THERAPEUTIC POTENTIAL OF SELENOCYSTINE AND 5-FU IN TUMOR PROGRESSION VIA MODULATION OF THE JAK/STAT AND IMMUNE CHECKPOINT PATHWAYS

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Abstract

Breast cancer continues to pose a substantial worldwide health concern, requiring the investigation of innovative therapeutic approaches. This research examines the anticancer efficacy of Selenocystine and 5-Fluorouracil (5-FU) in human breast cancer MCF-7 cell lines. The MTT assay was utilised to evaluate cell viability after treatment with different concentrations (2–15 μM) of Selenocystine, 5-FU, and their combination. The findings indicated a dose-dependent decline in cell viability, with the combination therapy exhibiting the most significant cytotoxic impact, attaining an IC50 of 5.37 μM, in contrast to 7.02 μM for Selenocystine and 8.01 μM for 5-FU alone. Flow cytometry analysis revealed a substantial rise in the Sub-G1 and G0/G1 cell cycle

populations, along with a notable decrease in the S and G2/M phases, indicating cell cycle arrest and the activation of apoptosis. Additionally, quantitative real-time PCR analysis demonstrated a significant downregulation of Janus Kinase/Signal Transducer and Activator of TranscriptionJ (AK/STAT) gene expression following treatment, with the combination therapy producing the most pronounced drop. The results indicate that Selenocystine augments the lethal effectiveness of 5-FU, offering a viable combinatorial approach for breast cancer therapy by facilitating apoptosis and inhibiting oncogenic signalling pathways. Subsequent research should concentrate on clarifying the fundamental molecular pathways and assessing in vivo efficacy to confirm the therapeutic potential of this combination.

Keywords : Breast cancer, MCF-7 cells, Selenocystine, 5-Fluorouracil, JAK/STAT signaling

Introduction

5-fluorouracil is a chemotherapeutic agent utilised globally for the treatment of metastatic colorectal cancer, either as monotherapy or in conjunction with irinotecan, a topoisomerase I inhibitor [1]. 5-FU is regarded as exclusively active during the S phase of the cell cycle, with little efficacy in G0 or G1 phases. Thymidylate synthase (TS) is the primary enzyme in 5-FU metabolism and is a crucial component of one of the three principal routes of 5-FU anabolism. Similar to other enzymes engaged in the anabolism or catabolism of 5-FU, they are frequently modified, facilitating 5-FU resistance [2]. Moreover, the primary cellular activities (apoptosis, autophagy, respiration, glucose metabolism, and cell cycle) may be modified in CRC cells subjected to 5-FU. Drug transporters are critical components in multidrug resistance (MDR). The development of epithelial-mesenchymal transition (EMT), along with 5-FU-induced epigenetic alterations and microRNA (miR) dysregulations, represents significant pathways of resistance to 5-FU. Treatment of cells with 5-FU is known to induce DNA damage, particularly double-strand and single-strand breaks, during the S phase as a result of the misincorporation of FdUTP into DNA [2, 3]. DNA damage can occur at all phases of the cell cycle in proliferating cells, and the repair mechanisms involved change across these phases. The DNA damage checkpoint pathways in G1, S, and G2 link DNA damage detection to the inhibition of cell cycle advancement, activation of DNA repair mechanisms, preservation of genomic stability, and, when damage is irreparable, the onset of cellular senescence [4].

Selenocystine, a diselenide oxidation product of selenocysteine, was demonstrated to diminish tobacco-derived nitrosamine-induced lung tumor in A/J mice [5] and boost hepatic chemoprotective enzyme activities (including NAD(P)H-quinone oxidoreductase and glutathione S-transferase) in CF-1 mice [6]. Selenocystine was discovered to provide protection against lead-induced toxicity in CHO cells and neurotoxicity in PC-12 cells [7].

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) system is an evolutionarily conserved and essential mechanism necessary for optimal cellular function [8]. Our understanding of this route has primarily been derived from initial research on the immune system. The JAK/STAT pathway connects various extracellular signals to numerous specific cellular responses, such as cell proliferation, motility, survival, apoptosis, inflammation, self-renewal, suppression of antitumor immune response, stress response, and other responses contingent on the

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target tissue [9]. The direct external activators comprise the pro-inflammatory IL-6 cytokine family, including IL-6, IL-11, IL-31, and Oncostatin M (OSM), together with non-IL-6 family cytokines such as IL-8, IL-10, IL-21, IL-32, and IFNγ. STATs may also be activated by diverse receptor tyrosine kinases (RTKs), including the human epidermal growth factor receptor (EGFR/HER/ErbB) and vascular endothelial growth factor receptor, as well as G-protein-coupled receptors, various cytokine receptors such as growth hormone receptors, toll-like receptors, non-RTKs including Src kinases, and other cytoplasmic kinases such as protein kinase C. The JAK/STAT pathway transmits a diverse array of signals from the external cellular environment to provoke the desired cellular response [10].

Immune checkpoint inhibitors (ICIs) have consistently advanced the treatment of malignant tumours since their introduction. Immunocheckpoint inhibitors have progressively supplanted chemotherapy as the primary treatment for malignant tumours; nonetheless, they are helpful in only a subset of patients and remain unsuccessful for the majority. Alterations in the tumour microenvironment prompted by immune checkpoint inhibitors (ICIs) facilitate the rapid proliferation of malignant tumour cells. The specific mode of tumour progression is referred to as the hyper-progressive disease state [11-15].

Considering the aforementioned data, the present study was conducted to check out whether Selenocystine and/or 5-FU can enhance the anticancer effects in MCF-7 cell lines.

Methodology

Cell culture

The MCF-7 breast cancer cells were obtained from HTB-22 (ATCC, USA) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) enriched with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 µg/mL streptomycin and maintained in humidified 5% CO2 air at 37°C.

Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16] has been employed to investigate the impact of Selenocystine and 5-FU on the viability of MCF-7 cells. on summary, 4 × 10^4 cells were cultivated on a 96-well plate and treated for 24 hours with concentrations of 0, 2, 5, 7.5, 10, and 15 µM of Selenocystine, 5-FU, and their combination. Cells treated with saline were utilised as controls. Following a 24-hour exposure period, the cells were treated with MTT in darkness for 3 to 4 hours at 37 °C to yield purple formazan crystals. Optical density (OD) was measured at 570 nm utilising a BioTek ELx800 microplate reader (Thermo Fisher Scientific, Inc., MA, USA). The subsequent equation was employed to determine cell viability:

$$\text{Cytotoxicity } \% = 1 - \frac{Mean\ absorbance\ of\ treated\ cells}{Mean\ absorbance\ of\ control\ clls} \times 100$$

$$\text{Viability } \% = 100 - \text{cytotoxicity}\%$$

The effects for each compound are expressed by IC50 (the concentration required for 50% inhibition of viability).

Cell cycle analysis

Flow cytometry was employed to assess alterations in the cell cycle and to quantify the percentage of cells in each phase of the cycle. To assess the cell cycle perturbations induced by the treatment of MCF-7 cells (2×10^5) to the IC50 concentrations of Selenocystine and 5-FU and their combination for 48 hours, DNA was stained with propidium iodide (PI) utilising the Propidium Iodide Flow Cytometry Kit (ab139418) following the manufacturer's guidelines. Cell cycle profiles were obtained utilising a BD FACSCalibur flow cytometer (Biosciences, USA) [17].

Gene expression assay

Total RNA was extracted from MCF-7 cells (2×10^5) using a High Pure RNA Isolation Kit (Cat. No. 11 828 665 001, Roche Diagnostics GmbH, Germany) following the manufacturer's protocol, after exposure to IC50 doses of SELENOCYSTINE, 5-FU, and their combination for 48 hours. The QuantiTects Reverse Transcription Kit (Qiagen, USA) was utilised to synthesise complementary DNA (cDNA) from the total RNA extracts of each sample for PCR amplification. The primers supplied by Sangon Biotech, Beijing, China were utilised to evaluate the expression of the JAK/STAT via quantitative real-time polymerase chain reaction (qRT-PCR). The relative fold change in gene expression was calculated using the 2-ΔΔCt technique (Ct: cycle threshold) as described by Livak and Schmittgen [18].

Statistical investigation

The data for each group and the percentage of pathological alterations were presented as mean ± standard error (SE). A one-way analysis of variance was utilised for pairwise comparisons, and the intergroup variance was assessed using Duncan's multiple range post hoc test. Variations were deemed significant at $p < 0.05$.

Results

Impact of Selenocystine and 5-FU on breast cancer MCF-7 cells viability

The viability percentage of MCF-7 cells was considerably diminished in a dose-dependent manner when treated for 24 hours with Selenocystine (2–15 µM) and 5-FU, both individually and in combination. The combination at a dose of 15µM resulted in the most significant reduction in MCF-7 cell viability (7%) compared to Selenocystine and 5-FU (Table 1, figure 1). Moreover, combination of Selenocystine and 5-FU produced highest cytotoxic effect on MCF-7 cells with an IC50 of 5.37 µM, followed by 7.02 µM for Selenocystine, and 8.01 µM for 5-FU

Table 1. Effects of Selenocystine and 5-FU on breast cancer MCF-7 cells viability.

Treatment	Concentrations (µM)					
	0	2	5	7.5	10	15
Control	1.00 ± 0.00	99 ± 0.58^{a}	97.33 ±0.33^{a}	97 ± 0.57^{a}	96.33 ± 0.33^{a}	95.33 ± 0.33^{c}
Selenocystine	1.00 ± 0.00	88 ± 0.6^{b}	81 ±1.15^{b}	75 ± 0.58^{b}	45 ± 1.15^{b}	22± 1.15^{c}
5-FU	1.00 ± 0.00	78 ± 1.15^{b}	70 ± 1.2^{c}	62 ± 1.15^{c}	33 ± 1.2^{c}	18 ± 1.2^{a}

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Combination	1.00 ± 0.00	67 ± 1.2^{d}	57 ± 1.15 ^{d}	44 ± 1.2^{d}	28 ± 1.15^{d}	7 ± 1.15^{b}

Values are represented as the mean ± SE (n=3). Mean values for the same parameter carrying different superscripts (a, b, c, d) are significantly different at $p<0.05$.

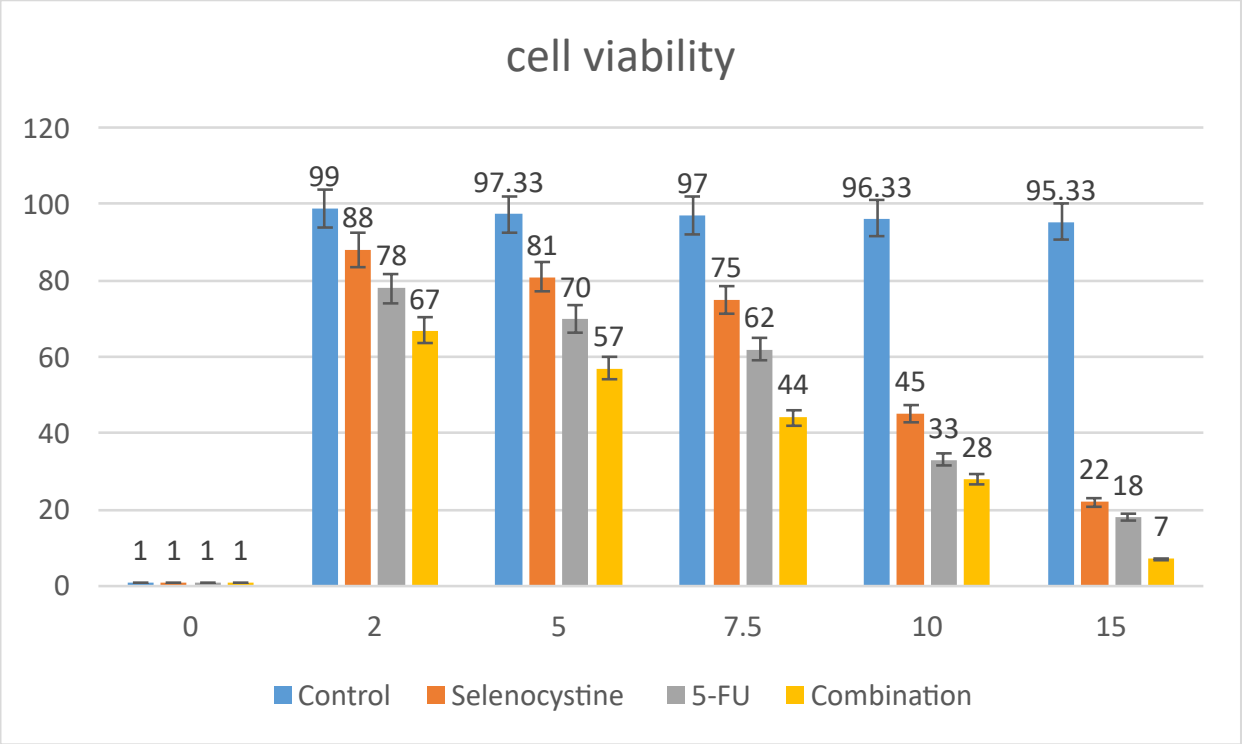

Figure 1. Effects of Selenocystine and 5-FU on breast cancer MCF-7 cells viability.

2. Impact of Selenocystine and 5-FU on cell cycle progression of breast cancer MCF-7 cells

MCF-7 breast cancer cells treated with Selenocystine, 5-FU, and their combination for 48 hours exhibited a significant ($p < 0.05$) rise in the percentage of the Sub-G1 and G0/G1 populations, with a significant ($p < 0.05$) drop in the percentage of the S and G2/M populations compared to control cells. The combination of Selenocystine and 5-FU resulted in a notable increase in the percentage of the Sub-G1 population and a considerable reduction in the G2/M population compared to each treatment administered individually (Figure 2).

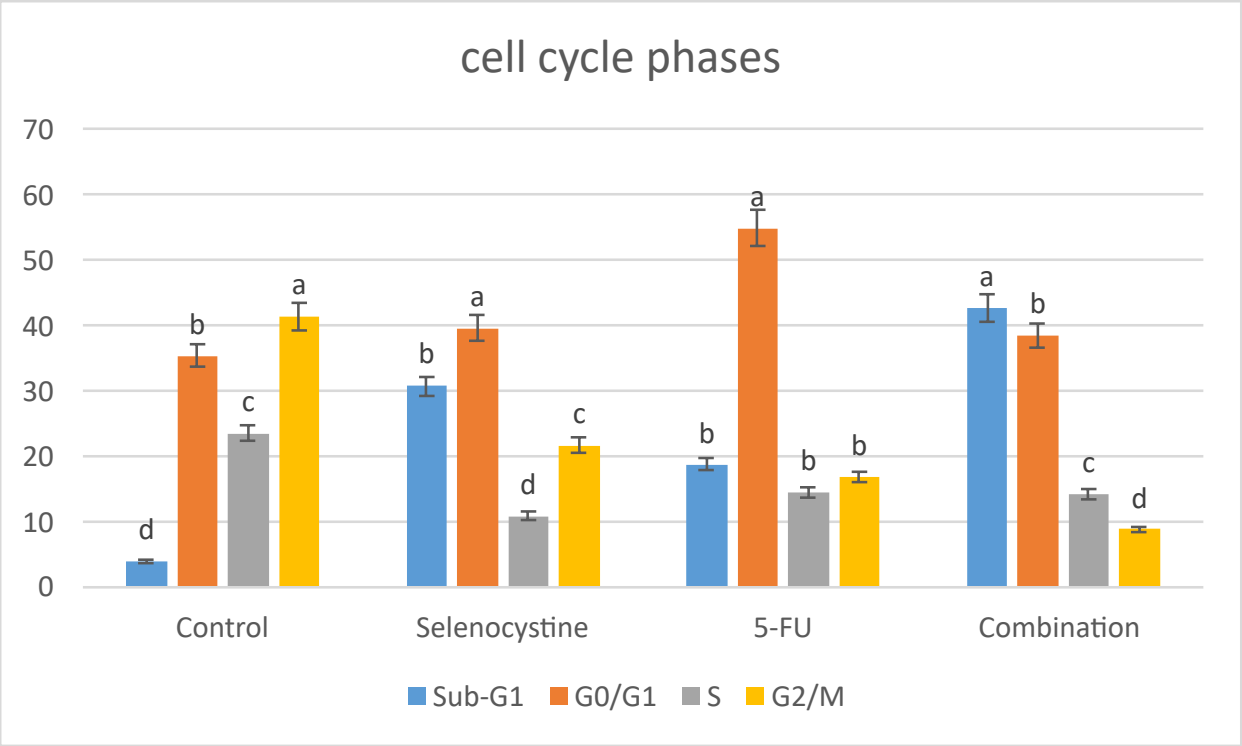

Figure 2. Effects of Selenocystine and 5-FU on cell cycle progression of breast cancer MCF-7 cells. Mean values for the same parameter carrying different superscripts (a, b, c, d) are significantly different at $p<0.05$.

3. Impact of Selenocystine and 5-FU on JAK/STAT gene expression of breast cancer MCF-7 cells

We observed a significant downregulation ($p < 0.05$) in the mRNA expression of the STAT and JAK geneS in MCF-7 cells incubated with Selenocystine and 5-FU when compared with control cells. A combination of Selenocystine and 5-FU significantly decreased JAK/STAT mRNA expression compared with each treatment alone (Figure 3).

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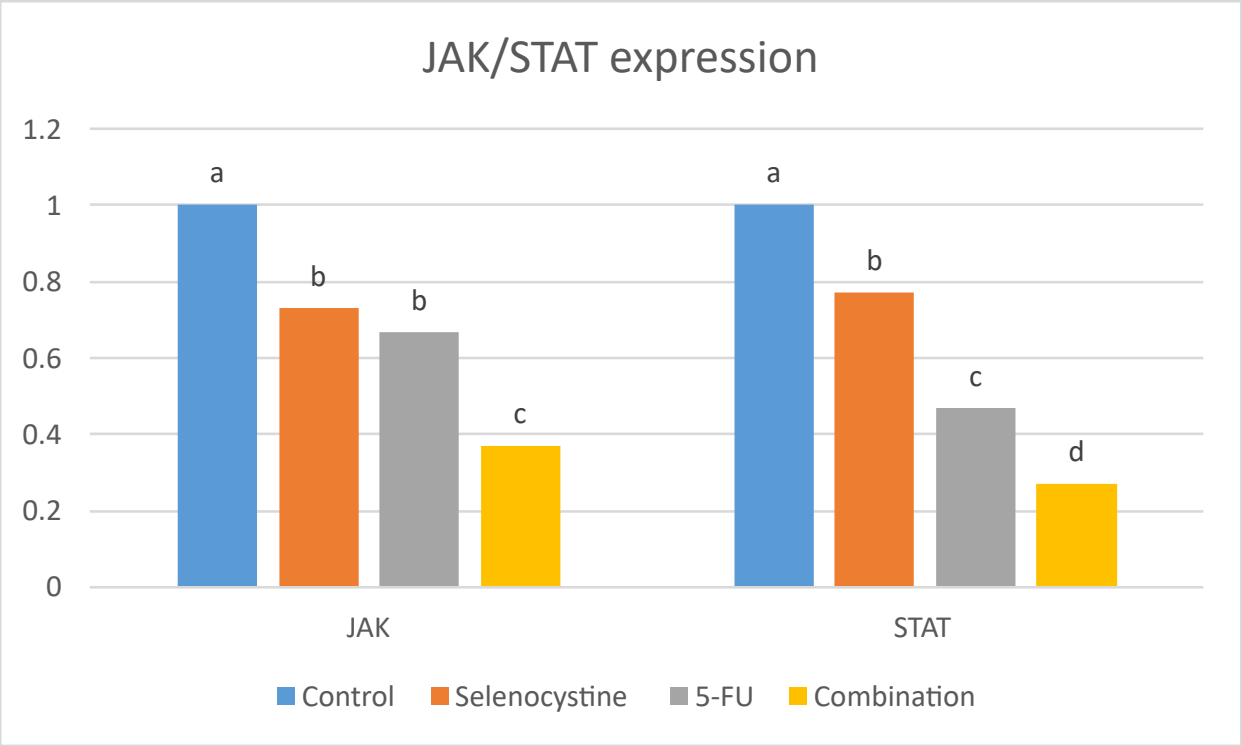

Figure 3. Effects of Selenocystine and 5-FU on JAK/STAT gene expression of breast cancer MCF-7 cells. Mean values for the same parameter carrying different superscripts (a, b, c, d) are significantly different at $p<0.05$.

Discussion

The study estimated the anticancer potential of Selenocystine and 5-FU independently and together against MCF-7 human breast cancer cell lines. The findings reveal significant cytotoxic activity, cell cycle perturbations, and inactivation of the JAK/STAT pathway, particularly when both compounds were used in combination. It may thus be reasoned that Selenocystine could act as an enhancer for 5-FU and open a new avenue for combinatorial therapy against breast cancer.

1. Enhanced Cytotoxic Effects and Reduction of Cell Viability

The MTT assay showed a dose-dependent decrease in MCF-7 cell viability by either Selenocystine or 5-FU treatments, with the combination treatment showing the most significant reduction. The combination treatment had the lowest IC50 (5.37 µM) as compared with 7.02 µM for Selenocystine and 8.01 µM for 5-FU. It strongly implies some kind of synergism between Selenocystine and 5-FU.

The increased efficacy may result probably due to divergent modes of action. Since Selenocystine acts as a redox modulator, cancer cells can be further sensitized towards chemotherapeutic agents by increasing oxidative stress. [19].

2. Induction of the Cell Cycle Arrest and Apoptosis

Flow cytometric assays showed an increase in the Sub G1 phase with a simultaneous accumulation of cells in G0/G1 or with a reduction in S and G2/M phases. [20][21]These observations point

toward the agents, together or singly, to interrupt normal cell cycle progression and lead to apoptosis.[22]

Such G0/G1 arrest may indicate the treatments' interference with early cell cycle checkpoints possibly through the inhibition of cyclins or cyclin-dependent kinases or by activating checkpoint proteins such as p21 or p27. [23]The increased population in Sub-G1 is indicative of DNA fragmentation characteristic of apoptosis, therefore suggesting that the combination therapy activates apoptotic pathways.[24]

Furthermore, the synergistic effect could result from their differential mechanisms of action since 5-FU acts on S-phase cells by incorporation of its metabolites into RNA and DNA, [25]whereas Selenocystine may exert cytotoxic actions against a wider spectrum of cell cycle phases by generating oxidative stress, modulating signal transduction, and possibly blocking mitosis.[26]

3. Downregulation of JAK/STAT Gene Expression

Quantitative real-time PCR results revealed that both agents, and more so their combination, led to a significant downregulation of JAK and STAT gene expression. This finding has considerable implications given the central role of the JAK/STAT pathway in tumor progression, including proliferation, anti-apoptosis, immune evasion, and inflammation.[27]

The JAK/STAT pathway is often constitutively activated in many cancers, including breast cancer, contributing to uncontrolled cell growth and resistance to apoptosis. By suppressing this pathway, Selenocystine and 5-FU may impair oncogenic signaling, promote pro-apoptotic gene expression, and sensitize cancer cells to immune system recognition. Importantly, the combination treatment led to the most profound suppression, suggesting a mechanistic basis for the synergistic cytotoxicity observed.[28]

Additionally, given the interplay between JAK/STAT signaling and immune checkpoint regulation, the suppression of this pathway could also imply enhanced anti-tumor immune responses. While this study focused on in vitro MCF-7 cells, future studies in vivo might reveal whether this combination also modulates immune checkpoint proteins such as PD-L1 or CTLA-4.

4. Clinical Relevance and Therapeutic Implications

The data provide strong preclinical evidence supporting the therapeutic potential of combining Selenocystine and 5-FU in breast cancer treatment. Selenocystine’s capacity to enhance the cytotoxicity of 5-FU and concurrently modulate critical cancer-related signaling pathways such as JAK/STAT supports its role as a chemosensitizer. [29][30]Given that 5-FU resistance remains a significant clinical challenge, especially due to alterations in DNA repair, drug metabolism, or cell cycle regulation, the combination with Selenocystine might offer a way to overcome resistance and improve patient outcomes.[31]

Additionally, the downregulation of JAK/STAT suggests that this combination may have applications beyond cytotoxicity, potentially affecting tumor immune evasion and inflammatory signaling. It opens avenues for combining this therapy with immune checkpoint inhibitors, potentially amplifying the therapeutic benefit.[32]

5. Limitations and Future Directions

While the results are promising, this study is limited to in vitro findings. Future investigations should:

Evaluate the combination in in vivo models of breast cancer to confirm the observed effects and assess toxicity.

Explore molecular mechanisms downstream of JAK/STAT, such as changes in pro- and anti-apoptotic gene expression (e.g., Bcl-2, Bax).

Assess the role of oxidative stress, mitochondrial dysfunction, and autophagy in the observed effects of Selenocystine.

Investigate potential synergy with immune checkpoint blockade, considering the link between JAK/STAT and PD-L1 expression.

Conclusion

The present in vitro investigation revealed that the combination of Selenocystine and 5-FU enhances anticancer efficacy in MCF-7 cells by augmenting cytotoxic activity, inducing cell cycle arrest, and promoting apoptosis. This study elucidates techniques to enhance the anticancer efficacy of CAP while mitigating its cardiotoxic effects. Additional preclinical and clinical investigations are necessary to assess the efficacy of combination treatments.

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