Preliminary Analysis of Anti-proliferative, Apoptotic, and Anti-migratory Effects Ilw-3-6 in Skov-3 Ovarian Cystadenocarcinoma Cell Line

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Abstract: Background: Ovarian cancer and ovarian related diseases affect reproductive health. Therapeutic molecules are needed to improve treatment outcomes and overcome drug resistance. The benzimidazole-based sulphonamide LLW-3-6 has both anti-apoptotic and anti-proliferative effects when used to treat prostate, breast, and brain cancer cells.

Objective: The study described herein evaluates the anti-proliferative and anti-migratory effects of LLW-3-6 in SKOV-3 ovarian cystadenocarcinoma cell line.

Methods: Studies were conducted using SKOV-3 cells treated with LLW-3-6. The cell line was propagated and proliferative activity was evaluated by hemocytometric and MTT colorimetric assays. Cellular apoptosis was assessed using caspase-3 spectrophotometric analysis. Lastly, a scratch wound assay was conducted at several concentrations and time points to assess the effect of LLW-3-6 on migration.

Results & Discussion: Proliferative studies suggest, SKOV-3 cells exposed to LLW-3-6 in culture resulted in decreased growth and proliferation of cells in a time and dose-dependent manner. The apoptotic effect of this agent was noted with the confirmed presence of Caspase-3 in a dose and time-dependent manner as well. Preliminary studies also suggest an anti-migratory effect of LLW-3-6, confirmed by scratch wound analysis.

Conclusion: LLW-3-6 is potentially a chemotherapeutic option for decreasing proliferation and inducing apoptosis in ovarian carcinomas. Additional biological analysis are ongoing to further assess the utility of the molecule and its mechanism of action.

Keywords: Anti-migratory, anti-proliferation, benzimidazole, LLW-3-6, ovarian cancer, SKOV-3.

1. INTRODUCTION

LLW-3-6 is a benzimidazole-based derivative of celecoxib. Initially designed to target PDK-1, the molecule can be synthesized in four steps using microwave synthesis and traditional heating methods [1]. In preliminary studies, LLW-3-6 (Fig. 1) was shown to reduce the growth of PC-3 human prostate cancer cells. The molecule later demonstrated the ability to induce COX-2 localization in PC-3 cells in a manner consistent with a stress response to toxic insult [2]. In the cell line, the molecule was able to inhibit growth by nearly 50% [1, 2]. The molecule differentially impacts human metastatic breast cancer cells (MCF-7 and MDA-231), reducing growth in non-estrogen responsive MDA-231 cell colonies by more than 85% [3]. The most impressive outcome involving LLW-3-6 relates to the ability of the molecule to decrease cell viability in glioma cells by 40% when administered independently and by 69% when given in combination with sulfasalazine [4]. The goal of this study is to further characterize the anti-proliferative nature of LLW-3-6 by examining the anti-migratory effect of the molecule on SKOV-3 cells, an ovarian cystadenocarcinoma cell line.

Fig. (1). LLW-3-6.

Ovarian cancer is the fourth leading gynecologic malignancy and the most lethal. The ovarian cancer coalition
reported that globally, 239,000 new cases of ovarian cancer were diagnosed in 2012, with those diagnosed succumbing to the disease at a rate of 50 to 70% [5]. Despite efforts to improve awareness and treatment options, it is predicted that there will be 22,530 new cases in 2019 in the US alone [6]. This is comparable to the trend in new cases observed between the years 1975 and 2011 [5, 7]. In recent years, incidence and mortality rates have stabilized. However, a significant number of women continue to be negatively impacted by the disease as a result of late-stage diagnosis [7, 8]. Effective drug therapies are essential to improving chemotherapeutic outcomes for these individuals.

The benzimidazole moiety has been exploited as a privileged pharmacophore in the development of innovative bioactive agents for a range of diseases. Researchers attribute the desirability of the benzimidazole to the structural similarities that exist between the molecule and naturally occurring substances [9]. Drugs having the fused aromatic core have been commercialized for their pharmacological utility (Fig. 2) [9]. Among these are structures with a free amine in the benzimidazole nucleus and those with aliphatic substituents at positions 2 and 3 on the heterocycle. Most structures contain substituents that contribute to the pi electron-donating ability of the molecule. LLW-3-6 is similar to these molecules containing both benzimidazole core and pi-electron density, features that were previously shown to contribute to the activity of the molecule.

Beyond commercial success and specific to work reported here, benzimidazoles have been effective in the treatment of ovarian cancer and towards overcoming drug resistance. C. Torres et al. developed a benzimidazole-based derivative that modulated tubulin to produce anticancer activity [10]. Similarly, benzimidazole-oxindole analogs were shown to interact with microtubules and inhibit the cell cycle at the G2/M phase [11]. Analogs developed by Xiang showed favorable growth inhibition of several cancer cell lines, including drug-resistant SKOV-3 ovarian cancer cells [12]. Some benzimidazole derivatives exhibit anti-proliferative and apoptotic effects on cancer cells by disrupting DNA structures within cells [13, 14]. The bis-benzimidazoles ABA13 and ABA833, for example, possess a strong affinity for adenosine thymine sites of duplex DNA. Adding to these in vitro studies, albendazole has shown favorable results in preclinical and clinical studies against a range of cancers [15, 16]. The molecule, when formulated in albumin nanoparticles, inhibited proliferation in paclitaxel resistant ovarian carcinoma.

Because of the known utility of benzimidazole-based molecules, it was of interest to investigate the activity of LLW-3-6, having demonstrated anti-proliferative properties, in SKOV-3 cells. This study provides preliminary insight into the anti-proliferative, anti-apoptotic, and anti-migratory properties of the molecule in an ovarian cancer cell line.

2. MATERIALS AND METHODS

2.1. Chemicals

LLW-3-6 was synthesized at Spelman College [1]. Unless otherwise stated, all other reagents were of analytical grade and were purchased from Sigma Chemical Co.

2.2. Analysis of Cell Morphology

The SKOV-3 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The SKOV-3 cells were cultured in McCoy’s 5A media
supplemented with 10% standard fetal bovine serum and 1% penicillin/streptomycin (both from Thermo Fisher Scientific, Waltham, MA, USA) and were maintained at 37°C with an atmosphere of 5% CO₂ and 95% air. SKOV-3 cells were seeded and grown to the desired confluence. Light microscopic images were obtained at a magnification of 100X and 200X.

2.3. Analysis of Growth Inhibition

Cell proliferation was also determined by a Modified Microculture Tetrazolium (MTT) assay. Cells were plated in a 6-well culture plate (4 x 10^3 cells/well) overnight followed by either the addition of serum-free media or treatment with LLW-3-6 (0.01, 0.1, and 1 nM). After culture for 24, 48, 72 hours, cells were washed twice with Krebs-HEPES buffer and then incubated in 1-mL of Krebs-HEPES buffer (0 mM glucose) with 0.5-mg/ml MTT (4 hours, 37°C). The cellular formazan was extracted with acidic isopropanol, and the absorbance of the converted dye was measured at a wavelength of 570-nm (with background subtraction at 650 nm), using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.4. Analysis of Apoptosis

SKOV-3 cells were seeded at 1 x 10^5 cells/T25 flask to 80% confluence under normal cell culture conditions. The complete medium was replaced with serum-free medium and incubated over a period of 0, 36, 60, 84 hours at 37°C under an atmosphere of 5% CO₂ and 95% air. Cells were cultured for 0, 36, 60, 84 hours in the presence of 1-nM LLW-3-6. After exposure to serum-free media or LLW-3-6, SKOV-3 cells were pelleted by centrifugation at 200 x g for 10 minutes, cells were washed twice with Krebs-HEPES buffer and then incubated in 1-mL of Krebs-HEPES buffer (0 mM glucose) with 0.5-mg/ml MTT (4 hours, 37°C). The cellular formazan was extracted with acidic isopropanol, and the absorbance of the converted dye was measured at a wavelength of 570-nm (with background subtraction at 650 nm), using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

2.5. Analysis of Cell Migration

To standardize 24 well plates, a centrally-located horizontal line was drawn along the bottom of each well. Then, SKOV-3 cells were seeded and allowed to grow for 5 days until 90% confluent. Cells underwent serum starvation for 24 hours to synchronize cells before treatment. After 24 hours, serum-free media was extracted and serum-rich media was added. A 200 µL pipet tip was used to create a wound along the north and south azimuth of each well. Images were obtained at 0 minutes, centering the horizontal line in the field of view. Then, SKOV-3 cells were exposed to 100 µL of LLW-3-6 at 0.01, 0.1, or 1-nM for 0 to 40.5 minutes. The wound area was traced and measured in ImageJ (National Institutes of Health, Bethesda, MD, USA) to determine the percentage of the wound occupied by cells at each time point [17].

2.6. Statistics

All experiments were replicated in triplicates. Data is expressed as Standard Error of the Mean (SEM) of three experiments. Statistical analysis was performed by using Microsoft Excel software, as well as t-test. Differences were considered significant at p < 0.05.

3. RESULTS

3.1. LLW-3-6 Alters the Morphology of SKOV-3 Cells

SKOV-3 cells were seeded in a flask and grown to a confluent monolayer to assess growth patterns and rate of proliferation. The morphologic appearance of these cell lines showed characteristics similar to ovarian cancer cells (i.e., OVCAR-3, OVCAR-8 and TOV112-D cells) Fig. (3A). When cells were exposed to LLW-3-6 for 24, 48, and 72 hours (Fig. 3B-D), both cellular morphologic and density changes were noted. SKOV-3 cells appeared to transition from a stellate appearance to a more spherical configuration, an observation consistent with cell morbidity. Cells appeared to have less cytoplasmic volume with the most apparent changes occurring 48 hours post-exposure. Cells were also less adherent after this time.

3.2. LLW-3-6 Inhibits Proliferation in SKOV-3 Cells

LLW-3-6 demonstrated nanomolar potency towards inhibiting cell viability (Fig. 4). In the presence of all concentrations of LLW-3-6 tested, cell proliferation decreased by at least 40% for all periods. When the concentration of the molecule was increased to 0.1 and 1-nM, there was little difference in the ability of LLW-3-6 to inhibit proliferation in comparison to what was observed at 0.01-nM. Nevertheless, at the largest concentration tested, cell growth was inhibited by 60% after 72 hours.

3.3. LLW-3-6 Induces Apoptosis in SKOV-3 Cells

The ability of the benzimidazole-based molecules to induce apoptosis was measured as a function of the upregulation of caspase-3 (Fig. 5). For cells cultured in 1-nM of LLW-3-6, caspase-3 activity was detected at 36 hours post-exposure. In comparison, the presence of the apoptotic marker increased by 65% after the cells were incubated with the molecule for 60 hours. However, the change between 60 and 84 hours of exposure was less than 5%.

3.4. LLW-3-6 Inhibits SKOV-3 Cell Migration

To determine the effect of LLW-3-6 on the migration of SKOV-3 cells, a scratch wound assay was conducted (Fig. 6). After the wound was created, cells not exposed to the drug continued to proliferate and migrate in a manner that decreased the area of the wound from 0.653 µm² to 0.156 µm².
Upon treatment with LLW-3-6, the wound size was preserved at 0.01-nM of LLW-3-6. When the concentration of LLW-3-6 was increased to 1-nM, the wound size increased by 12% in comparison to the control. In addition, there was a visible increase in cell death in all concentrations tested.

4. DISCUSSION

With a high incidence and mortality, ovarian cancer is the fourth most prevalent type of gynecological malignancy. Understanding how the disease could be modulated with small molecules is a critical step towards improving treatment options and survival rates of those impacted by the disease. Benzimidazole-based molecules have shown promise in treating ovarian cancerous cells [15, 16, 18] and have been used as a privileged scaffold in commercial pharmaceuticals [9]. Prior studies involving breast and
prostate cancer highlight the negative effect of the benzimidazole derivative on cell survival [1-3].

Similar to other benzimidazole derivatives, the data presented here suggest growth and survival of SKOV-3 ovarian cystadenocarcinoma cells is inhibited by LLW-3-6 at all concentrations of the drug tested. The antiproliferative and apoptotic activity of the molecule was complemented by its ability to prevent cell migration. At the highest concentration tested, LLW-3-6 decreased cell growth at the edge of the artificial wound.

Microscopic analysis of cellular morphology reflects an effect on ovarian cancer cells that could lead to abnormal cellular structure and cell death. Studies suggest that shrinkage of cells, fragmentation into membrane-bound apoptotic bodies, and DNA fragmentation lead to such morphologic changes. However, this mechanism has not been investigated for LLW-3-6.

The impact of the benzimidazole-based sulphonamide on apoptosis was also assessed. The molecule was able to upregulate caspase-3, at all times tested. The most significant change in activity was noted after 36 and 60 hours post exposure. However, the apoptotic activity showed little change after 84 hours, which may suggest that non-adherent cells were removed prior to the assessment of caspase-3 activity.

CONCLUSION

Given highly metastatic nature of SKOV-3 cells, it was of interest to characterize the anti-proliferative and potential of LLW-3-6. The molecules prevented the propagation of the SKOV-3 cells in a scratch wound assay. At the highest concentration tested, LLW-3-6 increased the size of the wound. This observation further illustrates the cytotoxic effects of the molecule and suggests that the molecule could potentially be useful at inhibiting metastasis and delaying the development of secondary malignant sites. Additional investigations will be geared towards ascertaining the mechanisms that undergird the biological response of LLW-3-6, with a particular focus on the anti-migratory properties of the molecule. Nevertheless, the current study provides additional insight into the pharmacological utility of LLW-3-6 and adds to the favorable outcomes observed for molecules containing a benzimidazole core. Collectively with data from previous studies, this work provides additional evidence that LLW-3-6 can be useful in the development of therapies that will improve outcomes for those diagnosed with various reproductive cancers.

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.
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