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# *In vitro* Cell Proliferation Assay for Measuring the Cytotoxic Effects of Spironolactone (SPIR) in Osteosarcoma Cell Line

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**ABSTRACT**

*Cancer cell lines are used to study malignancies, cell biology, and drug discovery. For this reason, osteosarcoma (OS) cell lines have the potential to be useful models for the investigation of osteosarcoma progression and treatment. Osteosarcoma is a primary malignant bone tumour affecting mostly children and adolescents. Spironolactone (SPIR), is an FDA-approved diuretic drug with a long-term safety profile used to treat hypertension and kidney disease. Previous research has found that SPIR also is able to up-regulate Natural Killer Group Member D (NKG2D) Ligand in multiple cancer cell lines by activating the ATM-Chk2-mediated checkpoint pathway, which in turn enhances tumour elimination by natural killer cells, supporting a role in cancer immune response. In addition, NKG2D is an activating receptor that can bind to a wide range of stress-induced ligands found in cancer or viral infection. Therefore, the purpose of this study is to determine the effect of SPIR on cell viability of highly metastatic osteosarcoma (HOS-143B) cells and human foetal osteoblast (hFOB) cells. SPIR was treated to HOS-143B cells at doses ranging from 5 to 40µM with human foetal osteoblast, hFOB as controls. Cytotoxicity level of SPIR was determined at post- 24, 48 and 72 hours using the cell proliferation assay. At post-24 and 48 hours, the SPIR exhibited an effect on HOS-143B as evidenced by the consistent pattern of high percentages (80% and greater) of viable cells at all doses. Meanwhile, during the incubation period, SPIR had a dose-dependent impact on hFOB cells, with viable cell percentages ranging from 95% to 35%. Taken together, these findings indicate that when SPIR was given at doses below its cytotoxic limit, it had an overall beneficial effect on the proliferation of OS cancer cells.*

**Keywords:** *osteosarcoma;spironolactone;cell proliferation assay;childhood cancer;in vitro study*

## 1. INTRODUCTION

Osteosarcoma (OS) is the third most common type of cancer in adolescents, which comes after lymphomas and brain tumour, with an increased occurrence of 5.6 cases per million children under the age of 15 [1]. It occurs most frequently in teenagers at the metaphysis of lower extremity long bones (75%), demonstrating a correlation between puberty hormonal changes and/or physiologic bone growth [2, 3]. To date, the majority management of OS patient undergo

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neoadjuvant chemotherapy, radiotherapy, followed by surgical resection [4]. However, the five-year survival rates for traditional OS were quite poor during most of the twentieth century [5].

Another strategy for cancer therapy, is immunotherapy, which utilizes our own immune system to eliminate cancer cells. Immunotherapy has been established as a critical component of cancer treatment, significantly improving the prognosis of a large number of patients with a variety of hematological and solid malignancies including OS [6]. It appears that cell lines may be useful in studying disease development and treatment. Therefore, *in vitro* assays serve as a starting point for the development of cancer medication discovery strategies [7]. As revealed by previous study, high-throughput screening of specific molecular compounds, notably FDA-approved pharmaceuticals, has emerged as a promising technology over the years, with the potential to repurpose existing therapies for use in novel disease states including cancer [8].

Drug repositioning or repurposing is a method that applies already-approved drugs to new indications [9]. Moreover, the effectiveness and potency of drugs are typically assessed in cells exposed to a drug for a period of up to 72 hours using drug-dose response assays [10]. All phases of clinical trials have been completed for already-approved drugs, and their safety profiles in humans are known. Thus, drug repositioning minimizes the time and expenses associated with drug development and is attracting greater interest by academia and industries. One mechanism by which the immune system detects abnormal cells in the body to avoid tumorigenesis is via natural killer (NK) cells and cytotoxic T cells, which recognize Natural Killer Group Member D ligands (NKG2DLs) expressed on the surface of tumor cells and then targets the cells for destruction [11].

Spironolactone (SPIR), an aldosterone antagonist, is increasingly prescribed to treat heart failure, hypertension, and liver disease. [12]. On top of that, SPIR is a good candidate drug for cancer therapy from the perspective of drug repositioning [13]. Several studies have suggested that the usage of SPIR has been demonstrated to be associated with certain types of cancer in humans including pharyngeal [14], thyroid [15] and renal cancers [16-18]. SPIR has recently been shown to have anti-cancer properties by inhibiting DNA damage repair and acts as a chemosensitizer in conjunction with DNA-damaging drugs like cisplatin [19-21]. In addition, SPIR appears to have an interesting pharmacological effect that may extend its potential uses in cancer treatment. One mechanism by which the immune system detects abnormal cells in the body to avoid tumorigenesis is via NK cells. Interestingly, SPIR has the ability to facilitate immune recognition by upregulating the expression of NKG2D ligands which could enhance the efficacy of killing mechanism by NK cells. However, these studies have either used small sample sizes or confounded spironolactone use with other medicines.

NKG2D ligands are only expressed selectively or at low levels by normal cells, but their expression is elevated in response to cellular stress and transformation [22]. Earlier finding reported that SPIR has been shown to upregulate NKG2D ligands in osteosarcoma cells [23]. In addition, previous study found that SPIR upregulated NKG2DL surface expression level on various cancer cells through activation of ATM-Chk2-mediated pathway [11]. Moreover, in chemo-resistant metastatic solid tumours, multiple myeloma, and myeloid leukaemia, induced expression of NKG2D ligands on tumours has been found to be a potential therapeutic approach [11, 24-26]. We hypothesized that SPIR could sensitize these highly metastatic cancer cell lines by upregulating the NKG2D ligands expression. It is significant as the initial stage in immunotherapy treatment to ensure more precise and targeted killing process by NK cells.

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Therefore, the present study was designed to investigate the cytotoxic effect of SPIR on cancer cell lines using cell proliferation assay in order to determine the time and concentration dependent effect of spironolactone (SPIR) on HOS-143B cancer cell line and using human foetal osteoblast (hFOB) as control.

## 2. METHODOLOGY

### *2.1 Chemicals*

Human osteosarcoma cell line, HOS-143B (ATCC® CRL-8303) and human foetal osteoblast, hFOB 1.19 (ATCC® CRL-11372™) cells was purchased from Bio-Focus Saintifik Sdn Bhd. Meanwhile, Minimum Essential Medium (MEM) in Earle's BSS, Dulbecco's Modified Eagle Medium (DMEM) Nacalai Tesque Inc, 0.015 mg/ml 5-bromo-2'-deoxyuridine, fetal bovine serum (FBS, Invitrogen, USA), 1% of *Penicilin Streptomycin* (Nacalai, USA) and Phospate Buffered Saline (PBS, Sigma Aldrich, USA) was purchased from Sigma Aldrich Sdn Bhd. In addition, CellTiter 96® AQueous One Solution Cell Proliferation Assay Promega Corporation (G3580) and 96-well microplate reader SPL was purchased from Life Sciences. Finally, Dimethyl sulfoxide (DMSO), ethanol and trypan blue reagent and spironolactone (SPIR) were obtained from Sigma-Aldrich, Inc. (St Louis, MO, USA).

### *2.2 Cell culture*

Human cancer cell line, HOS-143B were cultured in Minimum Essential Medium (Eagle) in Earle's BSS with 0.015mg/ml 5 bromo-2-deoxyyuridine, (Nacalai Tesque, Inc USA). The MEM was supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% of *Penicilin Streptomycin* (Invitrogen, USA). While human foetal osteoblast (hFOB) cells were cultured in Ham's F12 Medium Dulbecco's Modified Eagle's Medium with 2.5 mM L-glutamine (without phenol red). DMEM F12 were supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 0.3 mg/ml G418. All cells were cultured and maintained in 5% $CO_2$ at 37˚C and monitored closely where the medium was changed for subsequent 2 days under aseptic conditions. Cells were harvested when it reached 70 to 80% confluent for preparation at specific cell number for further experiment.

### *2.3 Cell Counting and Dilution*

Cells were harvested by removing the media, rinsed with PBS and incubated with fresh 0.25% trypsin, 0.02% EDTA solution and the cells were allowed to sit at at 37°C for 5 minutes until they detached. Then, media were added and collected into test tube for centrifuge. After centrifugation, the supernatant was discarded, and 1 mL of fresh cell culture media (CCM) was added into the tube containing cell pellet. It was mixed thoroughly using vortex until homogenous. Cell counting was done by using trypan blue dye (0.4%) (Gibco, USA) and counted using hemocytometer. Cell viability percentage (Equation 1) was determined by counting together the total number of live and dead cells to get the total cell count. Subsequently, the live cell count was divided by total cell count. Then cells were diluted into the desired concentration for each experiment.

$$\text{Percentage Viability} = \frac{\text{Number of Viable Cells}}{\text{Total Number of Cells}} \times 100 \qquad (1)$$

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### *2.4 Cell Morphology*

Cells were viewed using phase contrast light microscope (Leica, Germany) at the incubation period of 24, 48 and 72 hours to observe the morphology and growth of cells throughout the study period.

### *2.5 Cytotoxicity Assay*

Cell viability was examined by using the 3-(4,5-dimethylthiazol-2-yl)5-(3-carboxy methoxyphenyl) − 2-(4-sulfophenyl) − 2H-tetrazolium (MTS) assay. The cells were seeded in 100 µl medium/well in 96-well plates (HOS-143B cells, $1 \times 10^4$ cells/well; hFOB cells, $1\times 10^4$ cells/ well) and allowed to grow overnight. The cells were further treated with SPIR at different concentration (5, 10, 15, 20, 25, 30, 35, 40 µM) to assay cell viability. After incubation for 24h, 48h and 72 hours in a humidified incubator, 20µl of CellTiter 96® AQueous One Solution Reagent were pipetted into each well of the 96-well assay plate containing the samples in 100µl of culture medium. Finally, the plate was incubated at 37 °C for 2 hours in a humidified, 5% $CO_2$ atmosphere. The absorbance was measured by a microplate reader VICTOR Multilabel plate reader (PerkinElmer, USA) at a wavelength of 490 nm. All experiments were performed three times in triplicates.

Percentage of cell viability was calculated using formula (Equation 2) as follows:

$$\text{Cell viability}\,(\%) = \left[\frac{\left(\overline{A_{\text{sample}}} - \overline{A_{\text{blank}}}\right)}{\left(\overline{A_{\text{control}}} - \overline{A_{\text{blank}}}\right)}\right] \times 100\% \qquad (2)$$

$A_{sample}$ = Absorbance reading of samples
$A_{control}$ = Absorbance reading of control
$A_{blank}$ = Absorbance reading of blank

### *2.6 Data Analysis*

A statistical analysis was performed using SPSS software version 20. The data were presented as mean ± standard error of mean (SEM) of three replicates. The data is considered statistically significant at $p < 0.05$.

## 3. RESULTS AND DISCUSSIONS

### *3.1 Effect of Spironolactone Towards Osteosarcoma Cell Line (HOS-143B)*

Metastatic osteosarcoma cell lines (HOS-143B) and pharmaceutical agents (SPIR) in cancer research and drug screening were utilized in this study. To investigate the effect of this chemotherapeutic agent on cell viability in HOS-143B cells, we performed a cell proliferation assay using standard experimental protocols and calculated the percentage after three exposure time points at 24, 48 and 72 hours. It should be noted that this is the initial phase of the study prior to the in vivo immunotherapy treatment that will be performed subsequently to develop

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an osteosarcoma mouse model. Thus, it is crucial to determine the cytotoxicity of SPIR toward cancer cells.

Figure 1 shows the effect of all concentrations of SPIR from 5-40µM on the osteosarcoma cell line. This study showed that the effect of SPIR was exerted as early as 5µM and had the highest cell viability on HOS-143B cell at the concentration 25, 30, 35 and 40µM over the period of 24 hours; whereby the percentage of cells viability rate were 85.89%, 93.78%, 89.26% and 90.87% respectively. Correspondingly, the percentage of cell viability remains constant as evidenced by the consistent pattern of high percentages (80% and greater) of viable cells at all doses after 48 hours and the best cell viability was, 93.78% at the concentration of 30µM. This result is in contrast with a recent study [27] whereby they found that SPIR inhibits the proliferation of osteosarcoma cells. However, the researcher was using a different type of cell line. Whereas in this present study, HOS-143B cell lines were used and it is known to be highly metastatic cells. Thus, the effect was expected to be higher compared to other types of OS cell lines.

On the other hand, SPIR-treated cell line of HOS-143B exhibited a lower percentage of cell viability post-treatment at 72 hours in all concentrations with the percentage range around 65-74% compared to 24- and 48-hours treatment. It's possible that this is the result of prolonged exposure of SPIR in cancer cells which finally causes toxicity and leads to cell death. Furthermore, the media used were not changed during the incubation period. Thus, the cells could be aggregated abundantly. As a result, no more space for the cells to grow and finally lead to cell apoptosis. Alternatively, increasing the duration of exposure would slowly inhibit the growth of OS cells.

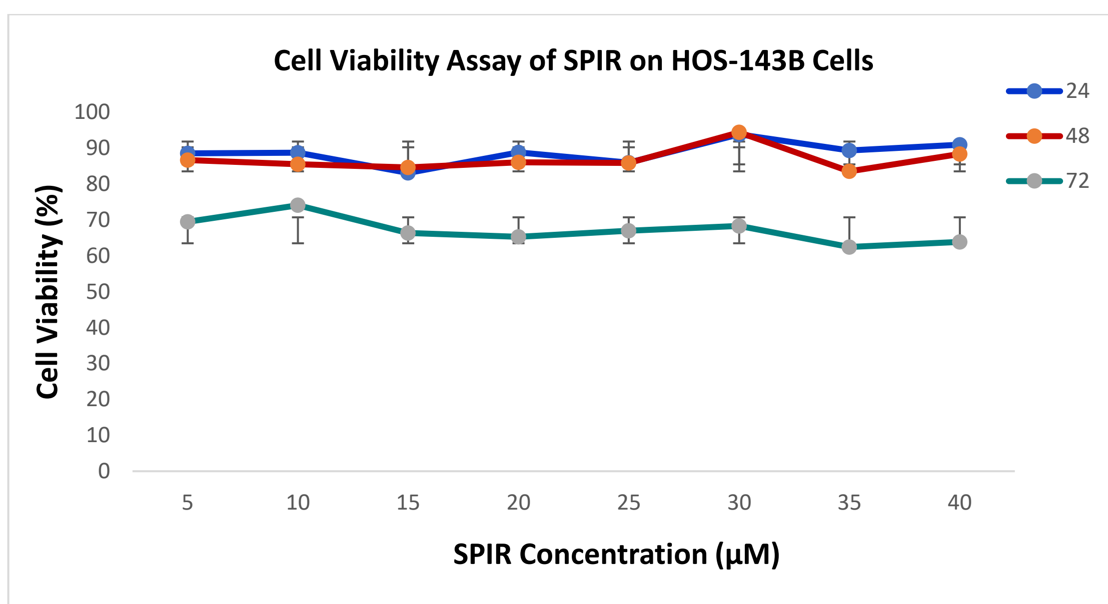


Figure 1: Effect of SPIR treatment on HOS-143B osteosarcoma cells viability. Cell viability was measured after 24-, 48- and 72-hours treatment with spironolactone (SPIR). The effect of SPIR had the highest cell viability on HOS-143B cell line after 24 hours (blue line). About the same pattern appears after 48 hours treatment (red line) and the lowest viability can be observed after 72 hours treatment (green line). Results expressed as percentage of cell viability treated cell normalized to untreated cell (Data: mean ± SEM of three independent experiments).

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### *3.2 Effect of Spironolactone Towards Normal Osteoblast (hFOB)*

Figure 2 shows the effect of all concentrations of SPIR from 5-40µM on human foetal osteoblast, whereby hFOB is represented as controls in this study. SPIR were treated at three exposure times which are 24, 48 and 72 hours. The results revealed that SPIR markedly reduced the viability of hFOB cells in a dose dependent manner. As observed, throughout the incubation period, SPIR had a dose-dependent impact on hFOB cells, with viable cell percentages ranging from 95 % to 35 %. The antiproliferative effect of SPIR on hFOB cells revealed that the SPIR had the highest inhibitory growth at the high concentration especially starting at 25, 30, 35 and 40 µM for the period of 24 hours and ranging from 67.48 %, 58.56 %, 54.65 % and 49.71 % respectively.

Meanwhile, the same pattern in percentage of cell viability can be seen after 48 hours of SPIR exposure towards hFOB cells. From the data, SPIR had the highest inhibitory growth at the high concentration as well starting at 25, 30, 35 and 40 µM after 48 hours and ranging from 57.83 %, 47.7 %, 35.98 % and 31.57 %. In addition, the same inhibitory effect was observed after 72 hours of exposure. There is a limited amount of research carried out on the effect of SPIR on normal cells. Indeed, it appears that increasing doses of SPIR produced proliferation inhibition effects. Nevertheless, since the use of hFOB purpose is as a control, it is important to note that our primary goal is to focus on the HOS-143B, cancer cells instead. Despite that, SPIR effects show less harmful towards hFOB at a lower dose compared to the high dose of SPIR.

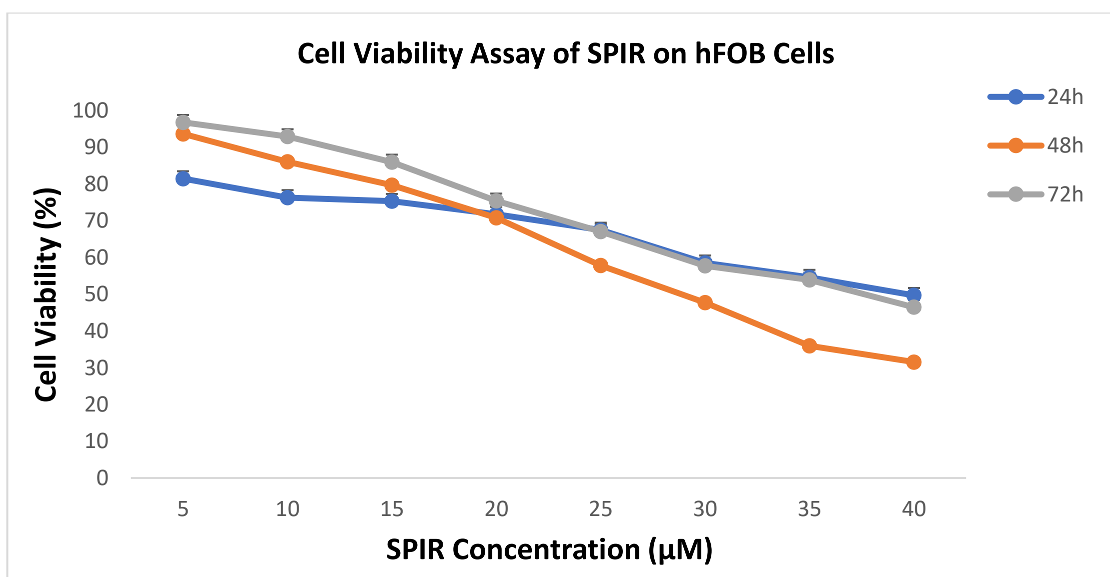


Figure 2: Effect of drug on cell viability of SPIR on hFOB cells. Cell viability was measured after 24-, 48- and 72-hours treatment with spironolactone (SPIR). The percentage of cell viability was decreased in dose dependent manner at each time point as the SPIR concentration increased. Results expressed as percentage of cell viability treated cell normalized to untreated cell (Data: mean ± SEM of three independent experiments)

Chemotherapy is frequently used in conjunction with radiotherapy and surgery to treat most types of cancer. Survival rates for patients with osteosarcoma rose exponentially after the introduction of chemotherapy, but have since reached a plateau. To date, the 5-year survival rate for all high-grade OS patients is about 60% –66 %, however it varies greatly by diagnostic stage. Patients with locally advanced disease have a 5-year survival rate of up to 60 %–78 %,

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however this percentage lowers to 20 %–30 % for those with metastatic disease [28-31]. SPIR, is an aldosterone antagonist drug that is used to treat hypertension and kidney disease. In addition, several studies reported that SPIR has been shown to be associated with certain types of cancer [32-34]. However, the mechanisms involved remain unclear.

Theoretically, the purpose of this approach is to assess the cytotoxic effects of SPIR on cancer cell lines in order to determine whether SPIR can sensitize cancer cells. As observed in the data presented, SPIR treatment has been shown to slightly reduce OS cell viability while remain non-cytotoxic to the OS cells. The cell viability percentage can be seen to be maintained throughout the incubation period. On the other hand, SPIR shows inhibitory effects on the cell viability of the normal cells. Even though this result contradicts the current study [13], there are a few factors that should be considered such as type of cells, drug concentration and combination with other types of drugs.

Overall findings, the effect of SPIR on the cells is stable throughout the dose in HOS-143B cells. Due to that, this is an advantage to immunotherapy treatment in using SPIR as a sensitizing factor as it can be used as an adjuvant at a very low dose with increasing effects on cancer cells death. Whereas, according to the hFOB data, it will gradually begin to affect the cells as the time period continues together with a high concentration of SPIR applied.

## 4. CONCLUSION

In conclusion, optimization is crucial for data reliability to evaluate drug sensitivity. Cell proliferation assays are commonly used by researchers to assess the efficacy of a drug on a specific cell line. Additionally, it gives precise and reliable data on the number of cells present, their multiplication, and their development. In addition, careful consideration of assay optimization of drug potency during the preclinical drug screening process may help to improve the success rate of cancer drug candidates that reach clinical trials. This procedure serves as an initial step to ensure that the dose of SPIR used will not kill the cancer cells completely. Thus, an optimization is needed prior to immunotherapy treatment that will be conducted on a later part of this study. Therefore, our present findings suggested that SPIR could serve as a potential candidate in enhancing the upregulation of NKG2D ligands. This will eventually enhance the immune sensitization and susceptibility of OS cells to NK cell-mediated lysis by up-regulating NKG2D ligands which are expressed on the tumour cells surface.

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## CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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