RESEARCH ARTICLE

Editorial Process: Submission:10/21/2021 Acceptance:09/17/2022

The Anticancer Effects of Novel Imidazo[1,2-a]Pyridine Compounds against HCC1937 Breast Cancer Cells

Akram M Altaher[1]*, Mohammed A Adris[2], Saeb H Aliwaini[3]*, Adel M Awadallah[4], Rami Y Morjan[4]

Abstract

Background: Anticancer drugs confront clinical obstacles such as drug resistance and adverse effects. Imidazo[1,2-a] pyridines (IPs) compounds have lately gained considerable interest as possible anticancer therapeutics due to their potent inhibitory function against cancers cells. This study was to determine the anticancer activities of three novel IPs (IP-5, IP-6, and IP-7) against the HCC1937 breast cancer cell line in vitro. **Materials and Methods:** The cytotoxic and anti-proliferative effects of IPs compounds in HCC1937 cells were determined by cell viability (MTT) assay, trypan blue assay, and clonogenic survival assay. Scratch motility assay was used to test the antimigration ability of the IPs. Western blot analysis was carried out to detect the level of apoptosis and cell cycle protein markers and to understand the mechanism of action of IPs compounds. **Results:** IP-5 and IP-6 have a strong cytotoxic impact against HCC1937 cells with IC50 values of 45μM and 47.7μM respectively. IP-7 possesses less cytotoxic effect against HCC1937 cells with IC50 of 79.6μM. Trypan blue assay showed that the three compounds induce significant cell death in the HCC1937 cells. Clonogenic and mammosphere assays demonstrated that IP-5 reduced the HCC1937 cells survival rate by more than 25.0% at 1000 cell concentrations. Western blotting analysis showed that IP-5 compound causes cell cycle arrest as noted by the increasing levels of p53 and p21 in treated cells. IP-5 induced an extrinsic apoptosis pathway as reveals from the increased activity of caspase 7, caspase 8, and the increasing level of PARP cleavage in treated cells. Also, IP-5 treated cells revealed segmented chromatin which is characteristic of apoptotic cells as shown by DAPI stain. Importantly, In comparison to control cells, IP-5-treated cells exhibited lower levels of pAKT. **Conclusions:** The novel three IPs compounds represent potential active anticancer compounds against HCC1937 breast cancer cells in vitro.

Keywords: Imidazo[1,2-a]pyridine- breast cancer- HCC1937- cytotoxicity- apoptosis- P53

Asian Pac J Cancer Prev, **23 (9)**, 2943-2951

Introduction

Cancer is a general term that refers to a group of diseases that can affect any organ in the human body, and it is the world's second major cause of death to date (World Health Organization, 2018). Breast cancer (BC) is still the second most common kind of cancer among women after the cancer of the skin (National Cancer Institute, 2021a). BC is a complex condition that is unlikely to respond to a single treatment and it is occasionally a consequence of inherited gene mutations (National Cancer Institute, 2021b). Being a woman and becoming older are the two main factors that increase the risk of creating breast cancer (Centers for Disease Control and Prevention, 2021).

Other BC risk factors include possess a family history of BC or ovarian cancer, possess thick breast tissue, physical inactivity, not breastfeeding, and hormone therapy, and chest treatment with radiation therapy for another cancer (Mørch et al., 2017). The stage of cancer describes the amount of cancer in the body, it enables to evaluate the seriousness of cancer and how best to treat it (Kalli et al., 2018). To plan the treatment schedule, it's crucial to know what stage breast cancer patients are in (National Breast Cancer Foundation, 2021). The early detection of BC and getting of most novel cancer treatments are the most important strategies to prevent its mortality. Early detection of breast cancer, when it is small and not spread, is better for effective treatment (Oeffinger et al., 2015).

The remedy rate BC stays low because of a variety of obstacles, of which drug resistance and severe side effects are the most significant. Cancers are capable of resisting conventional treatments, and the growing spread of resistance to these medications (Komeili-Movahhed

*[1]Department of Medical Sciences, University College of Science and Technology-Khan Yunis, Gaza Strip, Palestine. [2]Department of Biochemistry, Faculty of Medicine, University of AL-Butana, Ruffaa, Sudan. [3]Department of Biology and Biotechnology, Islamic University of Gaza, Gaza PO Box 108, Palestine. [4]Department of Chemistry, Faculty of Sciences, Islamic University of Gaza, Gaza PO Box 108, Palestine. *For Correspondence: a.taher@ucst.edu.ps, siwini@iugaza.edu.ps*

et al., 2015). While chemotherapy is the most commonly used cancer treatment, it also has adverse side effects ranging from mild such as weight loss, feeling exhausted, appetite change, and the sense of fatigue to the extreme as nausea, hepatotoxicity, lymphedema, and or swelling of limbs (El Kichaoui et al., 2016). A critical aspect of modern cancer research is the quest for effective, safe, and selective anticancer compounds. New types of therapies without or with a little harmful effect are required to treat various types of cancer (Narsimha et al., 2016).

Imidazo[1,2-a]pyridines (IPs) are nitrogen-based heterocycles that have a wide variety of biological responses and are usually made with natural ingredients (DeSimone et al., 2004). IPs-based compounds have obtained considerable attention as possible therapeutics against breast cancer due to their significant inhibitory action against diverse cancers cells proliferation and migration (Kim et al., 2014). Several in vitro investigations have found that IPs-based compounds (e.g. IP-Se-05; P-Se-06; HS-104; HS-106; HS-173; IPD-196; IP-6) can cause inhibitory effects against breast cancer cells proliferation, at relatively low concentrations, where they have potent dual inhibition against PI3K/Akt/mTOR signaling pathway (Kim et al., 2011; Li et al., 2013; Lee et al., 2013).

According to these studies, IPs-based compounds as a monotherapy exhibited antiproliferative effects against BC cell lines with IC50 values range between 0.01 and 10.0 μM. According to most previous studies, exposure of IPs-based compounds resulted in a considerable rise in cells number at the G2/M phase, and a decrease of its number in the G0/G1 phase, which resulted in delaying the progression of the cell cycle (cell cycle arrest), which was confirmed by lowered the expression of cyclin B1 and increase the expression levels of p-cdc2 and p-cdc25 (Hayakawa et al., 2018). Furthermore, the proportion of cells arrested with the combined treatment was much higher than the percentage of cells arrested with IPs-based compounds alone (Yun et al., 2013).

Based on these studies, it is reasonable to suggest that cancer cells proliferation is suppressed by IPs-based drugs, which cause cell cycle arrest at the G2/M phases. On the other hand, IPs-based compounds have been found to trigger apoptosis in a wide variety of cancer cells including breast, human hepatocellular carcinoma, liver, lung, Melanoma, cervical cancer cells. However, the apoptotic effect of IPs-based compounds in a variety of cancer cells was confirmed by an increase in PARP cleavage in cells treated with IPs-based compounds as compared to control cells. Also, the increased levels of Bax and cleaved caspases 3, and 9 and decreased the expression of Bcl-2 (Li et al., 2013; Lee et al., 2013; Jung et al., 2013; Hayakawa et al., 2018).

Overall, IPs-based drugs cause apoptosis in cancer cells by triggering the activation of caspases. Despite the fact that these agents have a wide range of therapeutic uses, none of them have been approved as anti-cancer medications. However, The anticancer abilities of three imidazo[1,2-a]pyridines (IP-5, IP-6, and IP-7) against the HCC1937 breast cancer cell line were tested in this study. Also, the ability of one of these compounds (IP-5) to cause cell cycle arrest, apoptosis, and inhibit the Akt signaling pathway was investigated in the present study.

Materials and Methods

Synthesis of Imidazo[1,2-a]pyridines compounds (IP-5, IP-6, and IP-7)

The IP compounds were chemically synthesized in the chemical laboratory of the Islamic University of Gaza by a scientific team headed by Prof. Dr. Adel Awadalah. These three compounds (IP-5, IP-6, and IP-7) were synthesized from the reaction of picoline derivatives 2-4 with hydrazonoyl chloride 1 in tetrahydrofuran in the presence of triethylamine at room temperature (Figure 1) (Morjan et al., 2014).

HCC1937 cells culture and treatments

The HCC1937 cells were a gift from prof. Adrienne Lesley Edkins, Department of Biochemistry and Microbiology, Rhodes University. The Dulbecco's Modified Eagle Medium (DMEM) was used to culture and maintained the HCC1937 cells (Biological Industries) in a 5% CO2 incubator adjusted at 37°C. Every 2-3 days, the DMEM was changed. The three compounds (IP-5, IP-6, and IP-7) were dissolved in dimethyl sulfoxide (DMSO) to produce a 10 mM stock solution. The stock solution was used within ten days only. Following that, further dilutions in DMSO media were made to reach the final concentration of 10 to 100 μM in ten Eppendorf tubes (Figure 2). Vehicle-treated cells were maintained in DMEM with DMSO.

Cell morphology

The HCC1937 cells were seeded at the appropriate count to reach a confluency of 60-70% at the treatment day. An inverted light microscope (Olympus, USA) was used to observe and photograph the morphological changes in the cells after they have been treated with the IP compounds.

Cytotoxicity assays

The HCC1937 cells were plated in 96-well plates at 6×10^3 cells per well and incubated for 48 hours in a 5% CO2 incubator adjusted at 37°C to reach a confluency of 70% at least. After 48 hours; Cells were treated with a graduated range of concentrations of IP compounds starting from 0 to100 μM for 48 hours. Then MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) with a concentration of 10 μmL was added to each well according to the instructions of the manufacturer (Roche Diagnostics, Mannheim, Germany) (Marie Kirwen et al., 2017). After 4 hours of incubation, a 100 μL of solubilizing buffer was added to the plate, which was then incubated in the dark for 16 hours under the same conditions. The absorbance for each well was read at 570 nm via the ELISA reader (CF-fiocchetti, Italy) (Van Meerloo, Kaspers and Cloos, 2011) and the average cell viability was expressed as a percentage of the average vehicle control. Five independent experiments were performed to determine the half-maximal inhibitory concentration values (IC_{50}).

Trypan- blue dye exclusion viability assay

Cells were cultured in a 6-well plate at a density of 2×10^5 - 3×10^5 cells per well and incubated for 24 hours in 5% CO_2 incubator adjusted at 37°C to reach a confluency of 70% at least. After that, cells were treated by IPs compounds at different concentrations and incubated for 24 hours. Afterward, the medium was removed and the cells were collected and washed twice with phosphate-buffered saline (PBS). Cells were stained with trypan blue stain (0.4%) and incubated for 5 minutes. Then, a Cell-counting chamber was used to calculate how many viable (unstained) cells in comparison to dead (blue-stained) cells under a light microscope (Olympus, Japan). The viability of cells was reported as a percentage by dividing total viable cells by the total cells (stained + unstained).

Clonogenic survival assay

HCC1937 cells were grown to 70-80% confluence and then treated for 24 hours with 25μM of IP-5. The treated HCC1937 cells were then re-cultured at a density of 500 cells in a 6 cm dish and placed for 2 weeks in a 5% CO2 incubator adjusted at 37°C. The DMEM media was altered every 48 hours. Survived cells were fixed in methyl alcohol for 2-3 minutes, then Giemsa stain in concentration 8-10% (Sigma, USA) was used to stain the cells at normal room temperature for 20 minutes. HCC1937 colonies that were stained by Giemsa were washed four times in phosphate buffer saline (PBS). The change in the percentage of colonies that have survived was calculated.

Mammosphere assay

Mammosphere forming assay was used to assess anchorage-independent growth as explained by the Aliwaini study (Aliwaini et al., 2015). To achieve a single cell suspension, cells were suspended with 0.26 % (v/v) trypsin and then passed thru a 40 M cell strainer (BD Biosciences). A 96-well ultralow attachment plate supplied with DMEM media was used to culture the obtained single-cell suspensions under mammosphere circumstances (1,000 cells/well). IP-5 treatment was done either before or after culturing. The DMEM media was altered every 48 hours. The ability of cells to forming of the mammosphere was calculated as sphere-forming efficiency after one week by viewed and imaged the culture under a light inverted microscope at 40x magnification (Olympus, Japan). The sphere-forming efficiency of cells was calculated as a percentage by dividing the total number of mammospheres created in 96 wells divided by the original number of single cells cultured and grown.

Nuclear fragmentation

IP-5 was applied to treat the HCC1937 cells for 24 hours, after that the Hoechst 33342 was used to stain the cells for 8-10 minutes. The cells were observed and photographed under a fluorescence microscopy microscope at 40x magnification (Zeiss, Germany).

Protein Extraction and Western Blotting

The HCC1937 cells were treated with vehicle or IP compounds for 48 hours, then harvested, for protein extraction in boiling blue. The extract was heated for 10 min to block protease action as earlier stated (Al-Qatati and Aliwaini, 2017). Gel Electrophoresis (SDS/PAGE) and western blotting assay were carried out using 20 μl of proteins from each concentration and 10 μl of protein ladder. Protein extracts were electrophoresed on 8% and 12% gel. Proteins that had been resolved were transported to nitrocellulose membranes and incubated in primary antibodies (1-1000) against PARP-1 (sc-7150), Tubulin (sc-5286), cyclin B (sc-sc-53236), P53(sc-126), P21(sc- 756) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), BAX (sc-7480), BCL2 (#2876), p-AKT (#9271) and Cas9 (##9502) (Cell Signaling, Boston, MA, USA).

After primary antibody incubation, Membranes were washed and incubated with suitable HRP-conjugated secondary antibodies (1:4000) (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK) for 1 hour. Antibody-reactive proteins were visualized as a band by means electrochemiluminescence reaction detection system (Thermo Scientific, Hudson, NH, USA) in the darkroom.

Statistical analysis

Results were presented as mean ± SD (standard deviation of the means) of three independent experiments. The collected data from all experiments were statistically analyzed using Microsoft excel 2013 (Redmond, WA, USA) and the statistical package of social sciences (SPSS ver. 24) software.

Results

IPs 5, 6, and 7 have antiproliferative effects against breast cancer cells

MTT assay was used to determine the cytotoxic effect of IP- 5, 6, and 7 on HCC1937 cells. The results showed that IP- 5, 6, and 7 compounds have cytotoxic effects with IC50 of 45, 47, and 79.6 μM respectively (Figure 2). At low concentrations (10, 20, and 30μM), all tested compounds inhibited cell growth similarly. At higher concentrations, IP-5 showed the most potent and continuous dose-dependent cytotoxic effect. Furthermore, IP-5 treated cells showed obvious stress morphological signs such as vacuolated, floating, and shrunken cells that appeared after 48 hours of the treatment. These results show that IPs 5, 6, and 7 display potent cytotoxicity against HCC1937 cells.

IP-5 induces breast cancer cell death

The effect of IP-5 on the viability of the HCC1937 cells was determined by treating cells with 50 μM of IP-5 for 48 hours and then staining it with trypan blue and the results were analyzed by Microsoft Office Excel 2013 (Figure 3.a). The results show that IP-5 killed more than 40% of breast cancer cells after 48 hours of the treatment. To see if IP-5 caused cell death through apoptosis, Cells were treated with IP-5 for 24 hours were fixed, DAPI stained, and visualized under the fluorescent microscope. In Figure 3 b-c, the frequency of apoptotic cells for every

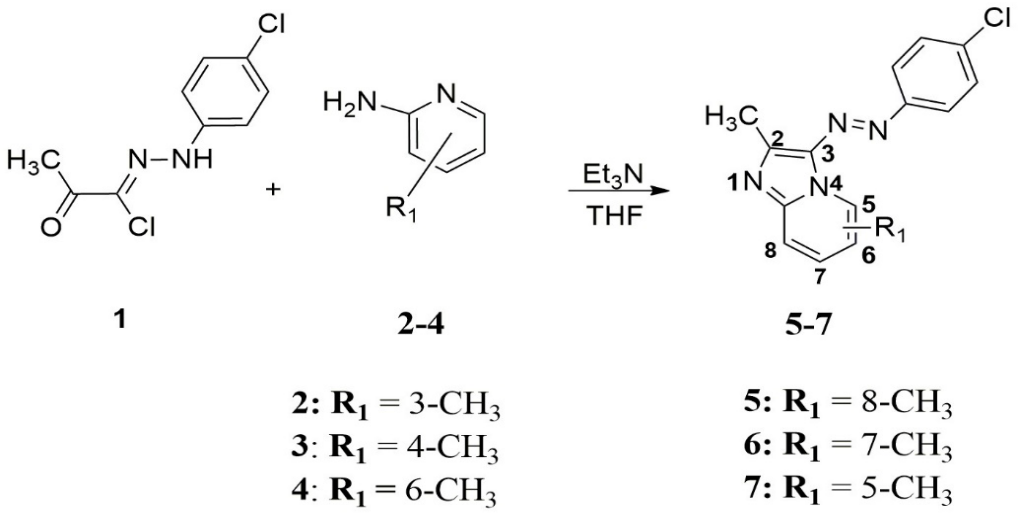

Figure 1. IP compounds 5-7 Structures and Synthesis (Morjan et al., 2014)

100 cells was counted and presented.

According to the findings, IP-5 therapy caused considerable rates of apoptosis in breast cancer cells (approximately 37%) (Figure 3 b-d). Taken together these data show that IP-5 induces apoptotic cell death in HCC1937 cells.

IP-5 inhibits the ability of HCC1937 cells to survive and proliferate

The cell survival rate of HCC1937 cells treated with IPs 5, 6, and 7 compounds was measured by colony formation assay. The findings illustrated that untreated HCC1937 cells (0/0) were survived at all cell concentrations, while IP-5 significantly decreased the HCC1937 survival rate (Figure 4 a, b, and f). Indeed IP-5 reduced the HCC1937 cell survival rate by more than 25.0%. Similarly, IP-5 inhibited the quantity and viability of HCC1937 mammospheres in a dose-dependent manner (Figure 4 c,d,e and g). Interestingly, a statistically significant drop in sphere-forming efficiency was seen at 25.0 µM of IP-5, also this dropping was even more at 50.0 µM of IP-5. When these data are joined collectively they

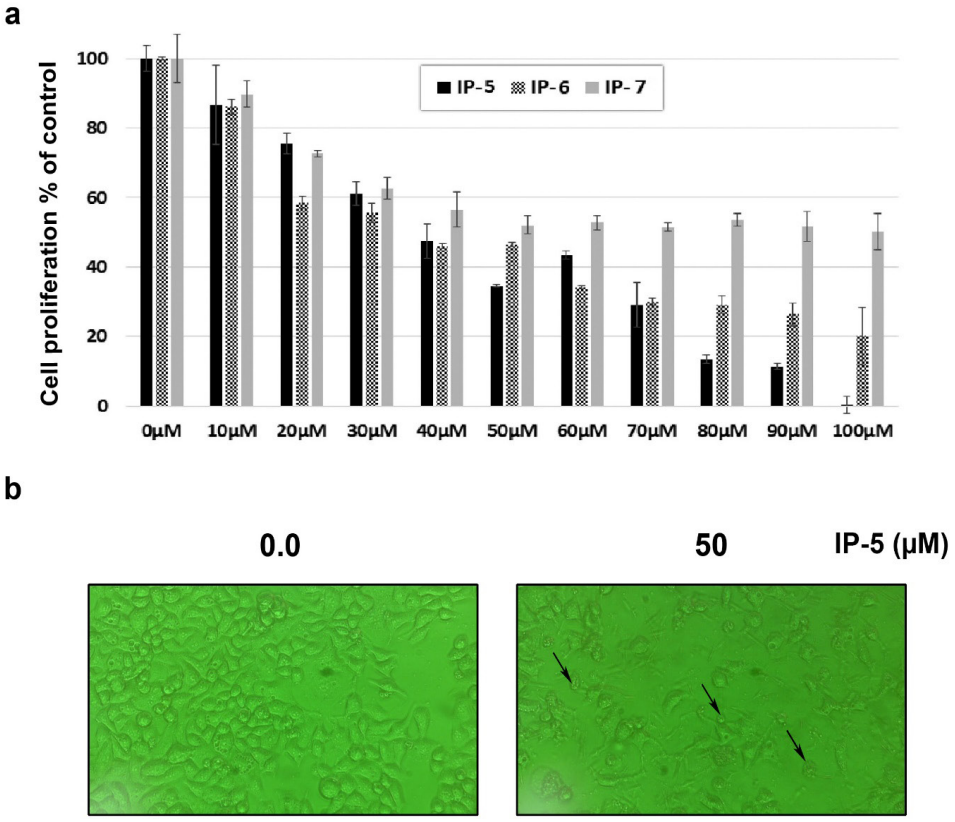

Figure 2. IP-5 Induces Cytotoxicity in HCC1937 Cells. (a) IP-5 was used to treat the cells at concentrations ranging from 0 to 50 µM. After 48 hours of treatment, cell viability was determined using the MTT test. (b) Representative photos of treated and untreated cells. Black arrows indicate dead cells.

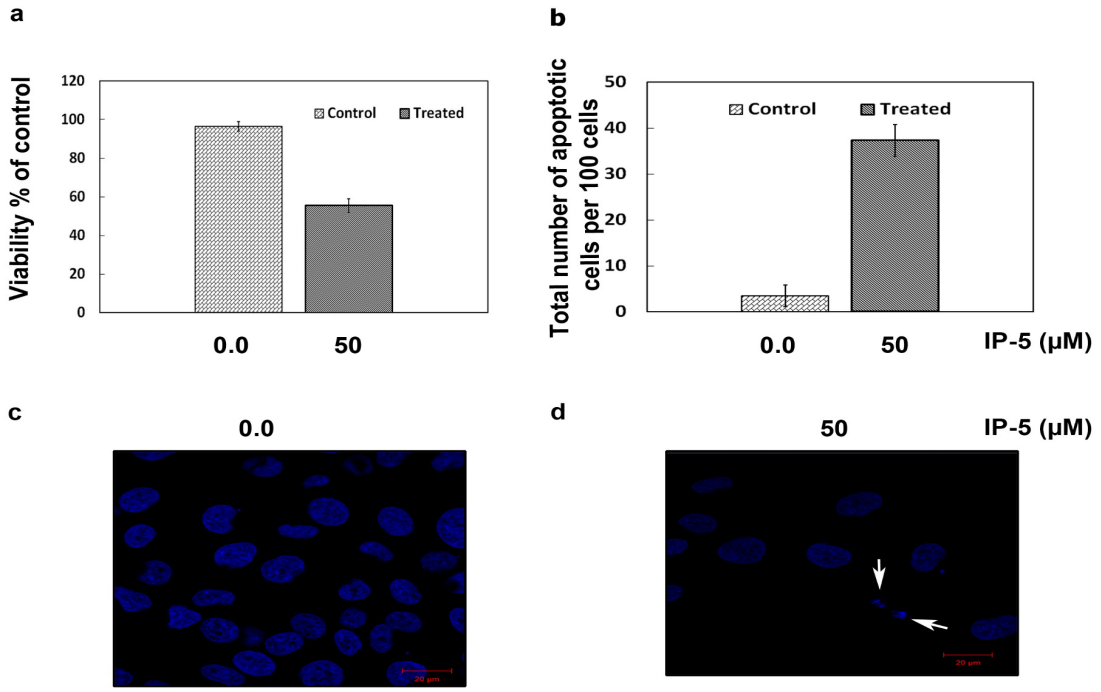

Figure 3. IP-5 Induces Cell Death in HCC1937 Cells. HCC1937 cells were treated 0 or 50μM and viability was determined by trypan blue in (a) and by DAPI staining in b-d. (b) The number of cells stained with DAPI nuclear stain per 100 cells in total cells treated with 50μM as compared to untreated (0.0) was counted

form a fact that the IP-5 has a strong anti-survival effect on HCC1937 cancer cells (Figure 4 a to f).

IP-5 induces cell cycle arrest and extrinsic apoptosis in Breast cancer cells

To investigate the mechanism by which IP compounds exert their anticancer effect, HCC1937 cells were plated and treated with IP-5 compound (0 and 50μM) for 24 and 48 hours. Protein lysate was analyzed by western blotting for key proteins of cell cycle arrest and apoptosis (p53, p21, cleaved PARP, caspase 7, caspase 8, and p-AKT). Results revealed that there was a remarkable increase in the level of p53 and p21 protein in a time-dependent manner in HCC1937 cells treated with IP-5 compound

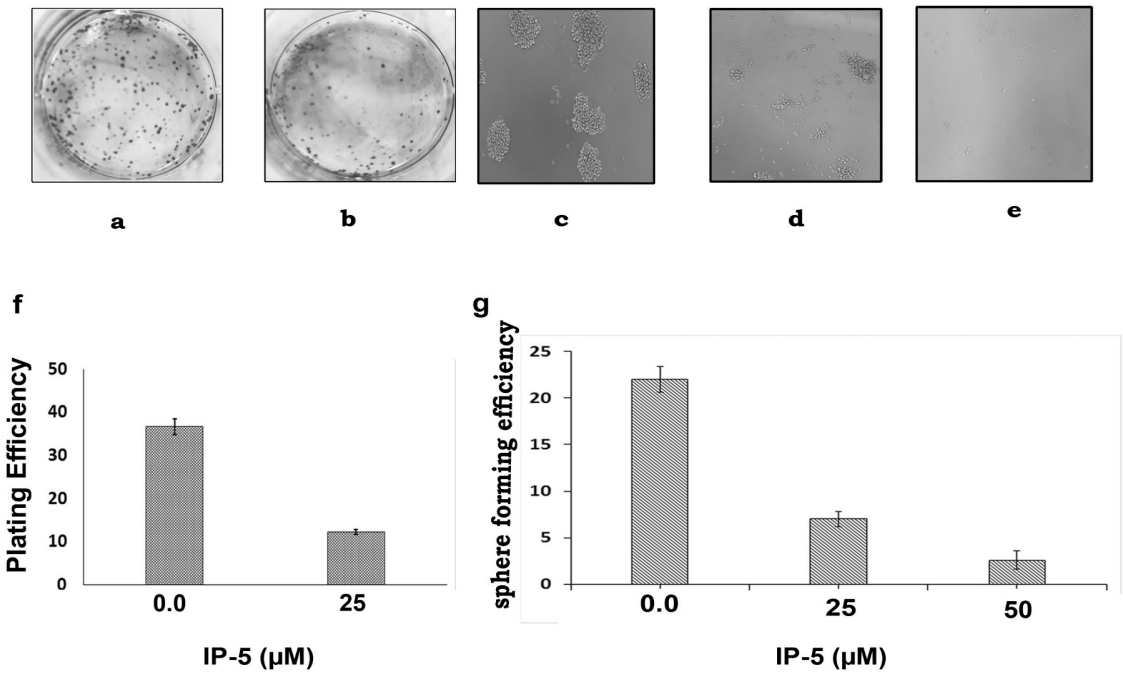

Figure 4. IP-5 Displays Antisurvival Activity against the HCC1937 Cells. (a, b and f) colony formation rate was quantified in HCC1937 cells treated without or with IP-5 for 24 h. Values were given as mean ± SD. (c,d and e). The number of mammospheres generated per a number of single cells seeded and computed in percentages was used to determine the efficiency of sphere formation (g).

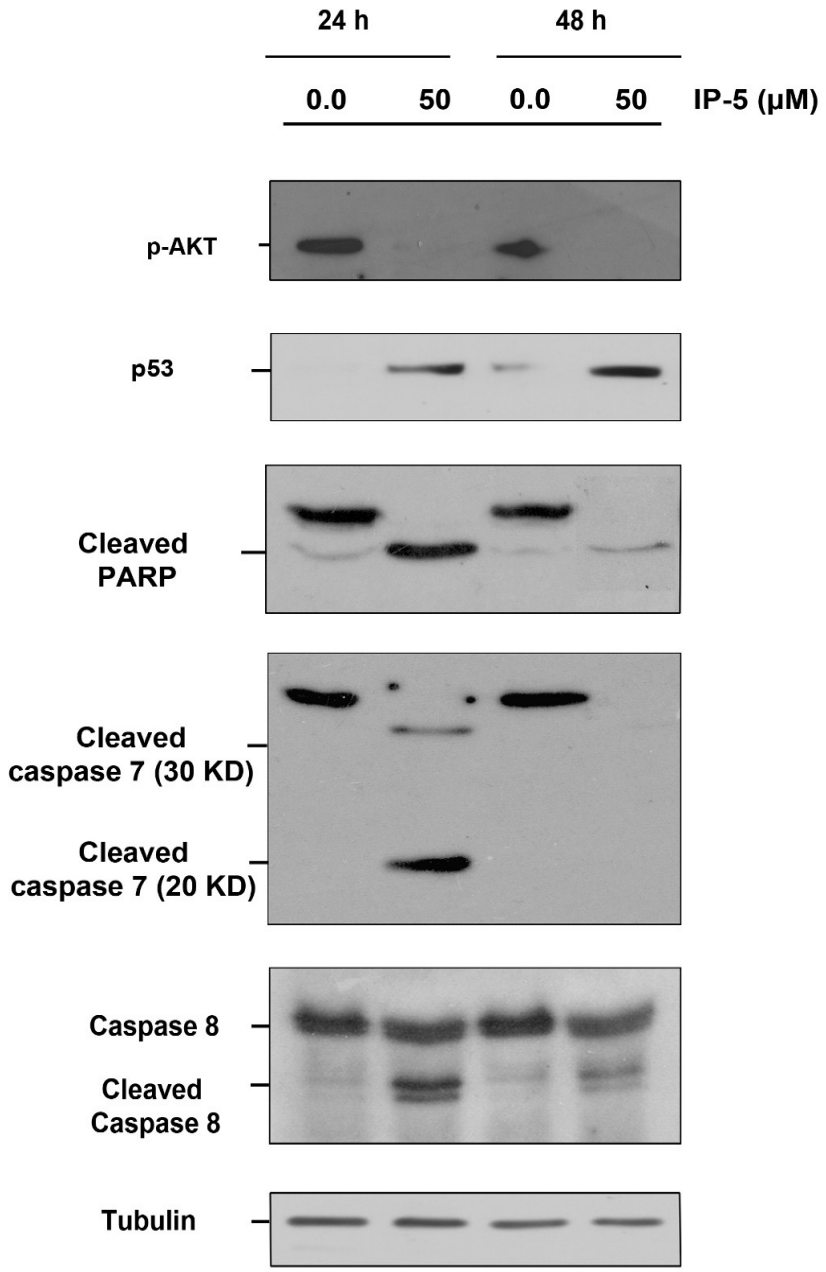

Figure 5. IP-5 Induces Cell Cycle Arrest and Apoptosis in the HCC1937 Cells. Western blotting of protein from the cells treated with 0 or 50μM of IP-5 and analyzed with antibodies to P-AKT, P53, p21, PARP, and caspase 7 and 8.

(Figure 5). On other hand, Figure 5 also shows that treatment with 50 μM of IP-5 for 24 hours and 48 hours induced high levels of cleaved PARP and active caspase 7 which are accepted markers of programmed cell death (apoptosis). Furthermore, the presence of active caspases 8 in the IP-5 treated cells indicates that the IP-5 compound stimulates the extrinsic apoptosis pathway (Figure 5). In summary, IP-5 causes its cytotoxic effect by inducing both cell cycle arrest and extrinsic apoptosis.

Discussion

In the current study, the antiproliferative effect of the newly synthesized IP-5, IP-6, and IP-7 have been experimented against the HCC1937 cancer cell line using the MTT assay. This anti-growth activity was determined after the treatment with different concentrations of compounds (0.0μM to 100.0μM) for 48 hours. Our findings reported that IP-5, IP-6, and IP-7 have a significant cytotoxic effect on HCC-1937 cell lines with IC_{50s} of 45 μM, 47.7 μM, and 79.6 μM respectively. The study findings present many clues and evidence that the tested novel IP compounds (5, 6, and 7) have the potential to be a therapy to treat breast cancer. On the other hand, the following electronic databases were searched: PubMed, Google Scholar, Web of Science, and Hinari to find out all relevant citations investigating the anti-cancer effect of IP-based compounds on the HCC1937 breast cancer cell line which were published between 1st Jan 2010 and 31st Dec 2021.

Unfortunately, we did not find any published work regarding this topic. But we found many studies have

tested the anti-proliferative effect of other breast cancer cell lines such as: MCF-7, T-47D, and SKBR3 and they proved that the IP-based compounds that have been tested by them have strong anti-proliferative effects against these cell lines, these compounds have a narrow range of IC_{50}s (0.1-10 μM) (Byth et al., 2006; Lee et al., 2012; Li et al., 2013; Gabriela et al., 2018). This could be due to that; the HCC1937 cell line is a TNBC subtype that has no effective molecular targeted therapies. Whereas the MCF-7 cell line is more famous and widely because it is ER+ cell line. Nevertheless, this argument cannot be proven.

However, to the best of our knowledge, the present study is the first one that shedding the light on the anti-cancer effect of IP-based compounds on the HCC1937 breast cancer cell line. We find it necessary to clarify that many previous studies have dealt with the effect of another compound rather than imidazo[1,2-a] pyridines such as: palladacycle complex (ASH10) against HCC1937 breast cancer cell line (Al-qatati and Aliwaini, 2020). The current findings were consistent with the majority of prior research that indicated IP-based compounds (e.g. IP-Se-05; P-Se-06; HS-104; HS-106; HS-173) have a significant antiproliferative effect on MCF-7, T-47D and SKBR3 breast cancer cell lines with IC_{50}s values ranging from 0.1 to 26.0 μM (Byth et al., 2006; Lee et al., 2012; Li et al., 2013; Lee et al., 2013; Gabriela et al., 2018).

In addition, another two studies investigated the cytotoxic effect of two novel imidazopyridine derivatives named HS-104 and HS-106 respectively against the MCF-7 breast cancer cell line in vitro, and they found that both HS-104 and HS-106 induced a strong reduction in the growth rate of the MCF-7 cancer cell lines in a dose-dependent manner with IC_{50} value 1.2 μM and < 10 μM respectively (Lee et al., 2012; Li et al., 2013). The variation in the IC_{50s} for the same compound against different cell lines of the same type of cancer might be attributed to the different content of receptors and enzymes for each cell line (Lee et al., 2013). The clonogenic survival assay has been set out to determine the anti-survival effects of anti-cancer treatments on colony-forming abilities in various cancer cell lines (Rafehi et al., 2011). In the present study, the cell survival rate of HCC1937 cells treated with IPs 5, 6, and 7 compounds were assessed by clonogenic survival assay.

The findings show that both IP-5 and IP-7 significantly decreased HCC1937 survival rate. Where IP-5 reduced HCC1937 cell survival rate by more than 94.0% at 500 cell concentration vs. 89.5% at 1,000 cell concentration. Furthermore, IP-7 reduced the HCC1937 cell survival rate by more than 67.0% at 500 cell concentration vs. 78.4% at 1,000 cell concentration. On the other hand, this was not the same for IP-6 where it has an anti-survival effect on HCC1937 cells only at low cell concentration; 22.0% at 500 cell concentration vs. 37.8% at 1000 cell concentration. Taken together these data demonstrate that all three IP-based compounds have potent anti-survival effects on HCC1937 cancer cells. This finding observed in the present study agreed with the more than one previous study that reported Imidazo[1,2-a]pyridines compounds (IP-Se- 5, IP-Se-6 and compound 5) in non-toxic concentration can inhibit colony formation ability in many cancer cell lines such as; MCF-10A-7 and HEK293 (Baviskar et al., 2011; Gabriela et al., 2018).

Regarding the mechanism of action of Imidazo[1,2-a] pyridines compounds on the HCC1937 breast cancer cell line, The present study findings suggested that IP-5 can indeed induce cell cycle arrest in HCC1937. This observation was obtained from seeing a high level of p53 and p21 proteins in western blot assays, which is a significant protein marker of the cell cycle arrest. However, this finding confirms that G0/G1 phase and G2/M phase cell cycle arrest are two mechanisms stimulated by IP-5 in HCC1937 cells. The activation of p53 and its target cyclin-dependent kinase inhibitors (p21) is commonly thought to be a cell cycle arrest signal.

The increases in the levels of p21 cell cycle regulator and cyclin D1 are commonly used as a markers for cell cycle arrest at the G1 stage (Aliwaini et al., 2019; Benzeno et al., 2004). Our results are similar to many previous studies which have indicated that the treatment of various cell lines (such as; Breast cancer, Human hepatocellular carcinoma, Liver cancer, Lung cancer, Melanoma, HeLa cervical cancer) by IPs-based compounds (e.g. IP-Se-05; P-Se-06; HS-104; HS-106; HS-173; IPD-196; IP-6) contributed in a cell cycle arrest in the G1 and G2 cell cycle stage, as well as elevated concentrations of p21, p53 proteins and cyclin D1 (Lee et al., 2013; Jung et al., 2013; Hayakawa et al., 2018; Aliwaini et al., 2019). In the current study, IP-5 compound has been proven to carry out its cytotoxic effect by triggering extrinsic apoptosis as evident by the high level of PARP cleaved, Caspases 7 and Caspases 8.

Our results were consistent with other studies that reported IPs-based compounds have been triggered apoptosis in a wide variety of cancer cells including breast, human hepatocellular carcinoma, liver, lung, Melanoma, cervical cancer cells. However, the apoptotic effect of IPs-based compounds in a variety of cancer cells was confirmed by an increase in PARP cleavage in treated cells as compared to control cells. Also, the increased levels of Bax and cleaved caspases 3, 7, 8, and 9 and decreased the levels of Bcl-2 (Li et al., 2013; Jung et al., 2013; Hayakawa et al., 2018). This proves IP agents' capacity to induce both extrinsic and intrinsic apoptosis in cancer cells. Despite their differences in the chemical composition of these compounds, they have a similar impact on our compounds.

A group of researchers synthesized various novel IP compounds and investigated apoptosis properties for each compound against MCF-7 breast cancer cell line. They reported that the compounds induced apoptosis of MCF-7 by influencing the mitochondria and induction of caspases (Lee et al., 2012; Li et al., 2013; Gabriela et al., 2018). Upon to our knowledge, to date, there is no data in the literature review regarding whether IP-based compounds trigger cell cycle arrest and or apoptosis in HCC1937 breast cancer cells and what is the actual molecular mechanism that causes it to have an inhibiting effect. However, the current study provides evidence that IP compounds induce cell cycle arrest and extrinsic apoptosis. Moreover, the findings of the current study

found that IP-5 causes inhibitory effects against cancer cells proliferation, at relatively low concentrations, where it has a potent inhibition against PI3K/Akt signaling pathway.

Our results agreed with the findings of numerous studies in the literature which suggested that IPs-based compounds (e.g. IP-Se-05; P-Se-06; HS-104; HS-106; HS-173; IPD-196; IP-6) have potent inhibition against PI3K/Akt signaling pathway (Kim et al., 2011; Li et al., 2013; Lee et al., 2013; Jung et al., 2013; Gabriela et al., 2018; Hayakawa et al., 2018; Aliwaini et al., 2019). According to these studies, IPs-based compounds as a monotherapy exhibited antiproliferative effects against many cell lines (such as; Breast cancer, Human hepatocellular carcinoma, Liver cancer, Lung cancer, Melanoma, HeLa cervical cancer) with IC50 values ranging from 0.01 to 44.6 μM. Ingersoll et al. (2015) have synthesized a group of novel IP compounds named HIMP and M-MeI and were examined its anticancer effects on various cell lines e.g. LNCaP C-81 cells and MDA PCa2b-AI cells. The findings registered that HIMP and M-MeI compounds were shown to be the most effective in a dose and time-dependent manner where both suppressed the PI3K/Akt signaling pathway and caused G2-stage cell cycle arrest. Where, both treatments inducing decreases in Akt protein and increasing pro-apoptotic proteins p53, p21, Bax, and down-regulated proteins which are involved in cell proliferation such as cyclin D1 (Ingersoll et al., 2015).

In conclusion, this is the first experimental in vitro study performing to investigate the anticancer abilities of three novel imidazo[1,2-a]pyridines (IP-5, IP-6, and IP-7) against HCC1937 breast cancer cell lines, and it is the first study to present the mechanism of action these compounds against this cell line. The present study provides several shreds of evidence that IP compounds are promising anticancer agents where the three novel IP compounds (IP-5, IP-6, and IP-7) induces a significant cytotoxic effect in a time and concentration-dependent manner in vitro against HCC1937 breast cancer cells, where these compounds lower the value of IC50 significantly.

Also, the trypan blue dye exclusion assay showed that these three compounds also induce cell death and anti-proliferative effect in the HCC1937 cell line. Moreover, these compounds also reduced the HCC1937 cells survival rate by more than 68% at 1000 cell concentrations and 60% at 500 cell concentrations. Furthermore, treatment of HCC1937 cells by the novel IP-5 induces cell cycle arrest and extrinsic apoptosis as noted by the increasing levels of p53, p21, and the activation of caspase 7, caspase 8, and the increasing level of PARP cleavage in treated cells.

Additionally, IP-5 can cause potent dual inhibition against PI3K/Akt signaling pathway. In light of these encouraging findings, we recommend investigating the anticancer activity of IP compounds is combined with other anticancer agents and determine the anticancer activity of these compounds on other cancers and normal cells. Further studies to understand the detailed nature of the effect IP compounds might have in vivo are required.

Author Contribution Statement

Altaher, A; conducted all experimental assays and was the substantial contributor to the manuscript's creation and writing. Aliwaini, S; supervised different steps for the conducted of experimental assays and was the second substantial contributor to the manuscript's writing. Adris, M; was the main supervisor of this study and he reviewed the writing and editing. Awadallah, A; He manufactured the IP compounds and put them to the test. Morjan, R; He was in charge of chemically analyzing of the manufactured compounds. All authors have approved the final version of the manuscript.

Acknowledgments

Special thanks and appreciation for the help and support of the dean of the Faculty of Science, and the head of the Biology and Biotechnology Department at the Islamic University of Gaza, where they allowed me to use instruments, equipment, and materials for tissue culture laboratory to perform the experiments of this study and preparation of results. I want to express my deep thanks to all the academic and administrative staff of the Faculty of Postgraduate Studies and Scientific Research at the University of AL-Butana for their guidance and support.

Ethical approval

Because this is an experimental in vitro investigation, no institutional ethics committee approval was required.

Availability of data and materials

This published article includes all of the data collected during this experimental study.

Conflict of interest

None.

References

Al-qatati A, Aliwaini S (2020). Anticancer activities of newly synthesized palladium (II) complex in breast cancer cells. *Int J Cancer Res*, **16**, 40-7.

Al-Qatati A, Aliwaini S (2017). Combined pitavastatin and dacarbazine treatment activates apoptosis and autophagy resulting in synergistic cytotoxicity in melanoma cells. *Oncol Lett*, **14**.

Aliwaini S, Awadallah A, Morjan R, et al (2019). Novel imidazo [1, 2-a] pyridine inhibits AKT/mTOR pathway and induces cell cycle arrest and apoptosis in melanoma and cervical cancer cells. *Oncol Lett*, **18**, 830–7.

Aliwaini S, Peres J, Kröger L, et al (2015). The palladacycle, AJ-5, exhibits anti-tumour and anti-cancer stem cell activity in breast cancer cells. *Cancer Lett*, **357**, 206-018.

Almeida G, Rafique J, Saba S, et al (2018). Novel selenylated imidazo[1,2-a]pyridines for breast cancer chemotherapy: Inhibition of cell proliferation by Akt-mediated regulation, DNA cleavage and apoptosis. *Biochem Biophys Res Com*, **503**, 1291-7.

American Cancer Society (2021). Breast Cancer Signs and Symptoms [Online]. Available: https://www.cancer.org/cancer/breast-cancer/screening-tests-and-early-detection.html [Accessed 18 June 2021].

Baviskar A, Madaan C, Preet R, et al (2011). N-fused imidazoles as novel anticancer agents that inhibit catalytic activity of topoisomerase IIα and induce apoptosis in G1/S phase. *J Med Chem*, **54**, 5013-30.

Benzeno S, Narla G, Allina J, et al (2004). Cyclin-dependent kinase inhibition by the KLF6 tumor suppressor protein through interaction with cyclin D1. *Cancer Res*, **64**, 3885-91.

Byth F, Geh C, Forder C, et al (2006). The cellular phenotype of AZ703, a novel selective imidazo[1,2-a]pyridine cyclin-dependent kinase inhibitor. *Mol Cancer Ther*, **5**, 655-64.

Centers for Disease Control and Prevention (2021). What Are the Risk Factors for Breast Cancer [Online]. Available: https://www.cdc.gov/cancer/breast/basic_info/risk_factors.htm.

Comşa Ş, Cîmpean M, Raica M, (2015). The story of MCF-7 breast cancer cell line: 40 Years of experience in research. *Anticancer Res*, **35,** 3147-54.

DeSimone R, Currie K , Mitchell S, et al (2004). Privileged structures: applications in drug discovery. *Com Chem High T SCR*, **7**, 473-93.

Ingersoll M, Lyons A, Muniyan S, et al (2015). Novel imidazopyridine derivatives possess anti-tumor effect on human castration-resistant prostate cancer cells. *PLoS One*, **10**.

Jung H, Zheng H, Jeong Y, et al (2013). Suppression of tumor proliferation and angiogenesis of hepatocellular carcinoma by HS-104, a novel phosphoinositide 3-kinase inhibitor. *Cancer Lett*, **328**, 176-87.

Kalli S, Semine A, Cohen S, et al (2018). American joint committee on cancer's staging system for breast cancer: what the radiologist needs to know. *Radiographics*, **38**, 1921-33.

El Kichaoui A, Ayesh B, Aliwaini S (2016). The use of some plant extracts as an alternative approaches for treatment of certain malignant cell lines. *J Med Plants Stud*, **4**, 174-88.

Kim O, Jeong Y, Lee H, et al (2011). Design and synthesis of imidazopyridine analogues as inhibitors of phosphoinositide 3-kinase signaling and angiogenesis. *J Med Chem*, **54**, 2455-66.

Kim Y, Kang C, Ranatunga S, et al (2014). Imidazo[1,2-a] pyridine-based peptidomimetics as inhibitors of Akt. *BMLC*, **24**, 4650-3.

Komeili-Movahhed T, Fouladdel S, Barzegar E, et al (2015). PI3K/Akt inhibition and down-regulation of BCRP re-sensitize MCF7 breast cancer cell line to mitoxantrone chemotherapy. *Iran J Basic Med Sci*, **18**, 472-7.

Lee H, Li G, Jeong Y, et al (2012). A novel imidazopyridine analogue as a phosphatidylinositol 3-kinase inhibitor against human breast cancer. *Cancer Lett*, **318**, 68-75.

Lee H, Jung K, Jeong Y, et al (2013). HS-173, a novel phosphatidylinositol 3-kinase (PI3K) inhibitor, has anti-tumor activity through promoting apoptosis and inhibiting angiogenesis. *Cancer Lett*, **328**, 152-9.

Li G, Jung K, Lee H, et al (2013). A novel imidazopyridine derivative, HS-106, induces apoptosis of breast cancer cells and represses angiogenesis by targeting the PI3K/mTOR pathway. *Cancer Lett*, **329**, 59-67.

Marie Kirwen E, Batra T, Karthikeyan C, et al (2017). 2,3-Diaryl-3H-imidazo[4,5-b]pyridine derivatives as potential anticancer and anti-inflammatory agents. *Acta Pharm Sinica B*, **7**, 73-9.

Van Meerloo J, Kaspers Gertjan J, Cloos J (2011). Cell sensitivity assays: the MTT assay. Cancer cell culture, Springer. pp 237-45.

Mørch L, Skovlund C, Hannaford P, et al (2017). Contemporary hormonal contraception and the risk of breast cancer. *N Engl J Med*, **377**, 2228-39.

Morjan R, Qeshta B, Al-Shayyah H, et al (2014). Reaction of Nitrilimines with 2-Aminopicoline, 3-Amino-1,2,4-Triazole, 5-Aminotetrazole and 2-Aminopyrimidine. *Int J Org Chem*, **4**, 201-7.

Narsimha S, Kumar S, Swamy K, et al (2016). Indole-2-carboxylic acid derived mono and bis 1, 4-disubstituted 1, 2, 3-triazoles: synthesis, characterization and evaluation of anticancer, antibacterial, and DNA-cleavage activities. *BMCL*, **26**, 1639-44.

National Breast Cancer Foundation (2021). Breast Cancer early detection, Diagnosies, Stages, Types and Treatment [Online]. Available: https://www.nationalbreastcancer.org/about-breast-cancer/breast-cancer-staging [Accessed 20 Feb 2021].

National Cancer Institute (2021a). Breast Cancer-Patient Version [Online]. Available: https://www.cancer.gov/types/breast [Accessed 20 March 2021].

National Cancer Institute (2021b). General Information About Breast Cancer [Online]. Available: https://www.cancer.gov/types/breast/patient/breast-treatment-pdq [Accessed 25 March 2021].

Oeffinger K, Fontham T, Etzioni R, et al (2015). Breast cancer screening for women at average risk: 2015 guideline update from the American Cancer Society. *JAMA*, **314**, 1599-614.

Rafehi H, Orlowski C, Georgiadis T, et al (2011). Clonogenic assay: adherent cells. *JoVE*, **49**, e2573.

World Health Organization (2018). Newsroom. Fact sheets. Cancer [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/cancer [Accessed 20 March 2021].

Yun M, Jung H, Lee H, et al (2013). Synergistic anticancer activity of HS-173, a novel PI3K inhibitor in combination with Sorafenib against pancreatic cancer cells. *Cancer Lett*, **331**, 250-61.