

Article

Cytotoxic and Cytostatic Effects of Nanoformulated Fenretinide on MG63 Osteosarcoma Cells

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Abstract

Background: Osteosarcoma is the most common primary malignant bone tumor in children and adolescents. At present, multi-agent chemotherapy and surgery provide only limited effects and the prognosis for patients with recurrent or metastatic disease remains poor, with 5-year survival rates below 30%. These challenges highlight the need for innovative therapeutic approaches targeting osteosarcoma more effectively. Fenretinide, a synthetic derivative of all-trans retinoic acid, has shown significant antitumor activity in various cancers. In a recent high-throughput drug screening study, fenretinide emerged as the most active molecule against diffuse midline glioma over more than 3500 compounds. Fenretinide also demonstrated cytotoxic activity against osteosarcoma cell lines in vitro and in preclinical models and is endowed with a favorable safety and toxicity profile. However, its poor water solubility and limited bioavailability have hindered its clinical translation. To improve fenretinide bioavailability and enhance tumor exposure, different nanotechnology-based drug delivery systems have been proposed. Here we propose a tertiary complex made of fenretinide, bovine serum albumin, and hydroxypropyl- β -cyclodextrin, indicated as BSAF. **Methods:** BSAF was evaluated for the main physico-chemical parameters such as hydrodynamic size, zeta potential, stability to drug leakage, and the biological effect on the osteosarcoma cell line MG63. **Results:** BSAF showed hydrodynamic size at the nanoscale, enhanced drug solubilization, high drug loading and size stability to dilution, characteristics that make this complex useful for targeted therapy. When tested on the MG63 osteosarcoma cell line, BSAF demonstrated significantly enhanced cytotoxicity, with half-maximal inhibitory concentration (IC₅₀) values ~50% lower than free fenretinide. The complex was more efficient than free fenretinide in inhibiting cell migration as demonstrated by wound healing assay. Live-cell imaging analyses revealed a cytostatic effect at sub-cytotoxic concentrations. Specifically, treatment with concentrations below the IC₅₀ resulted in significantly prolonged cell doubling time, decreased cell divisions, increased cellular sphericity and thickness, and decreased cell area. These morphological changes are more consistent with cell cycle arrest rather than apoptosis. These findings were corroborated by stable dry mass measurements, an indication of a cytostatic state rather than progressive cell death. In addition, cell motility parameters (e.g., instantaneous velocity, track speed, and displacement) at the single-cell and population level were markedly reduced at sub-IC₅₀ concentrations, further supporting a cytostatic phenotype. **Conclusions:** Collectively, the new BSAF complex showed promise as a potential therapeutic agent for treating osteosarcoma cancer, due to the favorable physico-chemical characteristics



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and the cytotoxic/cytostatic effects on MG63 cells. BSAF effects may be therapeutically valuable, particularly in preventing tumor recurrence by suppressing the proliferative and migratory potential of residual drug-resistant clones. Unlike conventional anticancer agents that mainly rely on cell death, fenretinide, when complexed, demonstrates a dual capacity to induce both cytotoxic and cytostatic responses, depending on concentrations, potentially overcoming multiple resistance mechanisms that are generally associated with tumor exposure to drug sub-cytotoxic concentrations.

Keywords: N-(4-hydroxyphenyl) retinamide; bovine serum albumin; hydroxypropyl-beta-cyclodextrin; tertiary complex; enhanced drug solubility; instantaneous velocity; track speed; displacement

1. Introduction

Osteosarcoma is the most common primary malignant bone tumor in children and adolescents, characterized by rapid local invasion and a high rate of pulmonary metastasis [1]. While standard treatment regimens combining surgical resection with multi-agent chemotherapy have improved outcomes, the prognosis for patients with recurrent or metastatic disease remains poor, with 5-year survival rates below 30% [2–6]. These challenges highlight the need for innovative therapeutic approaches to treat osteosarcoma more effectively.

N-(4-hydroxyphenyl) retinamide or fenretinide (Fen), is a synthetic derivative of all-trans retinoic acid that demonstrated significant antitumor activity in various cancers [7] including neuroblastoma [8–11], breast cancer [12], colon cancer, lung cancer, melanoma [13–15] and leukemia [16]. In a recent high-throughput drug screening on more than 3500 compounds, Fen emerged as the most active against diffuse midline glioma [17]: Fen demonstrated a potent cytotoxic activity and the ability to cross the blood–brain barrier, improving survival in animal models bearing diffuse midline glioma tumors.

The antitumor activity of Fen mainly relies on the generation of reactive oxygen species (ROS) leading to oxidative stress, disruption of mitochondrial function, accumulation of dihydroceramides due to inhibition of dihydroceramide desaturase (DES1), and modulation of stress-related signaling pathways in a p53-independent manner [18]. The simultaneity of all these mechanisms generates apoptosis in cancer cells in a way that differs from conventional chemotherapeutics and explains the lack of drug resistance. In addition, Fen is endowed with a favorable safety and toxicity profile, as proved in several phase I and II clinical trials [19–28] and in long-term administration in breast cancer chemoprevention trials [29].

However, in spite of its favorable pharmacological characteristics, Fen has not reached clinics yet since its poor water solubility strongly limits its bioavailability. To address these limitations, nanotechnology-based drug delivery systems, such as lipid [8–17] or polymer nanoparticles [30–34], have emerged as promising tools to encapsulate Fen, with the aim of improving bioavailability and tumor exposure by the enhanced permeability and retention (EPR) effect [35].

Here we propose an alternative nanoformulation based on Fen complexed with bovine serum albumin (BSA) and hydroxypropyl-beta-cyclodextrin (HPBCD). The antitumor effect of this complex (BSAF) was studied on the MG63 osteosarcoma cell line since very few studies have been conducted on the activity of Fen in osteosarcoma, despite the fact that, among different tumor types, osteosarcoma may be particularly susceptible to the activity of Fen due to its unique metabolic characteristics and its intrinsic vulnerability to oxidative

stress [2,4]. Indeed, osteosarcoma cells typically exhibit high basal levels of ROS due to their rapid proliferation, high metabolic activity, and mitochondrial dysfunction. The capacity of Fen to increase intracellular reactive oxygen species (ROS) levels is expected to elevate the overall oxidative burden in osteosarcoma cells beyond the threshold of cellular tolerance, thereby triggering cell death.

Furthermore, osteosarcoma cells have reduced expression or activity of antioxidant enzymes, such as glutathione peroxidase, catalase, and superoxide dismutase [36]. This impairs the cell's ability to neutralize the ROS induced by Fen, leading to accumulation of oxidative damage, DNA strand breaks, and cell death. In addition, osteosarcoma cells may have a dysregulated sphingolipid metabolism, improving their sensitivity to the accumulation of dihydroceramides induced by Fen, with the consequent induction of stress and apoptosis [37,38].

2. Materials and Methods

2.1. Chemicals

Fen was purchased from Olon Spa (Milan, Italy); BSA (heat-deactivated), HPBCD, potassium hydroxide (KOH) were from Sigma-Aldrich (Milan, Italy), while ethanol absolute anhydrous was purchased from Carlo Erba Reagents (Milan, Italy). Dulbecco's Minimal Essential Medium High Glucose (DMEM), glutamine, trypsin/EDTA solutions and heat-deactivated Fetal Bovine Serum (FBS) were from Gibco (Thermo Fisher, New York, NY, USA).

2.2. Preparation of BSAF

BSAF resulted from supramolecular assembly of Fen, BSA and HPBCD mediated by hydrophobic interactions in water of their hydrophobic portions. BSAF was prepared by dissolving Fen (0.1 mmoles) in ethanol (150 μ L) and KOH 10 N (10 μ L, 0.1 mmoles) and subsequently mixing with 1.5 mL of an aqueous solution of BSA (100 mg/mL) and HPBCD (0.1 mmoles) (Figure 1).

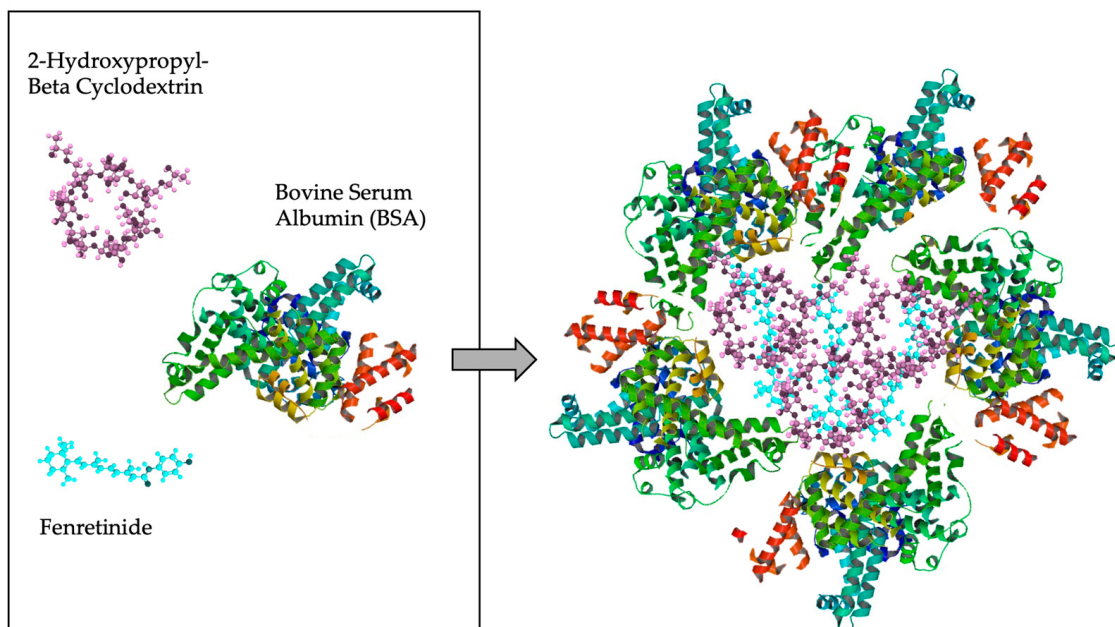


Figure 1. Components of the tertiary complex and schematic representation of a hypothetical arrangement of the tertiary complex. The presented model is intended solely for illustrative purposes and is not derived from or validated by structural data.

The resulting mixture was stirred at RT in the dark for 3 h to allow complexation and filtered through 0.2 µm filters. The filtrate was lyophilized, and the dry residue was reconstituted with water to obtain 10 mg/mL of the tertiary complex BSAF. This solution was stored at −22 °C until use. A dispersion of BSA and HPBCD was prepared employing the same procedure and used as drug-free control (BSA₀).

2.3. Characterization of BSAF

The loading and encapsulation efficiency of Fen within the nanosystem were determined as previously reported [13–16]. Briefly, the dispersions (10 mg/mL) were diluted at a 1:3 (*v/v*) ratio with an ethanol–water mixture (1:1, *v/v*). The Fen content was then quantified by UV-visible spectroscopy (Shimadzu UV-1601, Kyoto, Japan) at a wavelength of 360 nm, using the corresponding vehicle as a reference. Drug loading was calculated as the percentage by weight of Fen incorporated into the nanosystem, according to the following equation:

$$\text{Drug Loading } \left(\% \frac{w}{w} \right) = \left[\frac{\text{weight of Fen in 10 mg BSAF}}{10 \text{ mg BSAF}} \right] 100$$

Encapsulation efficiency was calculated as the percentage by weight of Fen incorporated into the nanosystems relative to the amount initially used in the formulation, according to the following equation:

$$\text{Encapsulation Efficiency } \left(\% \frac{w}{w} \right) = \left[\frac{\text{weight of Fen in 10 mg BSAF}}{\text{weight of Fen added to 10 mg BSAF}} \right] 100$$

Particle size was determined by using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA) and NanoSight Pro (Malvern Instruments and Malvern Panalytical, Malvern, UK) while zeta potential was assessed by using a Nicomp™ 380 ZLS instrument (Entegris/Particle Sizing Systems, Santa Barbara, CA, USA). Measurements were performed at 37 °C and Fen concentrations ranging from 100 to 6 mM in water. At least 12 measurements were collected for each sample, with data acquired from 3–10 min runs to achieve a total correlation function accumulation time of 30 min. Results were reported as volume-weighted distributions.

The stability of the nanosystem against drug leakage was assessed at 72 h by quantifying Fen leakage from BSAF [13–16]. Briefly, 1 mL of the complex solution (10 mg/mL) was placed in a release chamber separated from a receiving compartment by a dialysis membrane (molecular weight cutoff 5 kDa; Fisher Scientific, Milan, Italy). The receiving compartment contained 10 mL of water. The system was maintained at 37 °C, and sink conditions were ensured throughout the experiment.

Although water does not replicate physiological conditions, it was intentionally selected to maximize the concentration gradient across the diffusion cell, given the very low aqueous solubility of Fen. This approach represents a stringent experimental condition.

Therefore, if dissociation occurs in water, it can reasonably be expected to occur under physiologically relevant conditions as well.

Fen leakage was evaluated after 72 h by spectrophotometric analysis of the receiving phase at the drug's maximum absorption wavelength (360 nm), using the following equation:

$$\text{Drug Leakage at 72 h } \left(\% \frac{w}{w} \right) = \left[\frac{\text{amount of Fen in the receiving phase}}{\text{amount of Fen loaded in 10 mg BSAF}} \right] 100$$

The solubilization of Fen by BSAF, reflecting the solubility of the complexed Fen, was assessed by dissolving increasing amounts of BSAF in water at 37 °C under contin-

uous stirring. Fen solubility was monitored spectrophotometrically (Shimadzu UV-1601, Kyoto, Japan) by measuring the absorbance at its maximum absorption wavelength (360 nm). The Fen concentration increased with BSAF content until reaching a plateau, which corresponded to the solubility of Fen in its BSAF-complexed form.

2.4. Cell Lines

MG63 cells were provided by ATCC (Manassas, VA, USA). For the present study, the cells were grown in DMEM supplied with 10% FBS, penicillin (100 UI/mL), and streptomycin (0.1 mg/mL) at 37 °C in a 5% CO₂-humidified atmosphere. They were maintained in 25 cm² culture flasks (Corning, Tewksbury, MA, USA) and harvested using 0.25% Trypsin in 0.2 g/L EDTA solution in isotonic NaCl.

2.5. Alamar Blue Assay and IC₅₀ Calculation

The effect of BSAF on cell viability was evaluated by Alamar Blue assay that is based on the ability of metabolically active cells to reduce resazurin, a non-fluorescent blue dye, to resorufin, a pink fluorescent compound. This reduction occurs in the mitochondria of viable cells and serves as an indirect measure of cellular metabolic activity, which correlates with the number of living cells. The fluorescence of resorufin is proportional to cell viability and can be quantitatively measured using a plate reader.

Treatments were performed at increasing Fen concentrations, and the corresponding concentration of BSAF matched the equivalent Fen concentrations in the free drug. BSA₀ was also tested at the same concentrations used for BSAF.

To perform the assay, the cells were seeded at 1×10^4 cells/cm² in 96-well plates, and, after 24 h, they were treated with Fen or BSAF for 24, 48 or 72 h at concentrations of Fen ranging between 0.03 and 60 µM. Afterwards, 30 µL of a 600 µM resazurin solution was added to each well to reach a final concentration of 100 µM. After 4 h of incubation at 37 °C in the dark, the wells were analyzed by a microplate reader (Ensign Multimode Plate Reader, PerkinElmer Scientifica Italia, Milan, Italy), measuring fluorescence (excitation 560 nm, emission 590 nm). The data were normalized to untreated controls and expressed as a percentage of cell viability.

The half-maximal inhibitory concentration (IC₅₀) was determined using the log (inhibitory) vs. response–variable slope (four parameters) function of GraphPad Prism software, version 6.0c (GraphPad Software, San Diego, CA, USA). All the experiments were performed in a replicate of twelve.

2.6. Scratch Wound 96-Well Cell Migration Assay

MG63 cells were seeded at 2×10^4 cell/cm² and grown to confluence for 48 h in a 96-well Imagelock plate (Sartorius Italia, Varedo, Italy). The wounds were created by using the 96-pin Incucyte[®] Woundmaker (Sartorius, Italy) and the wells were washed three times with DPBS and then treated. Six replicates for each treatment were performed. BSAF and Fen were added with the concentration of Fen ranging between 5 and 40 µM. The plate was placed inside the Incucyte, allowed to equilibrate for at least 15 min before the first scan, and the software was set to scan the plate every 4 h for 48 h, using a 10× phase contrast objective. To analyze the acquired images, 4 representative images at different times were used to evaluate and refine the software analysis.

2.7. Quantitative Phase Imaging Microscopy

Quantitative Phase Imaging (QPI) experiments were performed by seeding cells in 96-well plates (Corning, Tewksbury, MA, USA) at a density of 1×10^4 cells/cm². After a 24 h incubation period, cells were treated with BSAF at the same concentrations used in the Alamar Blue assays. Time-lapse imaging was conducted using a Liveocyte[®] system

(Phasefocus, Sheffield, UK) equipped with a $10\times$ objective (NA 0.25). Images were acquired every hour over a 72 h period under standard culture conditions ($37\text{ }^{\circ}\text{C}$, 5% CO_2).

Data analysis was performed using the Liveocyte[®] software (version 2) to quantify BSAF-induced changes in cell proliferation, spreading, and morphology at both single-cell and population levels. The Liveocyte[®] system is based on ptychographic QPI, a label-free imaging technique that exploits light diffraction principles [39–41]. Briefly, the sample is sequentially illuminated with overlapping light spots, and the resulting diffraction patterns are captured by an sCMOS camera acting as a virtual lens. These data are computationally reconstructed to generate high-resolution phase images, eliminating the need for fluorescent labels or conventional imaging lenses that may introduce optical aberrations. Importantly, this technique enables the direct measurement of the cellular dry mass at the single-cell level.

2.8. Cell Cycle Evaluation

To analyze the effects of BSAF on cell cycle progression, MG63 cells were seeded at a density of 1×10^4 cells/cm². After a 24 h incubation period, cells were treated with BSAF at the IC₅₀ concentration. At 24, 48, and 72 h post-treatment, cells were detached, counted, and collected by centrifugation at $250\times g$ for 10 min.

DNA content was assessed by flow cytometric analysis of isolated MG63 nuclei following the method described by Nüsse and Marx [42], with minor modifications. Briefly, cell pellets were stained with propidium iodide (PI) at a final concentration of 50 µg/mL. Stained samples were incubated at $4\text{ }^{\circ}\text{C}$ in the dark and were analyzed by using a Bryte HS flow cytometer (Bio-Rad, Hercules, CA, USA) equipped with a Xe/Hg lamp and a filter set to obtain an excitation at 488 nm. PI fluorescence was collected on a linear scale at 600 nm, and the DNA distribution was analyzed by ModFit software (version LT 6.0, Verity Software House, Topsham, ME, USA).

2.9. Reactive Oxygen Species Production Assay

Intracellular ROS levels were assessed in intact cells using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFDA). This non-fluorescent compound is converted into the fluorescent molecule dichlorofluorescein (DCF) through the combined action of intracellular esterases and ROS, and the fluorescent product is retained within the cells.

MG63 cells were seeded at a density of 1×10^4 cells/cm² and allowed to adhere for 24 h. Cells were then treated with BSAF at the IC₅₀ concentration for 3 or 18 h. Following treatment, cells were detached and washed twice by centrifugation at $250\times g$ for 10 min in Hanks' balanced salt solution (HBSS). Cells were counted and resuspended at a concentration of 5×10^5 cells/mL. DCFDA was added to a final concentration of 2 µM, and samples were incubated for 1 h at $37\text{ }^{\circ}\text{C}$ in the dark. Negative controls consisted of unstained cells to assess sample autofluorescence.

After incubation, cells were washed and resuspended in HBSS containing propidium iodide (PI; 5 µg/mL) to counterstain non-viable cells, and kept on ice until analysis. Flow cytometric analysis was performed using a Bio-Rad S3 cell sorter, acquiring DCF fluorescence in the green channel (525 nm) and PI fluorescence in the red channel (600 nm), both on a logarithmic scale. The green DCF fluorescence of viable cells was quantified using FCSalyzer software (version 0.9.22-alpha).

2.10. Statistical Analysis

Data were derived from at least three independent experiments. Statistical analyses were performed using GraphPad Prism software (version 10.6.1; GraphPad Software, San

Diego, CA, USA). Group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test, with statistical significance set at $p < 0.05$.

3. Results

3.1. BSAF Preparation and Characterization

Complexation of Fen with BSA and HPBCD increased the drug solubilization in the aqueous phase. The physico-chemical parameters revealed that BSAF was endowed with high drug loading and encapsulation efficiency (Table 1). BSAF exceeded 16% (w/w) drug loading, demonstrating that the complexing molecules can effectively and efficiently embed Fen. The encapsulation efficiency being higher than 98% (w/w) indicates that the preparative process is thermodynamically favorable and proceeds efficiently without the need for an excess of Fen.

Table 1. Physico-chemical characteristics of BSAF complex.

Formulation	Drug Loading (% w/w)	Encapsulation Efficiency (% w/w)	Drug Leakage at 72 h (%)	Solubility of Fen as BSAF mg/mL
BSAF	16.4 ± 0.5	98.5 ± 1.4	7.3 ± 0.8	25.9 ± 1.3

Solubilization of Fen in water as BSAF was ~26 g/L. Fen, on the contrary, is classified as an insoluble molecule, its solubility in water being equal to 1.7 mg/L. Therefore, BSAF increased the drug solubility by about 15,300 times. This strong improvement is due to the high Fen loading in BSAF and the high aqueous solubility of both complexing agents, HPBCD and BSA.

Drug leakage was below 10% (w/w) at 72 h, indicating a good retention ability of the complex in an aqueous environment that is an essential feature in antitumor therapy to avoid drug release in circulation and maximize on-target release at the tumor site.

The mean size of BSAF (Figure 2A) ranged from 60 to about 100 nm in the whole concentration range examined (6–100 μ M). This indicated dimensional stability to possible concentration changes that could occur in vivo following administration. The concentration changes may be due to dilution in circulating blood or accumulation in permeable compartments due to extravasation through discontinuous capillaries in solid tumors. Size distribution is reported in the Supplementary Materials (Figure S1).

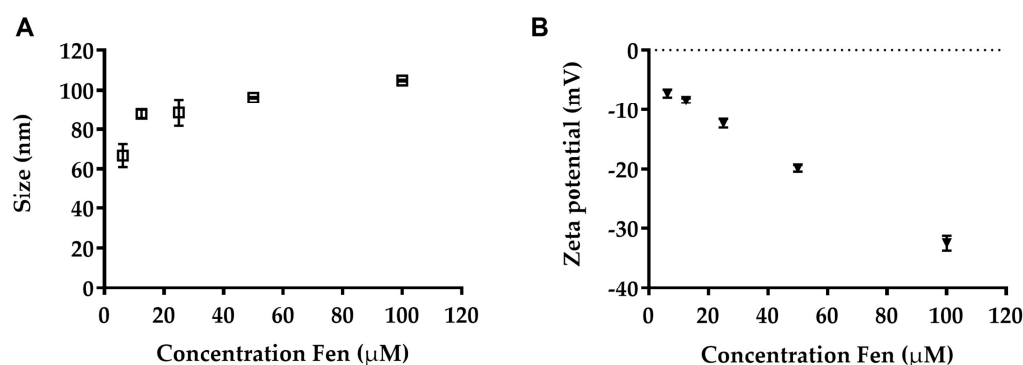


Figure 2. Mean size (A) and zeta potential (B) of BSAF as a function of concentration. $n = 12$.

BSAF zeta potential was negative at all the concentrations analyzed with values decreasing with an increase in BSAF concentration (Figure 2B). This is in accordance with the BSA isoelectric point, which is around five, generating a negative charge in water, with values becoming more negative with increasing BSA concentration.

Having a size under 100 nm makes BSAF particularly suitable for tumor-targeted therapy. Indeed, the extravasation of drug-carrying nanosystems through the discontinuities of tumor capillaries, which generates selective drug accumulation at the pathological site, has been demonstrated to be highly improved by decreasing the dimensions below 100 nm [43,44].

3.2. Effect of BSAF on MG63 Cell Viability

Treatment with BSAF and free Fen induced a concentration-dependent decrease in the cell viability of MG63 (Figure 3). BSA₀ did not show any activity in the concentrations analyzed (data reported in the Supplementary Materials, Figure S2).

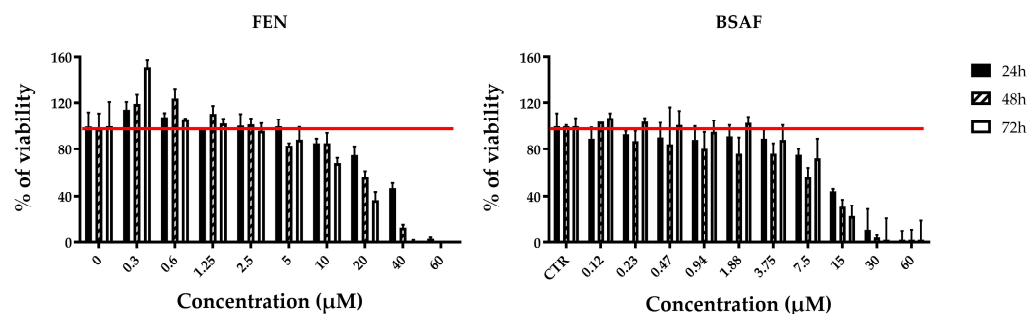


Figure 3. Viability of MG63 cells treated with increasing concentrations of Fen and BSAF. The red line is set at 100% viability. $n = 3$.

The IC_{50} values, reported in Table 2, indicated that BSAF was more active than Fen, the IC_{50} values of BSAF being lower than Fen at all the time points analyzed.

Table 2. IC_{50} of BSAF and Fen after treatment of MG63 for different time periods. The confidence intervals are reported in square brackets.

Time	BSAF IC_{50} and [Concentration Range] in μM of Fen	Fen IC_{50} and [Concentration Range] in μM of Fen
24 h	12.47 [10.19–15.26]	31.54 [27.18–36.59]
48 h	6.87 [5.00–9.43]	20.73 [17.29–24.86]
72 h	10.00 [8.56–11.68]	14.15 [10.37–19.30]

These results are in agreement with the high solubilization of Fen in the BSAF complex, which enhances drug availability to the cells. On the contrary, the lower availability of the free Fen towards the cells can be attributed to its intrinsic water insolubility, which promotes a tendency of the molecules to aggregate, hampering its diffusion through the cell membranes. The aggregation tendency is in part counterbalanced by the presence of ethanol, DMSO or other organic solvents, that are routinely used as solvents for insoluble drugs and mixed with the culture medium. However, when the organic solvent in the culture medium becomes progressively diluted, as occurs at the lowest concentration tested, the aggregation tendency is improved and the partition towards the cell membranes is further decreased.

3.3. Time-Lapse Microscopy

The evaluation of the cellular response to the treatments by resazurin is mainly based on mitochondrial activity and cell metabolic status. The results are averaged over the entire cell population and may therefore not discriminate among different metabolic effects. Furthermore, resazurin tests are performed at discrete time intervals with a loss of the

temporal dynamics of phenomena such as cell death and morphological alterations, which can lead to progressive cellular deterioration and unexpected outcomes.

Time-lapse microscopy, which uses microscopes equipped with incubators, can provide more accurate information. In this study we utilized two different time-lapse microscopy assays: the wound healing assay performed in phase contrast microscopy by Incucyte® (Figure 4) and the ptychograph microscopy by Livecyte® (Figure 5). Treatments were performed at the same concentrations as with resazurin assays.

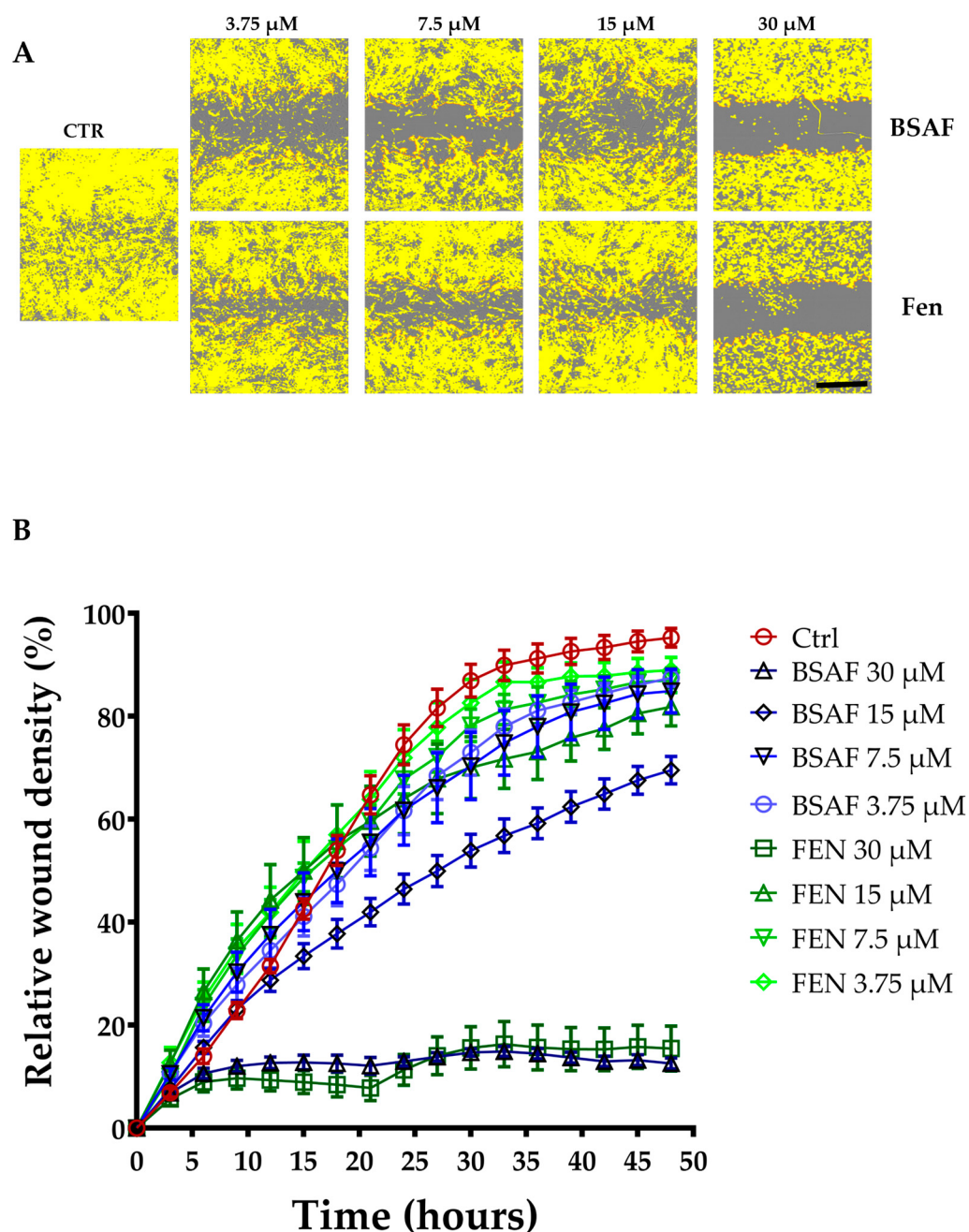


Figure 4. Representative images of the wound healing assay at 40 h in MG63 cells (A) and time course of wound closure (B). Photographs were taken at 10× magnification, scale bar 400 μm.

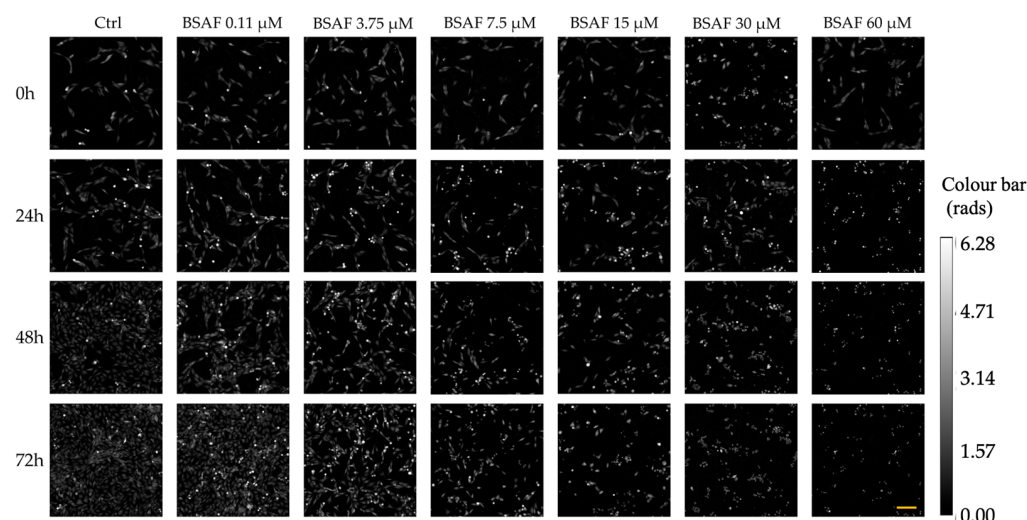


Figure 5. Representative images at different times of MG63 cells treated with BSAF at different concentrations. Photographs were taken at $10\times$ magnification, bar = 200 μ m. The color bar, expressed in radians, represents the intensity of the phase signals and is shown on the right.

3.3.1. Wound Healing Assay

Wound healing assay is a known means to evaluate the cell migration ability that is correlated to the metastatic potential of cancer cells. To compare the effects of BSAF to pure Fen, we performed the wound healing assay to evaluate the influence of nanoencapsulation on the antimetastatic effect of the drug (Figure 4). In particular, we reported in Figure 4A the images obtained after 40 h treatment and in Figure 4B the time course of wound closure. From the data, it is evident that BSAF induces a slower migration than Fen at all tested concentrations (Figure 4B). It is important to notice that the ability of antitumor drugs to hinder migration of the tumor cells that eventually survive their cytotoxic effect may increase the overall response to therapy [39].

3.3.2. Quantitative Phase Imaging

To better characterize the cellular response to BSAF treatment, QPI time-lapse microscopy was employed. This is a label-free imaging technique that measures the phase shift of light as it passes through a transparent biological sample. This phase shift arises from the reduced propagation speed of light in cellular material, which has a higher refractive index than the surrounding aqueous medium [40,41]. Importantly, the measured phase shift is directly proportional to the cellular dry mass, enabling the quantitative assessment of key biophysical properties. This capability is fundamental to the application of QPI in cell biology [45,46].

In this study, imaging was performed using a Liveocyte[®] microscope (Phasefocus, Sheffield, UK), which employs ptychographic QPI to generate high-contrast, label-free images suitable for long-term live-cell analysis. This approach allows the longitudinal quantification of multiple cellular parameters, including cell morphology, cell number, confluence, doubling time, shape, sphericity, cell displacement, and instantaneous velocity in response to treatment. Cells were monitored for 72 h, with images acquired at 1 h intervals, and the resulting data were analyzed to derive the parameters described in the following sections.

Cell Proliferation

Cell proliferation was evaluated by analyzing cell count, confluence, cell doubling time, and cell division obtained from the acquired images. The images of cells reported in Figure 5 were obtained at different time periods and different BSAF concentrations. They

confirmed the resazurin assay results, indicating a concentration-dependent decrease in the cell viability of MG63.

Cell count and confluence are complementary indicators of cell growth. Obviously, count represents the number of viable cells attached to the surface of the plate, while confluence is linked to the surface of the plate covered by the cells and can be influenced by cell dimensions and area.

The number of untreated cells increased about 3.5-fold within 72 h while the treatment with BSAF strongly reduced the cell count (Figure 6A). At concentrations lower than IC_{50} , only a 1.5–2-fold increase was obtained while at higher concentrations no increase was observed, suggesting cell death. Cell confluence increased about 2.5 times in 72 h. BSAF treatment completely hindered the confluence increase at concentrations lower than IC_{50} while at higher concentrations a decrease in confluence was observed, indicating cell death and detachment from the substrate (Figure 6B).

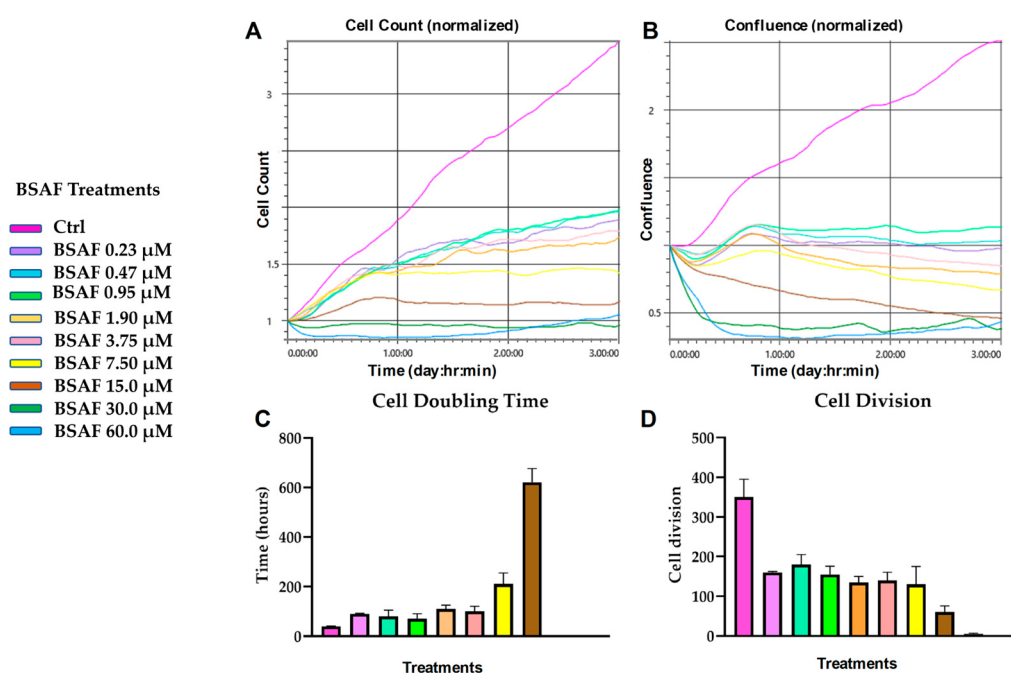


Figure 6. Cell count (A) and confluence (B) over time; cell doubling time (C) and cell divisions (D) of MG63 cells treated with BSAF at different concentrations.

Cell doubling time and cell divisions refer to the time required for a cell population to double in number and the number of cell divisions in a time frame, respectively. These are key quantitative parameters to evaluate cellular proliferation and to assess the cytostatic or cytotoxic effects of antitumor agents. Traditional methods often rely on endpoint assays, which may not accurately capture dynamic changes in cell behavior. In contrast, Liveocyte[®] enables real-time, non-invasive monitoring of live cells over extended periods. Treatment with BSAF increased cell doubling time in a concentration-dependent manner (Figure 6C). A higher value was obtained at 15 μ M corresponding to about 10 times with respect to the control. Cell divisions decreased with an increasing BSAF concentration until a minimum value at 15 μ M (Figure 6D). Beyond this concentration value no measures were obtained due to cell death. The increase in doubling time and the corresponding decrease in cell divisions observed under 15 μ M were both proportional to the concentration of BSAF, indicating that BSAF was active even at concentrations below its IC_{50} , providing a cytostatic rather than a cytotoxic effect.

Cell Morphology and Dry Mass

Sphericity, thickness, area, and dry mass were measured. The variation in these morphological parameters allows us to characterize cell behavior in response to therapeutic agents.

Area and sphericity are measures of cell attachment to the substrate. A decrease in area and increase in sphericity and thickness are usually associated with the cell's tendency to detach from the substrate due to cell death. Alternatively, these modifications may be due to cell progression towards a cytostatic phase. On the contrary, an increase in area, sphericity, and thickness may indicate cell differentiation [47,48]. Furthermore, area and thickness can be affected by cell senescence. Senescent cells do not duplicate but become larger and thinner than younger cells [41].

Dry mass refers to the total mass of cellular components like proteins, lipids, carbohydrates, and DNA, excluding water. It is a key indicator of cell growth and proliferation, therefore a decrease in dry mass is indicative of cell death.

Treatment with BSAF for 72 h provided a clear dose-dependent decrease in area (Figure 7A) and an increase in both sphericity (Figure 7B) and thickness (Figure 7C). The dry mass (Figure 7D) did not change at BSAF concentrations lower than IC_{50} while it sharply decreased at higher concentrations, indicating cell death. This behavior is in accordance with the tendency of the cells to detach from the substrate due to cell death at concentrations higher than IC_{50} , as indicated by the decreased area and increased sphericity. At concentrations lower than IC_{50} , the decrease in area and the increase in sphericity are less evident than in the cells treated at higher concentrations, indicating that the cells do not tend to detach from the substrate. Moreover, the thickness increases and the unchanged dry mass, with respect to the controls, indicates that below IC_{50} these cells are probably in a non-proliferative state rather than differentiated. Indeed, the differentiation of MG63 is associated with a transition from osteoblast-like to osteocyte-like features, providing morphological changes correlated with an increase in area and decrease in sphericity [47,48].

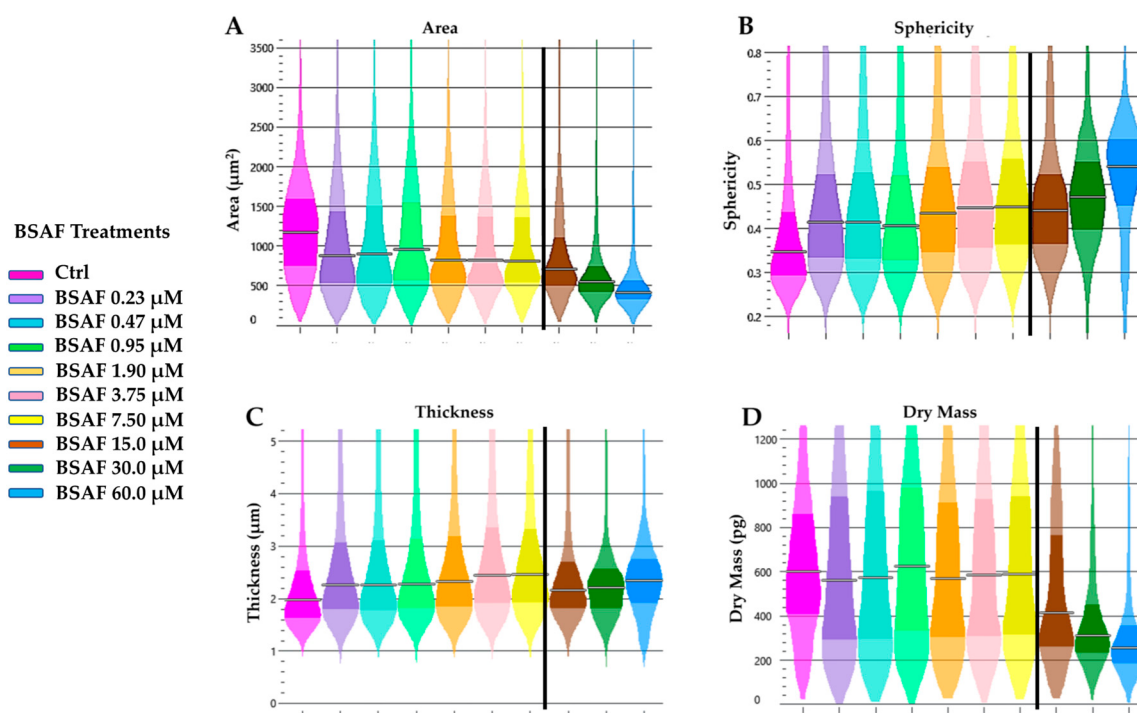


Figure 7. Morphology and dry mass of MG63 cells treated with BSAF at different concentrations: area (A), sphericity (B), thickness (C), dry mass (D). The black vertical line indicates the boundary separating treatments with concentrations below and above the IC_{50} .

Cell Motility

Osteosarcoma cell migration is a crucial step in its metastasis, involving cells detaching from the primary tumor, adhering to the extracellular matrix, and invading distant sites. Osteosarcoma is characterized by a high metastatic potential, with early metastasis to the lungs being a common complication. Treatment decreasing the motility of osteosarcoma cells could therefore reduce invasiveness and, consequently, metastatic potential.

BSAF led to a reduction in instantaneous velocity (Figure 8A), track speed (Figure 8B), and displacement (Figure 8C) at all the concentrations analyzed. The reduction in these parameters at concentrations below the IC₅₀ is in accordance with a transition of the cells towards a cytostatic condition, as already suggested by the morphological changes. At concentrations higher than the IC₅₀, the decrease is associated with cell death.

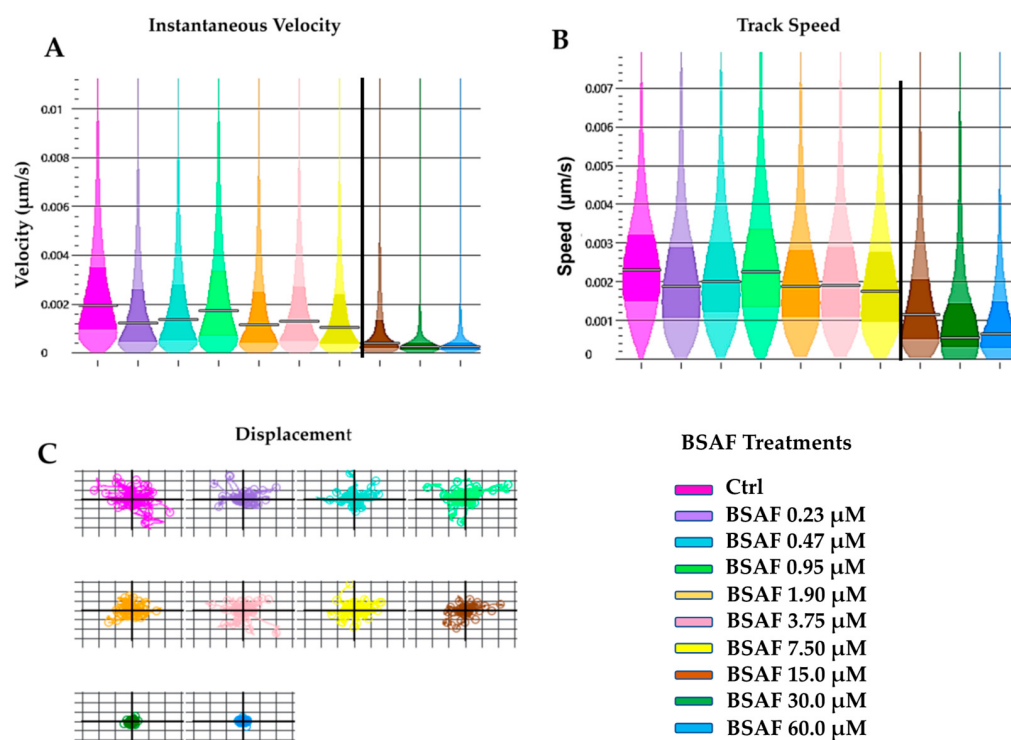


Figure 8. Cell displacement of MG63 cells treated with BSAF at different concentrations: Instantaneous velocity (A), track speed (B), displacement (C). The black vertical line in panels (A,B) indicates the boundary separating treatments with concentrations below and above the IC₅₀.

3.4. Cell Cycle Analysis

Cell cycle analysis indicated that fenretinide, administered either alone or in the tertiary (i.e., BSAF) complex, had only a modest effect on cell cycle progression, characterized by a slight increase in the proportion of cells in the G0/G1 phase (Figure 9). Representative flow cytometry profiles from a typical experiment are provided in the Supplementary Materials (Figure S3). These findings are consistent with the reduced cell proliferation observed by QPI analysis; however, the magnitude of the cell cycle alterations does not fully account for the pronounced proliferative arrest detected using the QPI approach.

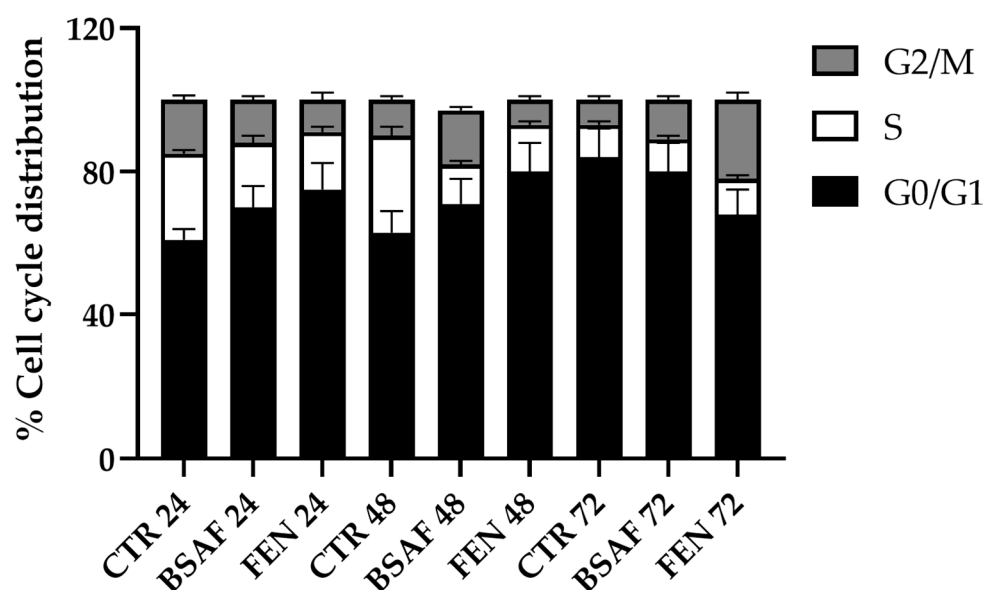


Figure 9. Percentage of MG63 cells in the different cell cycle phases after 24, 48 and 72 h of treatment.

3.5. Reactive Oxygen Species Assay

Flow cytometric analysis of ROS using DCFDA staining demonstrated that, in MG63 osteosarcoma cells, treatment with fenretinide alone or in the BSAF complex induced a marked increase in intracellular ROS levels after 24 h of exposure (Figure 10). Representative flow cytometry plots from a typical experiment are provided in the Supplementary Materials (Figure S4). These findings are consistent with results previously reported in multiple cell lines [8,13,16,17] and support the hypothesis that fenretinide exerts its biological effects, at least in part, through the induction of oxidative stress.

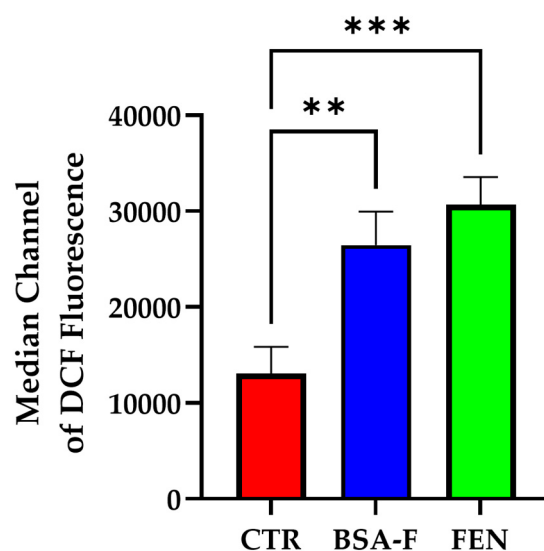


Figure 10. ROS production after 24 h of treatment analyzed by flow cytometric assay. The average mean channels of DCF fluorescence distributions are reported. ** $p = 0.05$; *** $p = 0.001$.

4. Discussion

Fen, a semisynthetic retinoid, represents a promising alternative to current chemotherapy (i.e., cisplatin, doxorubicin, high-dose methotrexate, and ifosfamide) due to its multi-targeted mechanism of action that is largely independent of common resistance-associated genetic alterations. Unlike conventional chemotherapeutics that primarily target DNA

replication or mitosis, Fen induces apoptosis through mitochondrial ROS generation, resulting in oxidative stress and caspase-independent cell death [49]. Importantly, this activity appears to be effective regardless of p53 status and extends to cancer stem-like cell populations implicated in therapy resistance and disease recurrence [12–15].

In addition to ROS-mediated cytotoxicity, Fen modulates multiple oncogenic pathways, including inhibition of PI3K/AKT/mTOR signaling, suppression of ceramide desaturase leading to pro-apoptotic dihydroceramide accumulation, and activation of retinoic acid and retinoid X receptors. These pathways converge on the mitochondrial translocation of Nur77 and displacement of Bcl-2, further enhancing apoptotic signaling [49]. Collectively, these complementary mechanisms position Fen as a strong candidate for targeting the molecular complexity of osteosarcoma.

However, despite its promising preclinical efficacy and favorable safety profile demonstrated in early-phase clinical trials [18–28,50], the clinical translation of Fen has been hampered by its extremely low aqueous solubility and poor systemic bioavailability.

Conventional formulations used in prior clinical studies failed to achieve therapeutically relevant plasma concentrations [20–28], limiting Fen's clinical utility, especially in poorly vascularized solid tumors, a category that includes osteosarcoma.

To address this critical limitation, various nanoformulation strategies have been investigated to improve Fen solubility and bioavailability. These include lipid-based nanoparticles, cyclodextrin inclusion complexes [14], and serum albumin nanocarriers [30,31]. Among these, complexes utilizing hydroxypropyl- β -cyclodextrin (HPBCD) or bovine serum albumin (BSA) have shown particularly promising results.

In the present study, we evaluated the antitumor efficacy of a novel Fen nanoformulation consisting of a ternary complex with HPBCD and BSA (BSAF). When tested on the MG63 osteosarcoma cell line, BSAF demonstrated significantly enhanced cytotoxicity compared to free Fen, with half-maximal inhibitory concentration (IC_{50}) values approximately 50% lower than those of the unformulated drug.

The cytotoxic effects of Fen and BSAF in MG63 cells have been attributed to increased ROS generation resulting from intracellular Fen accumulation, as previously reported in other cell lines [8,13,15,16]. Furthermore, wound healing assays demonstrated that BSAF was more effective than Fen at inhibiting cell migration.

This improvement can be attributed to enhanced drug solubilization and high drug loading in the complex that increases the drug availability for cellular penetration *in vitro* and is expected to translate into improved pharmacokinetic properties *in vivo*.

The complex also exhibits nanoscale dimensions, making it suitable for tumor-targeted therapy. In particular, the enhanced permeability and retention effect characteristic of primary solid tumors and their metastases enable nanosystems to preferentially accumulate following intravenous administration and to be retained within tumor tissues due to inefficient lymphatic drainage [35,44].

The size stability to dilution further makes this complex useful for targeted therapy since the dimensions are not affected by dilution in circulation.

Live-cell imaging analyses revealed that BSAF exerted cytostatic effects even at sub-cytotoxic concentrations. Specifically, treatment with concentrations below the IC_{50} resulted in significantly prolonged cell doubling time, decreased cell divisions, increased cellular sphericity and thickness, and decreased area, which are morphological changes consistent with cell cycle arrest rather than apoptosis.

These findings were corroborated by stable dry mass measurements, indicating a cytostatic state rather than progressive cell death.

In addition, cell motility parameters examined both at the single-cell and cell-population level were markedly reduced at sub-IC₅₀ concentrations, further supporting a cytostatic phenotype.

Such effects may be therapeutically valuable, particularly in preventing tumor recurrence by suppressing the proliferative and migratory potential of residual, drug-resistant clones.

Unlike conventional agents that rely solely on cell death, BSAF demonstrates a dual capacity to induce both cytotoxic and cytostatic responses, depending on BSAF concentrations, potentially overcoming multiple resistance mechanisms that are generally associated with tumor exposure to sub-cytotoxic concentrations of drugs [51].

This is a very important aspect for the efficacy of antitumor treatments since tumors may vary in dimensions and density, making the accessibility of administered drugs unpredictable. Resistance to therapy is likely to arise in the tumor compartments with poor drug penetration.

The ability of BSAF to also induce a cytostatic effect at very low concentrations suggests that it could provide an arrest of the tumor growth in the presence of tumors characterized by low drug permeability. Free Fen could not be used for targeted therapy since its insolubility in water would prevent its use in vivo as an injectable formulation. In contrast, BSAF markedly enhances the aqueous solubility of Fen, providing a feasible route for systemic administration and enabling the exploitation of its in vivo efficacy. Furthermore, by facilitating higher plasma and tumor-site drug concentrations, BSAF offers a promising platform for the clinical translation of Fen, either as a monotherapy or in combination with other therapeutic agents.

In conclusion, the Fen-HPBCD-BSA tertiary complex offers several critical advantages over the free drug, including enhanced solubility, greater cytotoxic potency, and the ability to exert cytostatic effects at subtherapeutic concentrations. These properties position BSAF as a promising candidate for further preclinical development and potential clinical evaluation in osteosarcoma treatment, particularly in overcoming the challenges posed by drug resistance, tumor heterogeneity, and poor drug bioavailability.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pharmaceutics18020278/s1>, Figure S1: Size distribution of BSAF; Figure S2: Viability assay on MG63 cells after 72 h treatment with BSA₀ at the same concentrations as BSAF; Figure S3: Cell cycle analysis in a typical experiment. The DNA distribution of MG63 nuclei stained with PI analyzed after 24 h of treatment with BSAF; Figure S4: Flow cytometric assay of ROS production in a typical experiment. The fluorescent distribution of intracellular DCF was obtained by gating the cells in the reported regions where dead, Red PI fluorescence stained cells, were excluded. Table S1: Solubility of Fen and BSAF in water and PBS pH 7.4.

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