and cell proliferation results were examined, we found that vinorelbine decreased the cell numbers due to its anti-proliferative effect. It was also numerically determined that vinorelbine caused cell death in two- and three-dimensional cell cultures (Figure 2,3). At a dose of 10 μM, it decreased the S-Phase fraction of cells in spheroids (Figure 5A). The values of BrdU-labelling ındex and the results of cell proliferation indicated compliance after vinorelbine treatment. LiCl inhibits cell proliferation in some cancers [28,29]. It inhibits myeloma proliferation due to its activation of the Wnt signaling pathway [28], decreases cell proliferation in the S-phase, proliferation in prostate cancer cells and the volume of tumor in thyroid cancers [19,20].

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GSK-3B activates NF-kappaB causing increased cell proliferation in cancer cells (19,20). It was shown that LiCl caused proliferation in ovarian cancer cells [23]. The activation of GSK-3B induced entry into S-phase by increasing cycline D1 expression thereby increasing proliferation of ovarian cancer cells [23]. We aimed to inhibit proliferation using a GSK-3B inhibitor in MDAH-2774 human ovarian tumor cell line.

In our study, LiCl inhibited cell proliferation and decreased cell numbers (Figure 1,2). In the BrdU-labelling ındex results, the ratio of S-phase cells decreased at all treated times, similarly in the three dimensional culture, the values of BrdU-labelling index also was decreased (Figure 8). Vinorelbine and LiCl proved to be very effective on the MDAH-2774 cell line. Vinorelbin, at 72 hours caused a significant level of inhibition of cell proliferation and decrease in cell numbers ($p < 0.05$) (Figure 1,2). Inhibitors of COX are thought to be potential tools in the therapy of a variety of tumors [32]. Acetaminophen caused cell death in neuroblastoma [30]. The preventive effect of its regular use was shown to be at the epithelial origin of colon and gastrointestinal tract tumors and breast and ovarian cancers [31]. NF-kappaB inhibits angiogenesis, tumor invasion and drug-resistance to apoptosis inducers of COX-2, and various proteases such as plasminogen activators, growth factors and gene transcription. It was found that acetaminophen prevented cancer formation by inhibiting the activity of NF-kappaB [31,32]. Acetaminophen inhibits prostaglandin formation by inhibiting COX enzymes. In the acetaminophen

 

group, the significant decrease ($p < 0.05$) observed in the number of cells at 24 and 48 hours can be related to this inhibition (Figure 1,2). Also, the increase in cell number observed at 72 hours when compared to control was consistent with similar works by Bilir et al 2002. In the three dimensional cell culture, acetaminophen showed a significant decrease in cell number ($p < 0.05$) (Figure 7). At 72 hours, the vinorelbine-acetaminophen combination inhibited cell proliferation, but compared to acetaminophen alone, it was observed that the actual interaction was more dependent on vinorelbine (Figure 6). The effect of this combination was parallel to that of vinorelbine. In the combination groups, both the BrdU-Labelling Index and cell proliferation decreased while cell cytotoxicity increased.

Various size, shaped and density of autophagic vacuols were observed ultrastructurally in the cell cytoplasms of the spheroids (Figure 5B). Additionally, many defective mitochondria were observed in the same cells treated with vinorelbine. These findings were evaluated as a sign of autophagic cell death (Figure 5B). After vinorelbine treatment, the incidence of autophagy, together with apoptosis or other cell death forms should be considered as a new therapeutic strategy modalities.

## 6. Conclusion

In conclusion, the treatment of MDAH 2774 tumor cell line with lithium chloride, vinorelbine, and acethaminophen or combination of these three drugs can be a useful treatment model. More research in gene expression will be needed in future tumor treatment plans.

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