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Preclinical Validation of ZF-148-2 Nanodrug for Breast Cancer: Antitumor Efficacy, Survival Benefit, and Histopathological Safety in the 4T1 Murine Model

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Abstract

Background: Triple-negative breast cancer (TNBC) is characterized by aggressive growth, early metastasis, and limited targeted therapeutic options. Our research on the ZnO quantum dot-based nanodrug was initially conducted using the first formulation, ZF-148, which was evaluated both *in vitro* in the 4T1 breast cancer cell line and *in vivo* in animal models using both oral and injectable administration approaches. The initial preclinical findings were published in 2022, and the formulation was subsequently protected by a patent. Following these initial findings, the preclinical investigation was continued through additional animal studies designed to obtain further evidence of antitumor activity, survival benefit, and histopathological effects. In the subsequent stage of this research program, the formulation was designated ZF-148-2 for the current series of preclinical studies. Thus, ZF-148 and ZF-148-2 refer to the same underlying therapeutic formulation and represent successive stages of its preclinical investigation rather than different drug formulations. The present study reports recent findings obtained with ZF-148-2, particularly in the 4T1 murine breast cancer model, with the aim of further expanding the preclinical evidence base and supporting the continued translational development of this nanodrug toward clinical evaluation for breast cancer. **Methods:** The cytotoxic effect of ZF-148-2 was assessed in MCF-7 breast cancer cells using the MTT assay after 48 h of treatment. For the *in vivo* study, 1×10^6 4T1 cells were inoculated subcutaneously into the flank of female BALB/c mice. Tumor-bearing animals were allocated to a vehicle control group (n = 4) or a

ZF-148-2 treatment group receiving 70 mg/kg by intratumoral injection every other day (n = 6). Tumor dimensions, clinical condition, and survival were monitored throughout the study. Tumors and major organs were subsequently examined histopathologically using hematoxylin and eosin staining. **Results:** In the recent preclinical studies of ZF-148-2, the nanodrug demonstrated concentration-dependent cytotoxic activity against breast cancer cells, with an estimated IC_{50} of approximately 4.0 mg/L. Tumor growth was markedly suppressed in treated mice. The mean individual tumor growth rate decreased from 0.491 ± 0.043 mm^2/day in the control group to 0.040 ± 0.068 mm^2/day in the treatment group ($P < 0.0001$). Treatment also significantly prolonged survival, with median survival increasing from 24 days in control animals to 42 days in treated animals (log-rank $P = 0.0018$). Control tumors were composed predominantly of densely packed viable malignant cells with high cellularity and limited spontaneous necrosis. In contrast, treated tumors exhibited marked architectural disruption, reduced viable tumor-cell density, extensive degenerative and coagulative necrotic changes, inflammatory-cell infiltration, and only residual islands of viable neoplastic cells. Histological examination of the liver, kidney, lung, spleen, heart, stomach, and brain revealed preserved tissue architecture and no significant treatment-related organ toxicity. **Conclusion:** Intratumoral administration of ZF-148-2 produced a pronounced antitumor effect in the 4T1 murine breast cancer model, as demonstrated by a highly significant reduction in tumor growth rate and a substantial extension of survival. The marked architectural destruction and extensive necrotic changes observed in treated tumors, together with the preservation of normal histological architecture in the examined major organs, provide complementary evidence of therapeutic activity without detectable major-organ toxicity under the conditions of this study. These findings demonstrate a favorable therapeutic profile of ZF-148-2 in this aggressive breast cancer model and substantially strengthen the current preclinical evidence supporting its continued development. Further adequately powered studies addressing dose-response relationships, pharmacokinetics, biodistribution, direct mechanistic validation, and local injection-site safety will be important to define the translational profile of the nanodrug and support its progression toward clinical evaluation for breast cancer.

Keywords

Preclinical Study, Breast Cancer, 4T1 Murine Model, BALB/c Mice, ZF-148-2 Nanodrug, ZnO Quantum Dots, Antitumor Efficacy, Tumor Growth Inhibition, Survival, Histopathology, Nanomedicine, Clinical Development

1. Introduction

Breast cancer remains one of the most important causes of cancer-related morbidity and mortality worldwide. Despite substantial progress in surgery, chemotherapy, endocrine therapy, targeted therapy, and immunotherapy, treatment of aggressive breast cancers remains challenging because of tumor progression, met-

astatic dissemination, recurrence, and therapeutic resistance. These limitations have stimulated the development of nanomedicine as a complementary and potentially alternative therapeutic approach. In particular, inorganic nanoparticles have attracted considerable attention because some nanomaterials can exert intrinsic biological effects rather than functioning exclusively as carriers for conventional anticancer drugs [1]-[3]. Metal and metal-oxide nanoparticles constitute an important group of experimental nanotherapeutics investigated in cancer research. Zinc oxide (ZnO), titanium dioxide (TiO_2), iron-oxide, gold, and other inorganic nanomaterials have been evaluated in breast cancer models because their nanoscale physicochemical properties can influence reactive oxygen species (ROS) generation, oxidative stress, mitochondrial function, apoptosis, cell-cycle regulation, cellular uptake, and interactions with the tumor microenvironment [2]-[5]. However, the biological activity of inorganic nanoparticles is strongly influenced by particle size, morphology, surface chemistry, formulation, dose, and administration route. Consequently, progression from *in-vitro* experiments to animal studies is an essential stage in determining whether a nanomaterial has sufficient therapeutic potential for further development. Among metal-oxide nanomaterials, ZnO has received considerable attention because of its physicochemical characteristics, relatively accessible synthesis, semiconductor properties, and nanoscale surface reactivity. Early investigations demonstrated preferential cytotoxic effects of ZnO nanoparticles (NPs) toward cancer cells and associated these effects with oxidative stress, ROS generation, mitochondrial dysfunction, and apoptotic pathways [4] [5]. These findings established a scientific basis for investigating ZnO as an active anticancer nanomaterial rather than merely as a drug-delivery vehicle. Further reduction of particle dimensions to the quantum-dot range provides ZnO with distinctive nanoscale characteristics, including a very high surface-to-volume ratio and size-dependent physicochemical behavior. These properties have stimulated investigation of ZnO quantum dots (ZnO QDs) for biomedical and anticancer applications. In breast cancer, human breast cancer cell lines MCF-7 and MDA-MB-231 cells have been among the important experimental models used to evaluate the biological activity of ZnO QDs. Roshini *et al.* reported that ZnO QDs produced concentration-dependent cytotoxic and antiproliferative effects against MCF-7 and MDA-MB-231 cells, accompanied by apoptosis, nuclear fragmentation, cell-cycle effects, and inhibition of cellular migration [6]. Independent studies using ZnO nanoparticles have likewise reported significant biological activity against MCF-7 breast cancer cells, further supporting the investigation of ZnO-based nanomaterials in breast cancer [7] [8]. The field has subsequently progressed from conventional breast cancer cell lines toward more complex experimental systems. The 4T1 murine mammary carcinoma model is particularly useful because it represents an aggressive mammary tumor that can grow rapidly and spontaneously metastasize in immunocompetent BALB/c mice [9]. Consequently, the 4T1/BALB/c model provides an important experimental bridge between *in-vitro* cytotoxicity and more advanced preclinical evaluation of

breast cancer therapeutics. Several independent investigations have examined inorganic nanomaterials in breast cancer animal models. Hong *et al.* developed red-fluorescent ZnO nanoparticles and evaluated their pharmacokinetic and tumor-targeting behavior in mice bearing 4T1 breast tumors, demonstrating tumor-associated localization of the ZnO-based nanosystem [10]. Iqbal *et al.* subsequently evaluated biosynthesized TiO_2 nanoparticles against 4T1 breast cancer cells and in BALB/c mice bearing subcutaneous 4T1 tumors. Their study demonstrated tumor-growth inhibition and incorporated investigations of oxidative stress, apoptosis, hemocompatibility, and systemic safety [11]. These studies provided important evidence that metal-oxide nanomaterials can progress beyond cellular breast cancer experiments into immunocompetent animal models. Other classes of inorganic nanomaterials have also been investigated in experimental breast cancer. Fe_3O_4 magnetic nanoparticles have been evaluated in 4T1 tumor-bearing mice for therapeutic delivery and immunomodulatory applications, including inhibition of tumor growth and spontaneous pulmonary metastasis [12]. Gold nanoparticle-based therapeutic systems have also been investigated in immunocompetent 4T1 models, including approaches combining tumor-directed radiation with immune checkpoint inhibition [13]. In addition, carbon nanotube-based nanocarriers have been investigated for anticancer drug delivery in animal models, demonstrating the broader potential of nanoscale inorganic platforms in breast cancer treatment [14]. Collectively, these studies establish that nanomedicine research in breast cancer has expanded from simple cellular cytotoxicity toward tumor targeting, therapeutic delivery, immune modulation, and *in-vivo* efficacy evaluation. Quantum-dot-based semiconductor approaches represent another important area of nanomedicine research. Their physicochemical properties can be strongly affected by nanoscale dimensions, and quantum dots have therefore been investigated for cancer imaging, detection, drug delivery, photodynamic applications, and experimental therapy. Recent literature has reviewed the application of quantum dots in breast cancer and emphasized both their potential therapeutic utility and the continuing importance of systematic biological and safety evaluation [15]. Within this broader field, ZnO QDs are particularly interesting because the nanomaterial itself can participate in biological responses and therefore may function as an active nanotherapeutic component. Within this scientific context, our research group has conducted a long-term program investigating ZnO quantum dots as active nanotherapeutic materials. The initial studies demonstrated the *in-vitro* cytotoxic effects of modified ZnO QDs against MCF-7 breast cancer cells and established an experimental foundation for the biological application of modified ZnO quantum dots [16]. This research was subsequently extended to new ZnO nanoformulations and Ag/Fe/ZnO nanocomposites, with their cytotoxic effects evaluated against MCF-7 breast cancer cells [17]. These investigations represented successive stages in the development and biological evaluation of ZnO-based nanomaterials. The research program was subsequently expanded to investigate ZnO QDs against several cancer-cell models together with

in-vivo biological evaluation [18]. Importantly, the breast cancer research was further developed through investigation of novel ZnO quantum-dot nanoparticles against breast cancer stem-like-cell-enriched mammospheres. This work examined biological responses associated with proliferation, apoptosis, stemness-related characteristics, and relevant signaling pathways [19]. The investigation of breast cancer stem-like cells represented an important extension of the earlier MCF-7 studies because these cellular populations are associated with tumor initiation, recurrence, metastatic behavior, and resistance to therapy. The next stage of the research program involved transition from cell-based experiments to an animal breast cancer model. A ZnO-QD-based nanomedicine was evaluated in BALB/c mice bearing 4T1 tumors without conventional chemotherapy [20]. This study represented an important progression of the ZnO-QD research from *in-vitro* breast cancer models toward *in-vivo* therapeutic evaluation and provided a preclinical basis for continued investigation of ZnO-QD-based nanomedicine in the aggressive 4T1/BALB/c model. The biological activity of the broader ZnO-QD research platform has also been investigated in other experimental malignancies. ZnO QDs were evaluated in acute promyelocytic leukemia cells, where apoptosis and PPARγ-associated effects were investigated [21]. More recently, a ZnO-based nanodrug was evaluated in a C57BL/6 mouse model of lung cancer and was reported to suppress tumor growth and metastasis in association with changes in $CD8^+$ T-cell infiltration and oxidative stress [22]. These studies provide evidence of the broader experimental investigation of the ZnO-based nanotherapeutic platform across different cancer models. Nevertheless, the translational focus of the present development program is specifically breast cancer. The present study represents a further stage in this long-term research pathway and specifically evaluates ZF-148-2, the designation used for the ZnO quantum dot-based nanodrug in the current stage of its preclinical investigation. ZF-148-2 is the same therapeutic formulation previously investigated as ZF-148; the change in designation reflects the successive stage of the research program rather than the development of a different drug formulation. The initial ZF-148 studies included evaluation in the 4T1 breast cancer cell line as well as *in-vivo* studies using both oral and injectable administration approaches. These earlier investigations provided the initial biological and preclinical foundation for the subsequent continuation of the research program. The current work therefore builds upon the accumulated sequence of studies involving ZnO QDs, MCF-7 breast cancer cells, breast cancer stem-like-cell populations, and the 4T1/BALB/c animal model [16]-[20]. An important feature of the present research program is the repeated evaluation of the ZF-148 platform *in-vivo*. The current investigation constitutes the fifth independent *in vivo* study in experimental breast cancer within this research program. Repeated animal investigations provide an important opportunity to examine the reproducibility of therapeutic responses and to strengthen the preclinical evidence beyond the findings of a single animal experiment. Accordingly, the present study was designed to further evaluate ZF-148-2 in the 4T1 murine breast cancer model

through longitudinal tumor-growth assessment, survival analysis, and detailed histopathological examination. Together with the accumulated evidence from previous investigations using oral and injectable administration approaches, the present work represents a further step in the long-term preclinical development of the ZF-148 platform. The continued evaluation of the formulation through complementary preclinical approaches is intended to increase confidence in the reproducibility and safety of the observed therapeutic effects and to provide a stronger scientific basis for progressing toward ethical approval and, subsequently, clinical evaluation in a hospital-based setting.

2. Experimental Section

2.1. Fabrication of a Water-Based Nanofluid Formulation as the Final Anticancer Agent (ZF-148-2 Nanomedicine)

The ZF-148-2 nanomedicine evaluated in the present study represents a continuation of our previously developed ZnO quantum-dot-based nanomedicine platform, originally reported as ZF-148 [20]. The nanomedicine used in the present investigation was prepared according to the previously established formulation and preparation procedure described in [20]. To prepare this (nanomedicine for breast cancer studies, zinc oxide quantum dots and ZnO/polyvinylpyrrolidone) (ZnO/PVP) nanopolymer are used as the principal active components, as described in our previous report [20]. These components are formulated into a hydrophilic nanofluid suitable for experimental use. An ethoxylated alcohol surfactant is used as a wetting agent, together with polyethylene glycol (PEG, molecular weight 6000) as a co-solvent and linking agent. In addition, an oil-in-water emulsifier from the sorbitan family is incorporated to facilitate stabilization of the nanoparticles in the aqueous phase. The pH = 7 - 8 of the nanofluid is adjusted appropriately, and the formulation is stirred on a magnetic hot-plate stirrer at low temperature until a homogeneous dispersion is obtained. The resulting nanofluid is subsequently subjected to sonication in an ultrasonic bath for 1 - 2 h. Based on our previous experimental investigations, the nanofluid has demonstrated favorable physicochemical and functional characteristics, including good dispersion stability, biocompatibility, and antibacterial activity [17]-[22]. No apparent treatment-related toxicity was observed under the doses and experimental conditions investigated in the reported studies [17]-[22]. The nanomedicine has also been investigated through different routes of administration in previous experimental studies [17]-[22]. The present study constitutes a further stage of the ongoing preclinical investigation of this established nanomedicine, focusing on its antitumor efficacy, survival benefit, and histopathological safety in the 4T1 murine breast cancer model.

2.2. Role of Zinc Oxide Quantum Dot Nanoparticles in Cancer Therapy

ZnO quantum dots (ZnO QDs) can interact closely with cancer cells because of

their ultrasmall size, high surface-to-volume ratio, and semiconductor nature. Their nanoscale surface facilitates close interaction with the cancer-cell membrane and, following cellular uptake, provides highly active sites for intracellular redox reactions. The most important anticancer mechanism is the ability of ZnO QDs to disturb the intracellular redox balance. Their semiconductor properties and highly reactive surface can promote electron-transfer reactions and, under appropriate conditions, generate reactive oxygen species (ROS). Excess ROS causes oxidative damage to cellular proteins, lipids, DNA, and particularly mitochondria. Mitochondrial dysfunction can then activate apoptotic signaling and ultimately lead to cancer-cell death. In parallel, ZnO can undergo partial dissolution in the intracellular environment and release Zn^{2+} ions. Excess Zn^{2+} can disturb intracellular zinc homeostasis, mitochondrial function, and redox regulation, thereby reinforcing oxidative stress and apoptotic signaling. Therefore, the distinctive anticancer action of ZnO QDs results from the combined effect of quantum-confined semiconductor behavior, high surface reactivity, ROS generation, and Zn^{2+} release, leading to oxidative stress, mitochondrial dysfunction, and programmed cancer-cell death. Their small quantum-dot dimensions are particularly important because they provide a very large reactive surface relative to the amount of material and allow the semiconductor properties of ZnO to become highly relevant at the cellular scale.

While the ultrasmall size of ZnO quantum dots facilitates close interaction with and penetration into the tumor microenvironment, their highly reactive semiconductor surface provides the therapeutic engine for redox reactions and ROS generation. Thus, the synergy between size-dependent accessibility and surface-mediated anticancer activity makes ZnO QDs promising candidates for cancer nanotherapy. Ultimately, their quantum-confined semiconductor properties, high surface reactivity, ROS generation, and Zn^{2+} release can converge to induce oxidative stress, mitochondrial dysfunction, and apoptotic death of cancer cells.

2.3. Synergistic Anticancer Mechanism of ZnO Quantum Dot Nanoparticles (NPs) and ZnO/PVP Nanocomposite in a Synthetic Nanofluid Medicine

The co-presence of ZnO quantum dots (ZnO QDs) and the ZnO/PVP nanocomposite in the nanofluid creates a complementary anticancer system in which two distinct ZnO-based nanostructures may act together to damage and ultimately destroy cancer cells. The ultrasmall ZnO QDs provide a highly reactive, quantum-confined semiconductor surface, while the ZnO/PVP nanocomposite provides an additional ZnO-based active component in which nanosized ZnO NPs are supported within a PVP (polyvinylpyrrolidone) matrix. Initially, both ZnO QDs and ZnO/PVP nanocomposite particles may establish close interactions with the cancer-cell membrane. Their nanoscale dimensions and accessible ZnO surfaces promote intimate contact with the cellular membrane and may facilitate cellular internalization. The PVP matrix helps maintain the ZnO nanoparticles in a stable

and dispersed state, while the ZnO nanoparticles remain available for interaction with the cellular environment.

Following cellular internalization, the two ZnO components can participate in complementary chemical processes. The ZnO surfaces may undergo partial dissolution under intracellular biological conditions, resulting in the release of Zn^{2+} ions. At the same time, the highly reactive semiconductor surface of the ZnO QDs may participate in electron-transfer and redox reactions and promote reactive oxygen species (ROS) generation. The ZnO nanoparticles incorporated into the ZnO/PVP nanocomposite may additionally contribute ZnO surface activity and Zn^{2+} release. The simultaneous presence of reactive ZnO surfaces, released Zn^{2+}, and ROS may therefore produce substantial intracellular chemical and oxidative stress. This stress can damage essential cellular structures, including membrane lipids, proteins, mitochondria, and DNA. Progressive structural damage may disrupt membrane integrity, mitochondrial function, intracellular homeostasis, and ultimately cellular viability. When the resulting damage exceeds the cellular repair and antioxidant capacity, apoptotic signaling may be activated and lead to programmed cell death. Beyond these established ZnO-associated processes, we propose an additional redox-mediated mechanistic hypothesis that may contribute to the anticancer activity of the nanofluid. The highly reactive ZnO surfaces and associated electron-transfer processes may interact with cellular redox-active functional groups, particularly thiol-containing biomolecules and protein disulfide bonds. Because thiol-disulfide chemistry plays an important role in protein structure, redox signaling, and intracellular redox homeostasis, perturbation of this dynamic equilibrium may provide an additional mechanism through which ZnO nanostructures alter cellular function. We therefore hypothesize that the ZnO QD/ZnO-PVP as a hybrid system may influence thiol-disulfide homeostasis through surface-mediated redox interactions, potentially shifting the balance between reduced thiol (-SH) and oxidized disulfide (-S-S-) states. Such perturbation could amplify cellular stress and compromise the structural and functional integrity of critical proteins. The proposed redox-mediated effect may operate together with Zn^{2+} release and ROS generation rather than independently of them. Their combined influence may progressively impair protein function, membrane integrity, mitochondrial activity, and intracellular homeostasis. When these disturbances exceed the capacity of the cancer cell to restore its normal biochemical state, mitochondrial dysfunction and downstream cell-death pathways may ultimately result in loss of cancer-cell viability and apoptosis. The synergistic effect therefore arises from the complementary functions of the two ZnO-based nanomaterials. ZnO QDs provide the ultrasmall, quantum-confined, highly reactive semiconductor component, whereas the ZnO/PVP nanocomposite provides a separate ZnO-containing nanostructure in which PVP supports the dispersion and availability of ZnO nanoparticles. Consequently, both components contribute ZnO-dependent surface reactions and Zn^{2+}-related activity, while the exceptional nanoscale surface reactivity of the QDs provides an additional source of redox activity and

ROS generation. The combined effects may consequently produce a greater disturbance of the tumor-cell redox and biochemical environment than either component alone.

Overall, the proposed mechanism can be summarized as: ZnO QDs + ZnO/PVP nanocomposite hybrid → membrane interaction → cellular internalization → ZnO surface reactions + Zn^{2+} release → ROS generation and oxidative/chemical stress → interaction with thiol-containing biomolecules and disulfide bonds → disturbance of thiol-disulfide redox homeostasis → structural and mitochondrial damage → loss of cellular integrity → activation of cell-death pathways → cancer-cell death. This proposed mechanism extends the conventional ROS- and Zn^{2+}-centered interpretation of ZnO-mediated anticancer activity by suggesting that perturbation of cellular thiol-disulfide redox homeostasis may represent an additional mechanistic layer contributing to the activity of the ZnO QD/ZnO-PVP nanofluid. This mechanistic concept is proposed as a testable hypothesis and requires direct experimental validation.

2.4. The Synergistic Role of Wetting Agents, PEG, and Sorbitan Esters in ZF-148-2

The integration of a specialized wetting agent within the nanofluid formulation is a critical factor in overcoming the biological barriers of the tumor microenvironment. Tumor tissues and cancer cell membranes often exhibit hydrophobic characteristics and irregular surface topographies that can repel aqueous-based treatments. The wetting agent effectively lowers the surface and interfacial tension of the nanofluid, ensuring uniform spreading and maximum contact area between the therapeutic agents and the tumor surface. By promoting complete "wetting" of the lipid bilayer, it ensures that the ZnO Q-dots are not just suspended in the medium but are in direct, intimate contact with the target cell. This enhanced coverage acts in synergy with the Sorbitan esters and PEG agents. While the wetting agent ensures the nanofluid "grips" the cell surface, the sorbitan esters begin to fluidize and destabilize the membrane's lipid packing, creating nanostructural defects. Simultaneously, PEG maintains the stability and mono-dispersity of the particles, preventing them from clustering at the surface. Together, these components transform the cell membrane from a rigid barrier into a permeable interface. This facilitated "opening" allows the ZnO/PVP nanoparticles and ZnO Q-dots to rapidly penetrate the cytoplasm. Once internal access is achieved, the chemical mechanism driven by ROS generation and Zn^{2+} release is triggered, leading to the efficient destruction of the tumor cell from the inside out [17]-[22].

2.5. Proposed *in Vivo* Trajectory, Tumor Selectivity, and Microenvironmental Action of ZF-148-2

Following oral administration, ZF-148-2 must pass through a sequence of biological environments before reaching systemic circulation. During gastrointestinal transit, the nanofluid encounters variations in pH, ionic strength, digestive com-

ponents, mucus, and biological macromolecules. These conditions may dynamically influence the dispersion, interfacial organization, and biological availability of the nanostructures. The ability of the formulation to maintain an appropriate dispersed state during this process is therefore an important determinant of subsequent absorption and systemic exposure. Following intestinal uptake, the nanostructures enter a protein- and electrolyte-rich biological environment in which their surfaces can interact dynamically with plasma proteins, peptides, lipids, and other biomolecules. Exposed amino-acid residues within these proteins, including polar and redox-active functional groups, may participate in hydrogen bonding, electrostatic interactions, and other non-covalent or interfacial interactions with the nanomedicine. In particular, redox-active protein groups may provide chemically responsive sites through which the biological identity of the nanostructures can be modified. This concept is consistent with the redox-mediated nano-bio interface previously proposed by our group, in which nanoparticle-biomolecule interactions were considered active chemical processes involving electron transfer and thiol-disulfide chemistry rather than passive adsorption [23]. These dynamic interactions may result in the formation and continuous remodeling of a biomolecular corona around the nanostructures. Rather than representing a fixed coating, this corona can be regarded as a dynamic biological interface that changes according to the surrounding molecular environment. Such interfacial conditioning may influence dispersion, cellular recognition, tissue distribution, clearance, and accessibility of the active nanostructures. Consequently, the biological behavior of ZF-148-2 after administration is expected to reflect not only the formulation itself but also the continuously changing molecular identity acquired during interaction with the biological milieu. After entering systemic circulation, the nanomedicine is exposed to the vascular endothelium, circulating proteins, immune cells, and multiple physiological clearance mechanisms. Its subsequent distribution among tissues is determined by the combined effects of these interactions and the properties of the vascular and extracellular environments encountered along the circulation pathway. Importantly, ZF-148-2 does not incorporate a conventional receptor-targeting ligand. Its proposed tumor selectivity therefore cannot be attributed to classical ligand-receptor targeting and is instead considered here as a potentially intrinsic physicochemical and microenvironment-dependent selectivity. Tumor tissue presents a biological environment substantially different from most normal tissues, including abnormal vascular architecture, heterogeneous vascular permeability, disorganized extracellular matrix, altered interstitial pressure, hypoxic regions, metabolic alterations, and spatially heterogeneous biochemical conditions. These characteristics can influence the extravasation, local retention, and cellular accessibility of nanostructures. The abnormal tumor microenvironment may therefore provide physicochemical conditions under which ZF-148-2 can interact more extensively with tumor tissue than with physiologically organized normal tissues. Following access to the tumor interstitium, the nanostructures may interact with extracellular-matrix compo-

nents, interstitial proteins, electrolytes, and the surface of tumor cells. Hydrogen bonding, electrostatic forces, hydrophobic interactions, and other non-covalent interactions can contribute to this interfacial association. The transition from extracellular tumor localization to direct tumor-cell interaction is particularly important because it brings the nanomedicine into proximity with membrane lipids, proteins, glycoproteins, and accessible functional groups. These interactions may facilitate membrane association and subsequent cellular internalization, allowing the nanostructures to progress from the extracellular tumor compartment into the intracellular environment. Once internalized, ZF-148-2 encounters a sequence of intracellular compartments with different pH values, ionic compositions, protein environments, and membrane properties. These conditions can modify nanoparticle-biomolecule interactions and expose the nanostructures to intracellular functional groups and redox-active molecular systems. The resulting physicochemical interactions may initiate the intracellular disturbances described previously in this manuscript, including redox imbalance, ionic dysregulation, membrane injury, mitochondrial impairment, and ultimately irreversible tumor-cell damage. The present section therefore considers these events as downstream consequences of tumor-cell access rather than repeating their detailed molecular mechanisms. The apparent selectivity toward tumor tissue is particularly important in light of the repeated histopathological observations obtained during the ZF-148/ZF-148-2 research program. The experimental findings described in this manuscript demonstrate pronounced tumor destruction while showing no corresponding pathological injury in the major examined normal organs under the investigated conditions. The earlier 4T1 investigation also demonstrated marked tumor necrosis, tumor-growth inhibition, absence of detectable metastasis, and immune-cell infiltration within treated mammary tissue. The favorable response observed with oral administration in that study provided an important experimental basis for continued development of the same formulation ([20], part 13). The presence of immune-cell infiltration following tumor treatment provides an additional mechanistic dimension. Extensive tumor-cell destruction can alter the tumor microenvironment by releasing tumor-associated antigens, intracellular molecules, and damage-associated signals. These changes may increase the biological visibility of the tumor and influence local immune-cell recruitment and activation. Accordingly, the antitumor effect of ZF-148-2 may not terminate with direct destruction of individual tumor cells; the resulting modification of the tumor microenvironment may potentially contribute to reduction of local immunosuppression and facilitate secondary immune-mediated clearance of residual malignant cells. This interpretation remains a mechanistic hypothesis and should be distinguished from direct demonstration of specific immune pathways. Overall, the proposed trajectory of ZF-148-2 can therefore be considered as a continuous sequence extending from oral administration and gastrointestinal conditioning to systemic distribution, biomolecular interfacial remodeling, tumor-microenvironment access, tumor-cell membrane interaction, intracellular entry, direct tumor-

cell injury, and subsequent modification of the local immune environment. The absence of a conventional targeting ligand, together with repeated evidence of tumor destruction and limited pathological injury in examined normal tissues, supports consideration of an intrinsic tumor-selective physicochemical response rather than classical receptor-mediated targeting. The conceptual pathway can be summarized as: Oral administration → gastrointestinal conditioning → systemic exposure → biomolecular corona/interfacial remodeling → tumor-microenvironment access → tumor-cell interaction and internalization → intracellular injury → tumor destruction → modification of the immunosuppressive microenvironment → potential immune-mediated tumor clearance. Direct pharmacokinetic, biodistribution, tumor-accumulation, and immunophenotyping studies will ultimately be required to quantitatively validate the individual steps of this proposed trajectory.

3. Materials and Methods for ZF-148-2 Nanomedicine

3.1. Cell Culture and *in Vitro* Cytotoxicity Assay

The cytotoxic activity of the ZF148-2 nanomedicine was evaluated using the human breast adenocarcinoma cell line MCF-7. Cells were obtained from the Pasteur Institute (Karaj, Iran) and cultured in Ham's F-12K medium supplemented with 10% fetal bovine serum (FBS) under standard culture conditions (37˚C, 5% CO_2, and humidified atmosphere). Approximately 5×10^3 cells/well were seeded into 96-well plates and allowed to attach for 24 h before treatment.

The stock solution of the ZF148-2 was prepared at 128.6 g/L, followed by serial ten-fold dilutions ranging from 128.6 g/L to 12.86 μg/L. Cells were exposed to each concentration for 48 h.

Cell viability was determined using the MTT assay (Cibbiotech Cell Proliferation Assay Kit) according to the manufacturer's instructions. Briefly, after drug treatment, MTT reagent (10 μL/well) was added and plates were incubated for 2 h at 37˚C. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Triton X-100-treated cells served as the positive control, whereas untreated cells served as the negative control. Cell viability was calculated relative to untreated controls, and the IC_{50} value was determined from the dose-response curve.

3.2. Experimental Animals

All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and complied with the institutional and national guidelines for the ethical use of laboratory animals. The experimental protocol was reviewed and approved by (Zist Faravard Pars, Tehran, Iran) company based on moral code (ethical principles). Every effort was made to minimize animal suffering and reduce the number of animals used.

Female BALB/c mice (6 - 8 weeks old; average body weighs approximately 18 g) were obtained from the Pasteur Institute (Karaj, Iran). Animals were maintained under standard laboratory conditions with free access to food and water

under a 12-h light/dark cycle. All experimental procedures were conducted according to institutional guidelines for laboratory animal care and use.

3.3. Tumor Induction

The murine breast cancer model was established by subcutaneous inoculation of 1×10^6 4T1 cells into the right flank of each mouse. Tumor development was monitored by palpation, and treatment was initiated after tumors reached approximately 7 mm in diameter.

Tumor-bearing mice were randomly assigned into two experimental groups:

Group A: Control: vehicle without ZnO NPs (100 μL) (n = 4 per group)

Group B: ZF148-2 nanodrug (70 mg/kg) (100 μL) (n = 6 per group)

The formulations were administered by intratumoral injection every other day throughout the treatment period. Tumor size, body condition, and clinical signs were monitored throughout the study.

3.4. Tumor Growth Assessment

Tumor dimensions were measured periodically using a digital caliper, and tumor size was recorded throughout the experimental period. Animals were observed daily for changes in activity, grooming behavior, body condition, and survival. Animals reaching humane endpoints or natural death were subjected to necropsy.

3.5. Histopathological Examination

At the end of the experiment, or following spontaneous death, major organs including the liver, kidney, lung, spleen, heart, and brain and tumor residue tissue were collected and fixed in 10% neutral buffered formalin. Following routine tissue processing, paraffin embedding, sectioning (4 - 5 μm), and hematoxylin and eosin (H&E) staining, tissue sections were examined by light microscopy for evidence of treatment-related pathological changes, including inflammatory lesions, necrosis, degeneration, fibrosis, hemorrhage, and alterations in tissue architecture.

Statistical Analysis: Statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA). Quantitative data are presented as the mean ± standard deviation (SD). Tumor growth was analyzed using an individual slope-based approach. For each animal, tumor area (mm^2), calculated as length × width from two perpendicular caliper measurements, was plotted against study time, and the individual tumor-growth rate (mm^2/day) was estimated by linear regression using all available measurements obtained while the animal remained alive. Individual tumor-growth slopes were compared between the control and ZF-148-2 treatment groups using Welch's independent-samples t-test. The Mann-Whitney U test was additionally performed as a nonparametric sensitivity analysis. Measurements after death were not imputed or carried forward. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank (Mantel-Cox) test. A P value < 0.05 was considered statistically significant.

MTT Cytotoxicity Assay: The cytotoxic activity of the ZF148-2 against MCF-7 breast cancer cells was evaluated using the MTT assay. Treatment with the compound resulted in a typical sigmoidal dose-response relationship was observed in cell viability. Dose-response analysis demonstrated an estimated IC_{50} value of 4 mg/L, indicating potent in vitro cytotoxic activity against MCF-7 cells. Cell viability progressively decreased with increasing drug concentration, confirming the antiproliferative effect of the ZF148-2 (Figure 1).

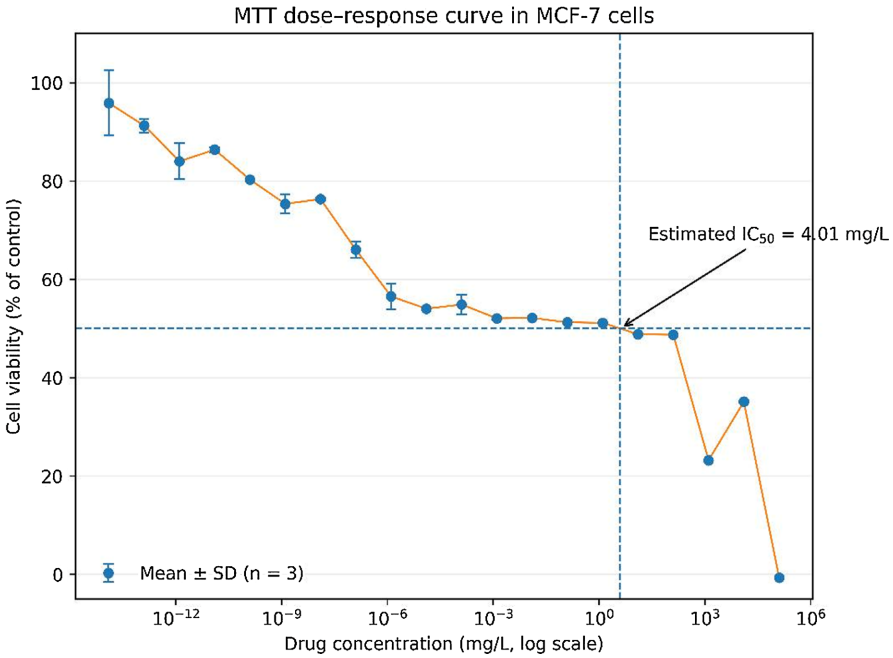


Figure 1. Dose-response curve of the investigational compound in MCF-7 cells determined by the MTT assay. Cell viability was measured after 48 h of treatment using serial 10-fold dilutions. The IC_{50} value was estimated by nonlinear regression (four-parameter logistic model).

Effect of Treatment on Tumor Growth: Tumor size increased progressively in the control group, whereas tumor growth was markedly suppressed in treated animals. Individual tumor growth rates were calculated as the slope of tumor size against study time using linear regression. The mean tumor growth rate was 0.491 ± 0.043 mm^2/day in the control group and 0.040 ± 0.068 mm^2/day in the treatment group. The reduction in tumor growth rate was statistically significant according to Welch's independent-samples t-test ($t = 12.85$, $df = 8.00$, $P < 0.0001$). This finding was also supported by the Mann-Whitney U test ($U = 24$, $P = 0.0095$) (Figure 2).

Animals were monitored daily for clinical signs throughout the study. Control mice progressively developed reduced activity, decreased mobility, lethargy, and rapid tumor enlargement before death. Mice receiving the ZF148-2 remained clinically stable during most of the treatment period. Mild reductions in activity were observed only during the terminal stages in some treated animals. No treatment-related signs of systemic toxicity, including severe weight loss, neurological abnormalities, respiratory distress, or gastrointestinal complications, were

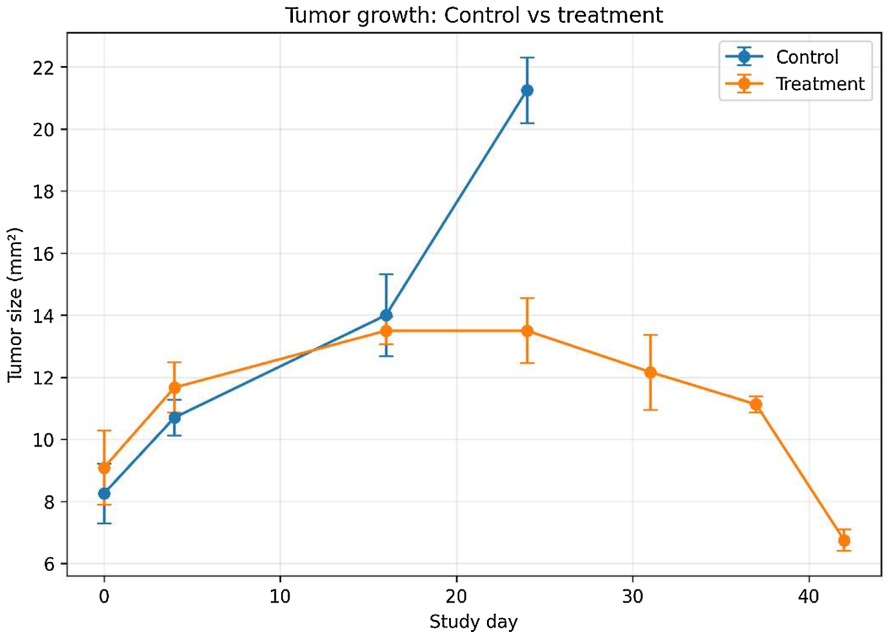


Figure 2. Tumor growth in control and treated mice. Mean tumor size (± SD) measured throughout the study in the control (n = 4) and treatment (n = 6) groups.

observed. Gross observations together with histopathological examination of the heart, liver, kidney, lung, spleen, and brain revealed no evidence of significant treatment-related organ toxicity.

3.6. Histopathological Findings

Histopathological examination was performed on the liver, kidneys, lungs, spleen, heart, stomach, brain, and tumor tissues using hematoxylin and eosin (H&E) staining. In the ZF-148-2-treated animals, the major organs generally showed preserved tissue architecture, with no treatment-attributable histological lesions detected under the conditions of this study. Hepatic lobular architecture and hepatocyte morphology were preserved, without hepatocellular necrosis, fibrosis, or significant inflammatory infiltration. Renal glomeruli and tubular structures were well preserved, although occasional minimal tubular epithelial degeneration was observed. Cardiac muscle fibers exhibited normal morphology without inflammatory infiltration or myocardial degeneration. Lung sections showed preserved alveolar architecture with only minimal focal inflammatory-cell infiltration. The spleen retained normal organization of the white and red pulp, with mild reactive lymphoid hyperplasia. Brain tissue showed intact cortical and cerebellar architecture without neuronal degeneration, hemorrhage, or inflammatory lesions. Gastric tissue maintained normal mucosal and glandular architecture without ulceration or significant inflammatory changes. Histopathological examination of tumor tissues revealed clear differences between the control and ZF-148-2-treated groups. Tumors from the control group were composed predominantly of densely packed viable malignant cells with high cellularity, hyperchromatic nuclei, a high nuclear-to-cytoplasmic ratio, preserved tumor architecture, and limited sponta-

neous necrosis. In contrast, tumors from ZF-148-2-treated mice showed marked disruption of tumor architecture, reduced viable tumor-cell density, extensive areas of coagulative necrosis, widespread degenerative changes, and inflammatory-cell infiltration. In some treated samples, only small residual islands of viable neoplastic cells remained within or adjacent to broad necrotic areas. These histopathological findings are consistent with a marked local tumor response to ZF-148-2 treatment (Figure 3, Figure 4).

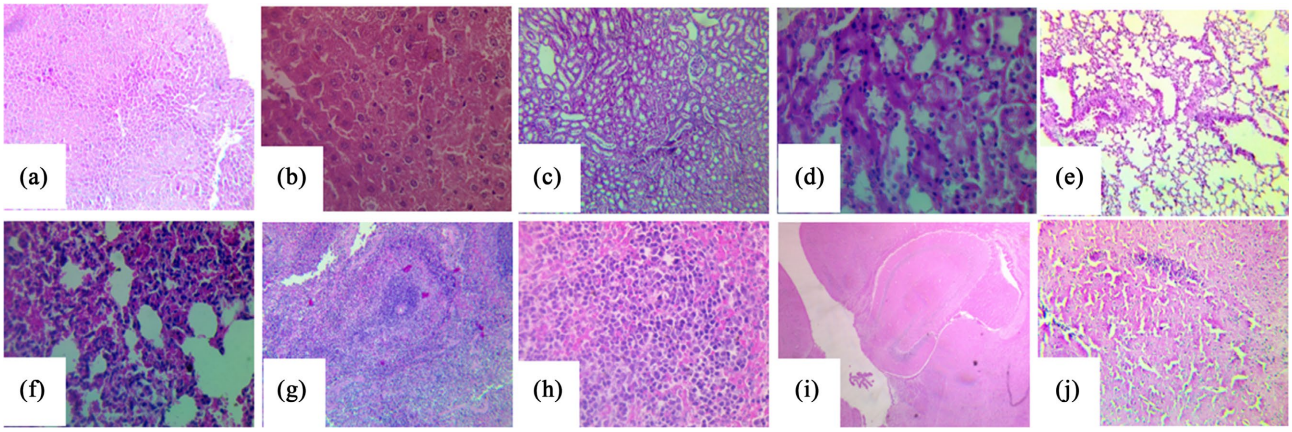


Figure 3. Histopathological evaluation of major organs following treatment with the ZF-148-2 nanodrug in tumor-bearing mice. Representative hematoxylin and eosin (h) & (e)-stained sections of (a), (b) liver, (c), (d) kidney, (e), (f) lung, (g), (h) spleen, (i), (j) brain, from treated mice. The liver exhibits preserved lobular architecture with normal hepatocyte morphology and no evidence of hepatocellular degeneration or necrosis. Renal tissue demonstrates intact glomeruli and renal tubules without tubular necrosis. Lung sections reveal preserved alveolar architecture with only minimal focal inflammatory infiltrates. Splenic white and red pulp remain well organized with mild reactive lymphoid hyperplasia. Cardiac muscle fibers exhibit preserved morphology without inflammatory infiltrates or myocardial degeneration. Brain tissue shows intact cortical and cerebellar architecture with preserved neuronal morphology. Gastric mucosa demonstrates intact glandular architecture without ulceration or significant inflammatory changes. Collectively, these findings indicate the absence of significant treatment-related histopathological toxicity.

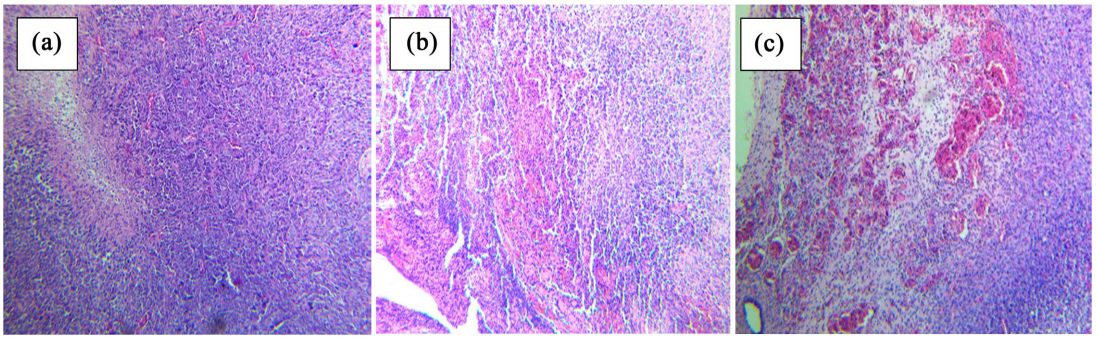


Figure 4. H&E staining revealed densely packed viable tumor cells with minimal necrosis in the control tumor (a). In contrast, treated tumors (b), (c) showed marked architectural disruption, reduced viable tumor-cell density, extensive degenerative changes, and increased areas of necrosis with inflammatory-cell infiltration, indicating a substantial treatment-associated antitumor effect.

4. Results and Discussion

4.1. Characterization of the Nanomaterials in the ZF-148-2 Nanomedicine

The comprehensive physicochemical characterization of the nanomaterials con-

stituting the ZF-148 nanomedicine was previously performed and reported in our 2022 publication [20]. The characterization included transmission electron microscopy (TEM), scanning electron microscopy (SEM), UV-Vis spectroscopy, Fourier-transform infrared spectroscopy (FTIR), and photoluminescence (PL) analysis. The corresponding experimental data, spectra, images, and detailed interpretations are fully presented in the previously published work [20]. The morphological analysis demonstrated that the modified ZnO quantum-dot nanoparticles exhibited a mixture of small nanosphere- and nanorod-like structures associated with fatty-acid groups. The UV-Vis absorption spectrum of the ZnO quantum dots showed a maximum absorption wavelength of approximately 193 nm, while the corresponding nanodrug formulation exhibited a slight shift to approximately 194.3 nm. These characteristics were consistent with the nanoscale nature of the ZnO quantum dots and their blue-shifted absorption edge. FTIR analysis supported the characteristic chemical and structural features of the ZnO quantum dots, consistent with the hexagonal wurtzite structure of ZnO (space group $P6_3mc$) and its association with the surrounding fatty-acid components. PL analysis also demonstrated the characteristic visible-emission behavior of the ZnO quantum dots [18] [20]. The ZF-148-2 nanomedicine evaluated in the present study corresponds to the same established nanomedicine platform previously characterized as ZF-148 [20], with the same principal nanomaterial structure and physicochemical characteristics. Accordingly, the comprehensive characterization reported in [20] provides the physicochemical basis for the nanomedicine evaluated in the present study. The present work focuses on the subsequent preclinical evaluation of this established nanomedicine, rather than repeating its previously reported physicochemical characterization.

4.2. Physicochemical Mechanism and Preclinical Development of the ZF-148-2 Nanodrug for Breast Cancer

The following mechanism is proposed as a conceptual and testable hypothesis based on the physicochemical characteristics of the ZnO quantum-dot system and observations from previous investigations. It is not presented as an experimentally demonstrated mechanism of the present study. The nanopharmaceutical formulation may interact preferentially with the abnormal physicochemical environment surrounding tumor tissue. Tumor-associated fluids and the altered biochemical environment may provide conditions that favor interaction of highly active nanoparticles with cancerous tissue. Because ZnO quantum dot nanoparticles are extremely small and possess a high surface-to-volume ratio and accessible surface states, they may establish close contact with tumor-cell membranes and membrane proteins. At this nano-bio interface, interactions with polar amino acids through hydrogen bonding, electrostatic interactions, and other surface-mediated interactions may contribute to nanoparticle-cell contact and subsequent cellular uptake. The possibility that smaller nanoparticles can interact with cell membranes more readily is also consistent with the physicochemical characteristics ob-

served for the ZnO Q-dot system [3] [5] [6] [16] [18] [20]. An important chemical hypothesis concerns the interaction of the nanoparticle surface with sulfur-containing amino acids. Cysteine contains a thiol group, and two cysteine residues can form a disulfide bond that contributes to protein folding and structural stability. We therefore propose that interactions between an electronically active ZnO surface and thiol-containing biomolecules may perturb local redox chemistry and, under appropriate conditions, influence thiol-disulfide equilibria. Such interactions could affect the structure or function of susceptible proteins and contribute to cellular dysfunction. This concept is proposed as a chemical hypothesis rather than as a demonstrated mechanism of the present study.

The ZnO@PVP component of the formulation may further influence the nano-bio interface by maintaining dispersion of the ZnO QDs in the aqueous formulation and providing additional functional groups capable of participating in hydrogen bonding and electrostatic interactions. The combined physicochemical properties of ZnO QDs and the polymer-supported formulation may therefore increase the effective interaction of the nanodrug with biological interfaces. Previous studies of ZnO-based nanomaterials have also demonstrated their relevant physicochemical and biological activity in cancer-related systems [3]-[8] [16] [18] [20] [24]. The semiconductor properties of ZnO QDs provide another important component of the proposed mechanism. Reduction in QD size can alter the electronic structure, band-gap energy, and optical behavior of semiconductor nanoparticles. Surface defects, oxygen vacancies, and trapping states can contribute to surface electronic activity. The photoluminescence and optical characteristics previously observed for the formulated ZnO QDs are therefore consistent with the presence of electronically active surface and defect states [3] [6] [16] [20]. These characteristics may permit localized electron-transfer and redox interactions at the nano-bio interface. Under biologically permissive conditions, such processes could contribute to reactive oxygen species (ROS) formation and oxidative modification of susceptible cellular components, including membrane lipids and proteins. ZnO-associated oxidative stress and cytotoxicity have been reported in cancer-cell systems [4] [5] [24]. The semiconductor properties of ZnO QDs may provide an additional physicochemical basis for the proposed nano-bio interactions. Their defined electronic structure, quantum confinement, and surface defect states, particularly oxygen vacancies, may enable the ZnO QDs to function as a nanoscale electron pump, facilitating localized charge transfer and redox interactions at the tumor-cell interface. In this framework, the interface may serve as an active physicochemical reaction zone rather than a passive site of nanoparticle adsorption. Such interfacial electronic activity could contribute to oxidative modification of membrane components, membrane destabilization, and subsequent cellular entry, followed by interaction with intracellular proteins and other critical cellular structures. These processes may ultimately contribute to disruption of membrane, protein, mitochondrial, and redox homeostasis and to tumor-cell injury and death. This mechanism remains a proposed and testable hypothesis re-

quiring direct experimental validation. Accordingly, we propose that the nano-bio interface may function not merely as a site of passive nanoparticle adsorption but as an active physicochemical reaction zone. The sequence may involve enhanced nanoparticle-cell contact, surface-mediated electron-transfer reactions, disturbance of local redox balance, ROS-associated molecular damage, and subsequent cellular injury. In parallel, ZnO dissolution may increase local Zn^{2+} availability, which could disturb intracellular zinc homeostasis and contribute to additional cellular stress. The combined effects of redox disturbance, ionic imbalance, protein dysfunction, membrane perturbation, and mitochondrial stress could ultimately exceed the capacity of tumor cells to maintain cellular homeostasis. This proposed model does not require a conventional receptor-targeting ligand. Instead, the physicochemical differences between tumor and normal tissues may influence nanoparticle-biomolecule interactions and thereby contribute to reactivity-based or microenvironment-responsive selectivity. Previous investigations of ZnO-based nanomedicine, including our studies of the ZF-148 platform, provide experimental observations that motivate this hypothesis [16] [18] [20]. The proposed mechanism can therefore be summarized as:

ZnO QDs/ZnO@PVP → tumor-associated nano-bio interface → surface-mediated molecular interactions and electron transfer → redox perturbation and ROS-associated effects + Zn^{2+} availability → thiol-disulfide/protein and ionic imbalance → membrane/mitochondrial dysfunction → tumor-cell injury and death.

The Q-Dot NPs possess a high surface-to-volume ratio, discrete quantum states, and high surface reactivity, which can favor therapeutic-drug loading, controlled release, enhanced cellular delivery, and diffusion within tumor microenvironments. Their ultrasmall size (typically 1 - 8 nm) and size-dependent electronic structure can further influence the stability, transport, and interfacial reactivity of associated therapeutic agents. In ZnO Q-Dots, abundant surface and defect states, including interstitial Zn^{2+} sites and oxygen vacancies, can generate electron-trapping centers and facilitate electron-hole pair formation. The resulting holes (h^{+}), together with the associated redox-active surface states, may promote oxidative reactions at aqueous and cellular interfaces. In an aqueous tumor microenvironment, these processes may contribute to water activation and the generation of reactive species, providing a plausible physicochemical basis for redox-mediated interactions with cellular components and, consequently, for the therapeutic activity of ZnO Q-Dot-based systems. Importantly, membrane interaction or opening, cellular uptake, intracellular Zn^{2+} changes, ROS generation, thiol-disulfide perturbation, mitochondrial injury, and selective interaction with the tumor microenvironment were not directly established as mechanisms in the present study. They should therefore be regarded as testable hypotheses. Dedicated studies of nanoparticle localization and uptake, intracellular Zn^{2+}, ROS and redox status, thiol-disulfide chemistry, protein changes, mitochondrial function, pharmacokinetics, and biodistribution will be required to determine the relative contribution of these proposed processes.

The ZF-148 nanomedicine has been developed through a continuous research program since 2013, encompassing successive *in vitro* and *in vivo* investigations. The first technological milestone was the registration of Iranian Patent No. 84862 on 5 February 2015 (05/11/1393), entitled *"Production of a Zinc Oxide Quantum Dot Composite Nanodrug and Evaluation of Its In Vitro Cytotoxicity Against KB44 Oral Cancer, MCF-7 Breast Cancer, HT29 Colon Cancer, and HeLa Cervical Cancer Cell Lines, together with an In Vivo Mouse Ear-Swelling Standard Test."* [25] This patent established the original ZnO quantum-dot-based nanodrug platform. A second technological milestone was the registration of Iranian Patent No. 100349 on 15 February 2020 (26/11/1398), entitled *"Formulation and Preparation of a Water-Based Nanofluid Containing Zinc Oxide Quantum Dots for the Treatment of Breast Cancer in BALB/c Mice Using the 4T1 Cell Line."* [26]. The associated 4T1 animal-model research was subsequently reported in 2022 [20]. The later designation ZF-148-2 refers to the same underlying nanodrug formulation and was used to identify subsequent stages of its preclinical investigation. Together, these patents and the successive experimental studies document a sustained preclinical development pathway for the ZnO quantum-dot-based nanodrug and provide a scientific and technological basis for its continued evaluation toward future clinical development.

4.3. Study Limitations and Further Preclinical Requirements

Several limitations should be considered. First, the number of animals was small, with four animals in the control group and six animals in the treatment group. Although a significant difference in tumor growth and survival was observed, larger studies are warranted to further confirm these findings. Second, only one treatment dose was investigated, and therefore the optimal therapeutic dose and dose-response relationship remain to be established. Third, ZF-148-2 was not compared with an established anticancer treatment. Such comparison would help determine the relative magnitude of its therapeutic effect. Fourth, pharmacokinetic, biodistribution, hematological, and biochemical toxicity assessments were not performed in the present study. Fifth, although the 4T1 model can develop metastatic disease, metastatic burden was not quantitatively evaluated. In addition, the *in vitro* experiments were performed using MCF-7 cells, whereas the *in vivo* experiments were performed using the 4T1 model. Future studies using complementary breast cancer models would therefore strengthen the biological interpretation of the findings. Taken together, the present study demonstrated a consistent antitumor response to ZF-148-2 based on tumor-growth inhibition, prolonged survival, and histopathological destruction of tumor tissue, without significant treatment-related injury in the examined major organs. These findings support further preclinical investigation of ZF-148-2 as a ZnO quantum dot-based nanotherapeutic for breast cancer. Further studies should include larger experimental groups, different dose levels, comparison with standard treatments, pharmacokinetic and biodistribution analyses, comprehensive toxicity assessment,

metastatic evaluation, and additional mechanistic studies. Such investigations will further define the therapeutic potential, pharmacological characteristics, and safety profile of ZF-148-2 and will strengthen the evidence base for its continued translational development and subsequent clinical evaluation through the appropriate ethical and regulatory pathway.

5. Discussion

Triple-negative breast cancer (TNBC) remains a major therapeutic challenge because of its aggressive behavior, metastatic potential, and limited targeted treatment options. In the present study, the ZnO quantum-dot-based nanomedicine ZF-148-2 produced a marked antitumor response in the syngeneic 4T1 breast cancer model, characterized by substantial inhibition of tumor growth, prolonged survival, and extensive histopathological destruction of tumor tissue. These findings provide further preclinical evidence for the therapeutic potential of the established ZF-148 nanomedicine platform and represent a continuation of the research program previously reported under the ZF-148 designation [20]. The biological activity of ZF-148-2 may be related to the distinctive physicochemical properties of ZnO quantum dots, including their ultrasmall dimensions, high surface-to-volume ratio, quantum-confined electronic characteristics, and chemically active surface. We propose that these properties become particularly relevant at the nano-bio interface, where ZnO QDs interact closely with tumor-cell membranes. At this interface, semiconductor surface states may facilitate localized electron-transfer and redox processes that could contribute to ROS generation and oxidative modification of membrane lipids and proteins. We further hypothesize that this interface may function as an active physicochemical reaction zone, linking nanoparticle-membrane interaction to subsequent membrane perturbation and cellular uptake. Following internalization, exposure to acidic intracellular compartments may promote ZnO dissolution and Zn^{2+} release according to: $ZnO + 2H^{+} \rightarrow Zn^{2+} + H_2O$.

Together, ROS generation and altered intracellular Zn^{2+} availability may increase oxidative and metabolic stress and contribute to mitochondrial dysfunction and cell death. A complementary hypothesis concerns the possible involvement of protein thiol-disulfide redox homeostasis. Cysteine-containing proteins are particularly sensitive to changes in cellular redox conditions, and the reactive surface of ZnO QDs, together with ROS generation and local Zn^{2+} availability, may perturb susceptible thiol-disulfide equilibria. Such perturbation could affect protein conformation, enzyme activity, membrane integrity, intracellular signaling, and mitochondrial function. We therefore consider thiol-disulfide disruption a possible additional biochemical component of the overall mechanism rather than a single established molecular target. The antitumor effect of ZF-148-2 may consequently arise from the convergence of interconnected physicochemical and biochemical processes rather than from a conventional receptor-specific mechanism. The pronounced histopathological response observed in treated tumors

provides direct tissue-level evidence of the biological effect. Compared with the densely cellular and viable malignant tissue observed in controls, ZF-148-2-treated tumors showed marked architectural disruption, extensive degenerative and coagulative necrosis, inflammatory-cell infiltration, and a substantial reduction in viable tumor-cell density. These findings are consistent with severe and sustained tumor-cell injury and support the biological relevance of the proposed multifactorial mechanism. The present findings also extend previous observations concerning the administration of the ZF-148 platform. Previous *in vivo* studies demonstrated antitumor activity following oral administration [20], while the present research program further evaluated oral delivery under controlled experimental conditions. The observed therapeutic response following oral administration is particularly relevant to the continued preclinical development of the nanomedicine, as it demonstrates that its antitumor activity is not restricted to direct local administration. Differences in the rate of response between controlled gavage and administration through drinking water may reflect differences in dose consistency and exposure over time. An important finding across the experimental studies conducted to date is the absence of treatment-related evidence of systemic toxicity. In the present study, treated animals remained clinically stable, and histopathological examination of the liver, lung, spleen, brain, heart, kidney, and stomach revealed no detectable treatment-associated pathological alterations under the investigated conditions. These findings are consistent with the favorable tissue-tolerance observations reported in previous *in vivo* studies of the ZF-148 platform [20]. The absence of detectable toxicity, together with the marked antitumor response, provides an important preclinical safety-efficacy observation for further development of the nanomedicine. The present work should therefore be viewed as a further stage in an established preclinical research pathway rather than as an independent development of a new nanomedicine. Over the recent three-year period, additional animal studies have been undertaken to strengthen the experimental evidence for antitumor efficacy, treatment response, survival, and tissue safety. Together with the previously published findings and the intellectual-property development of the platform [26], the accumulated evidence provides a broader preclinical basis for its continued translational evaluation and progression toward subsequent regulatory and clinical-development steps. Although the proposed nano-bio interface, redox, and thiol-disulfide mechanisms provide a coherent physicochemical and biochemical framework for interpreting the observed antitumor activity, these mechanisms remain hypotheses requiring direct experimental verification. Future studies involving pharmacokinetics, biodistribution, dose-response relationships, intracellular Zn^{2+} measurements, ROS and redox profiling, and dedicated thiol-disulfide analyses will be important for further defining the mechanism and pharmacological profile of ZF-148-2. Overall, the present findings demonstrate a consistent combination of antitumor efficacy, tumor destruction, survival benefit, oral therapeutic activity, and absence of detectable treatment-related systemic toxicity under the experimental conditions investi-

gated, supporting continued preclinical and translational development of this ZnO quantum-dot-based nanomedicine for breast cancer.

6. Conclusion

The present study provides further preclinical evidence supporting the antitumor efficacy, survival benefit, and favorable safety profile of ZF-148-2, the ZnO quantum-dot-based nanodrug investigated in the present breast cancer model. Importantly, ZF-148-2 does not represent a newly modified formulation, but the same nanodrug formulation that has been investigated throughout the preceding research program. The present work therefore constitutes a further stage in a long-term and progressively expanding preclinical investigation of the same nanodrug platform. In the 4T1 murine breast tumor model, ZF-148-2 produced a pronounced inhibition of tumor progression, reducing the mean tumor growth rate from 0.491 ± 0.043 to 0.040 ± 0.068 mm^2/day ($P < 0.0001$). This antitumor effect was accompanied by a significant extension of median survival from 24 to 42 days (log-rank $P = 0.0018$). Histopathological examination further demonstrated extensive tumor destruction, and extensive necrotic changes in treated tumors. Importantly, no detectable histopathological evidence of toxicity was observed in the major organs examined, and were detected under the experimental conditions. Considered together with the safety observations accumulated during the preceding preclinical studies, these findings provide consistent evidence of a favorable preclinical tolerability profile for the formulation. An important translational advantage of this nanodrug platform is its demonstrated activity following oral administration. In the earlier 2022 investigation using the same ZF-148 research platform, oral administration was specifically evaluated in the 4T1/BALB/c breast tumor model and produced a pronounced therapeutic response, with favorable histopathological findings reported in treated animals. This observation is particularly significant for the future development of the nanodrug because oral administration can offer important practical advantages, including greater patient convenience, improved treatment acceptability, and the feasibility of repeated administration without dependence on invasive delivery procedures. Thus, the previously demonstrated oral activity of ZF-148 should be regarded as an important translational characteristic of the ZF-148-2 platform and not merely as an alternative route of administration. The accumulated experimental observations obtained during the long-term development of this nanodrug also provide a basis for proposing a broader mechanistic model. The conceptual foundation of this model was previously introduced by the authors in 2020 in *The Mirror Strategy of Nanoparticles Against the Coronavirus* [27], in which nanoparticle-biological interactions were considered from a redox-chemical perspective, including oxidation-reduction processes and thiol-disulfide interactions. The proposed role of ZnO-based nanoparticles as redox-active nano-oxidizing agents and the possible relevance of this concept to cancer cells were also discussed in that work. The present research program provides an opportunity to extend this earlier chemical

concept into a mechanistic framework for interpreting the antitumor activity of the ZnO-based nanodrug. Based on the cumulative observations obtained over approximately 13 years of investigation of the same formulation, we propose that the antitumor activity of ZF-148-2 is not attributable to a single biological event, but rather to a coordinated physicochemical and redox-mediated interaction between the ZnO quantum-dot-based nanostructure and the altered biochemical environment of tumor cells. Within this proposed model, the nanodrug may interact directly with the tumor-cell environment through its nanoscale surface and physicochemical properties, followed by electron-transfer and redox processes, reactive oxygen species generation, and Zn^{2+} release under intracellular conditions that favor ZnO dissolution. These events may collectively disturb cellular redox balance, protein and membrane integrity, mitochondrial function, and other essential processes required for tumor-cell survival, ultimately promoting apoptotic and extensive tumor-cell death. A central element of this proposed mechanism is the interaction with cellular thiol-disulfide homeostasis. Tumor cells exist under a substantially altered redox state and frequently operate close to the limits of their antioxidant and redox-buffering capacity. We therefore propose that interaction of the ZnO-based nanostructure with biologically important thiol-containing molecules and disulfide bonds may further perturb this already vulnerable redox system. Such redox perturbation may alter the equilibrium between reduced thiol and oxidized disulfide states, interfere with essential protein functions, and increase oxidative stress beyond the compensatory capacity of malignant cells. In this framework, the therapeutic effect would arise not simply from nonspecific toxicity, but from the ability of the nanodrug to exploit the altered physicochemical and redox characteristics of the tumor-cell environment. This proposed redox-mediated mechanism also provides a potential conceptual link between several independently observed biological outcomes of the nanodrug, including cytotoxicity, tumor-growth suppression, extensive tumor necrosis, reduced cellular proliferation, and inhibition of tumor-associated angiogenic features. The model further provides a chemically coherent explanation for why a ZnO quantum-dot-based formulation can exert substantial biological effects without requiring a conventional receptor-targeting ligand. Rather than relying exclusively on molecular recognition by a predefined cellular receptor, the proposed mechanism emphasizes direct physicochemical and redox interactions with the distinctive biochemical environment of malignant cells. Importantly, this mechanistic model is not presented as a mechanism conclusively established by a single experiment. Rather, it represents a scientific hypothesis developed from the cumulative experimental observations obtained throughout the long-term research program and from the redox concept previously proposed by the authors. The consistency of the biological findings across successive preclinical investigations provides a rationale for this model, while direct molecular studies of intracellular redox changes, thiol-disulfide exchange, electron-transfer processes, Zn^{2+} release, and downstream signaling pathways will be required to experimentally validate each component of

the proposed mechanism. Overall, the reproducible antitumor activity, significant survival benefit, absence of detectable major-organ toxicity under the conditions investigated, previous demonstration of oral therapeutic activity, and the mechanistic framework developed from the long-term research program collectively support ZF-148-2 as a promising candidate for further translational development in breast cancer. Particularly noteworthy is the combination of therapeutic activity with the potential for oral administration, which may provide an important practical advantage for future pharmaceutical development and clinical implementation. The next stage should include adequately powered confirmatory studies, dose-response characterization, pharmacokinetic and biodistribution studies, formal repeated-dose toxicological and safety assessment, and direct molecular validation of the proposed redox-mediated mechanism before clinical efficacy and safety can be established in humans.

Author Contributions

Conceptualization, Z.F. (creator and formulator of the breast-cancer nanodrug and holder of the 2019/1398 domestic patent for ZF-148); methodology, Z.F., M.E., and A.M.; validation, Z.F., M.E., A.M., and F.A.; formal analysis, Z.F., M.E., and A.M.; investigation, Z.F., M.E., and A.M. (Dr. Massiha conducted the laboratory and animal model experiments for the original ZF-148, and Dr. Eghtedardoost conducted the laboratory and animal model experiments for the updated nanodrug version, ZF-148-2); resources, Z.F. and M.E.; data curation, Z.F., M.E., and A.M.; writing—original draft preparation, Z.F.; writing—review and editing, Z.F., M.E., A.M., and F.A.; visualization, Z.F. and M.E.; supervision, Z.F. and F.A. (with Z.F. and F.A. also responsible for obtaining the medical ethics code and preparing the clinical trial phase for hospital application); project administration, Z.F.; funding acquisition, Z.F. and M.E. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare that the research was conducted without any commercial or financial relationships, which could be construed as a potential conflict of interest. In addition, the authors declare no potential conflicts of interest and are responsible for the content and writing of the manuscript and publication of this paper.

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Supplementary Material

Historical Preclinical Development and Intellectual Property of the ZF-148 Nanodrug

Supplementary background to the present ZF-148-2 preclinical study

S1.1. Experimental Evaluation of the Original Nanodrug ZF-148 in a 4T1 Murine Breast Cancer Model

The development of the ZnO quantum dot-based nanodrug has followed a long-term research pathway spanning approximately 13 years, progressing from initial *in-vitro* investigations to repeated *in-vivo* studies in experimental cancer models. The early stages focused on the biological activity of ZnO quantum dots and their potential as active nanotherapeutic materials rather than merely as drug-delivery carriers. The initial studies demonstrated the *in-vitro* cytotoxic effects of modified ZnO quantum dots against MCF-7 breast cancer cells, establishing an experimental foundation for their biological application. The research was subsequently extended to additional ZnO nanoformulations and Ag/Fe/ZnO nanocomposites, with their cytotoxic effects evaluated against MCF-7 breast cancer cells. The ZnO quantum dot platform was then investigated in several cancer-cell models together with *in-vivo* biological evaluation. Further studies examined novel ZnO quantum-dot nanoparticles against breast cancer stem-like-cell-enriched mammospheres, including investigations of proliferation, apoptosis, stemness-related characteristics, and relevant signaling pathways. These studies extended the research beyond conventional breast cancer cell models toward cellular populations associated with tumor initiation, recurrence, metastatic behavior, and therapeutic resistance. The research subsequently progressed to animal breast cancer models. The original ZnO quantum dot-based nanodrug, ZF-148, was evaluated in a 4T1 murine breast cancer model over a two-year experimental period (2018-2020). During the first year, different concentrations of the nanodrug were prepared to assess potential side effects, and clinical and laboratory investigations were conducted over six months in laboratory mice at the laboratory of Zist Faravard Pars Company, located in the industrial city of Rasht. The formulation was protected by patent in 2015, and the findings from this phase were subsequently published in Artificial Cells, Nanomedicine, and Biotechnology in 2018 [18].

The 4T1 cell line, obtained as a vial from the Pasteur Institute of Tehran, was used in this animal study. This highly aggressive cell line is derived from a spontaneous mammary tumor in BALB/c mice and exhibits characteristics that make it a suitable model for human breast cancer, including efficient tumor establishment in the mammary gland and spontaneous metastatic progression to lymph nodes, blood, liver, lungs, brain, and bones [9]. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For tumor induction, 1×10^6 cells suspended in 50 µL PBS were injected subcutaneously into the region between the fourth and fifth mammary glands. Following confirmation of tumor development, the animals were randomly divided into five groups of nine mice each: Group A, healthy BALB/c female mice receiving no treatment; Group B,

tumor-bearing BALB/c female mice receiving no treatment; Group C, tumor-bearing BALB/c female mice treated intraperitoneally with 20, 40, and 60 μg/mL of the nanodrug in a 0.5-mL volume every other day after palpable tumor formation; Group D, tumor-bearing BALB/c female mice treated by intravenous (IV) injection; and Group E, tumor-bearing female BALB/c mice receiving the drug orally for two weeks. Throughout the treatment period, vitamin C (1000 mg/day) was added daily to the drinking water of all investigated groups.

Laboratory evaluations included cytological, nutritional, and behavioral studies, as well as measurements of body weight, tumor weight, and serum cholesterol levels. Primary biopsy samples were collected from the affected area upon tumor observation, and a second biopsy was performed after treatment for comparative histopathological analysis. At the end of the study, the tumor-control group showed significant body-weight loss during the experimental period, whereas treated groups partially recovered from tumor-induced weight loss. Post-mortem tumor-weight measurements demonstrated a statistically significant reduction in all nanodrug-treated groups compared with the tumor-control group. Growth-inhibition analysis further demonstrated marked suppression of tumor growth in treated groups relative to untreated controls. Histopathological examination showed that tumors in the control group consisted predominantly of viable tumor cells with only a small necrotic region, whereas the highest degree of necrosis was observed in the nanodrug-treated groups. Immunocyte infiltration within mammary tissue, assessed on hematoxylin and eosin-stained slides, revealed that neutrophils, plasma cells, and lymphocytes were absent in tumor-control tissues but clearly present in the mammary tissue of nanodrug-treated animals. Among the administration approaches evaluated in this initial study, the 40 μg/mL oral regimen administered by gavage for two weeks produced the most favorable response in tumor volume and tumor size reduction compared with the intraperitoneal and intravenous routes. Under the conditions of that study, this regimen resulted in complete tumor eradication and prevented detectable metastasis to other tissues and organs in the treated mice. These findings provided important initial *in-vivo* evidence supporting the antitumor potential of ZF-148 in the aggressive 4T1 breast cancer model. Importantly, this initial 4T1 animal study provided the experimental and preclinical foundation for the subsequent animal investigations. The observed tumor-growth inhibition, tumor eradication, absence of detectable metastasis, and histopathological responses supported continuation of the research program through further *in-vivo* studies designed to obtain additional evidence on therapeutic efficacy, survival, pathological responses, and treatment-related safety. Following this initial study, the research was continued through additional preclinical investigations. For the subsequent animal studies and additional experiments, the same formulation was designated ZF-148-2.

ZF-148-2 is the same formulation as the original ZF-148; the designation "ZF-148-2" was used to distinguish the subsequent studies from the earlier investigations conducted under the name ZF-148 and to indicate the continuation of the

research program.

The recent three-year research phase has therefore continued the original ZF-148 research pathway through further animal investigations. The present study represents a further step in this continuing preclinical investigation of the same ZnO quantum dot-based nanodrug, providing additional evidence on its antitumor efficacy, survival benefit, histopathological response, and treatment-related safety, and contributing to its continued development toward clinical evaluation for breast cancer.

S1.2. Technology Development and Intellectual Property

Since 2013, more than thirteen years of continuous research and development have been devoted to the ZF-148 nanomedicine, encompassing extensive in vitro and *in vivo* biological evaluations conducted in different experimental settings and through research collaborations. The first technological milestone was achieved through the registration of Iranian Patent No. 84862 on 5 February 2015 (05/11/1393), entitled "Production of a Zinc Oxide Quantum Dot Composite Nanodrug and Evaluation of Its *In Vitro* Cytotoxicity Against KB44 Oral Cancer, MCF-7 Breast Cancer, HT29 Colon Cancer, and HeLa Cervical Cancer Cell Lines, Together with an *In Vivo* Mouse Ear-Swelling Standard Test." [25] This patent established the original ZnO quantum dot nanodrug platform and documented its initial anticancer potential through in vitro evaluation against several cancer cell lines and preliminary *in vivo* biological assessment. The findings from the earlier research were reported in the scientific literature in 2014 [14], providing an early published record of the biological investigation of this nanomedicine. The research program subsequently progressed to further biological investigations of the same nanodrug, including studies in the 4T1 breast cancer animal model, as well as studies examining its antitumor effects and different routes of administration in BALB/c mice. A further technological milestone was achieved through the registration of Iranian Patent No. 100349 on 15 February **2020** (26/11/1398), entitled "Formulation and Preparation of a Water-Based Nanofluid Containing Zinc Oxide Quantum Dots for the Treatment of Breast Cancer in BALB/c Mice Using the 4T1 Cell Line." [26] The associated 4T1 animal-model research was subsequently published in 2022 [20] in Advances in Nanoparticles (ANP) under the title "*In Vivo* Animal Model Evaluation of a Powerful Oral Nanomedicine for Treating Breast Cancer in BALB/c Mice Using 4T1 Cell Lines without Chemotherapy." The study reported the evaluation of the ZnO quantum dot-based nanodrug in BALB/c mice bearing 4T1 breast tumors and provided further *in vivo* evidence of its antitumor activity and biological effects.

Following the publication of these 4T1 animal-model findings in 2022 [20], the results attracted considerable attention from scientific and professional communities, including relevant pharmaceutical and medical forums, conferences, and scientific journals. This scientific interest provided further impetus to continue the investigation of the nanodrug in the 4T1 model. Accordingly, additional animal studies were undertaken in collaboration with other experienced research

groups to obtain further and independent evidence regarding its antitumor efficacy, reproducibility, and biological safety. The designation ZF-148-2 was subsequently used for these later animal studies to distinguish the continuing work from the previously registered and reported studies. This designation refers to the same nanodrug formulation investigated throughout the research program. Collectively, the two registered patents and the successive body of published and unpublished preclinical investigations conducted over more than thirteen years document a sustained research program focused on the systematic biological evaluation of this ZnO quantum dot-based nanodrug. The accumulated evidence, including reproducible antitumor activity, favorable histopathological findings, and the absence of detectable major-organ toxicity in the relevant studies, provides a substantial scientific and technological foundation for the continued translational development of ZF-148-2 toward future clinical evaluation.

S2.

"Historical Preclinical *In Vivo* Evaluation of the Original ZF-148 Nanodrug Using the 4T1 Murine Breast Cancer Cell-Line Model (2018-2020; Basis for Publication [20] and the Second Patent Registration in 2020)"

The development of the ZnO quantum dot-based nanodrug has followed a long-term research pathway spanning approximately 13 years, progressing from initial *in-vitro* investigations to repeated *in-vivo* studies in experimental cancer models. The early stages focused on the biological activity of ZnO quantum dots and their potential as active nanotherapeutic materials rather than merely as drug-delivery carriers. The initial studies demonstrated the *in-vitro* cytotoxic effects of modified ZnO quantum dots against MCF-7 breast cancer cells, establishing an experimental foundation for their biological application. The research was subsequently extended to additional ZnO nanoformulations and Ag/Fe/ZnO nanocomposites, with their cytotoxic effects evaluated against MCF-7 breast cancer cells. The ZnO quantum dot platform was then investigated in several cancer-cell models together with *in-vivo* biological evaluation. Further studies examined novel ZnO quantum-dot nanoparticles against breast cancer stem-like-cell-enriched mammospheres, including investigations of proliferation, apoptosis, stemness-related characteristics, and relevant signaling pathways. These studies extended the research beyond conventional breast cancer cell models toward cellular populations associated with tumor initiation, recurrence, metastatic behavior, and therapeutic resistance. The research subsequently progressed to animal breast cancer models. The original ZnO quantum dot-based nanodrug, ZF-148, was evaluated in a 4T1 murine breast cancer model over a two-year experimental period (2018-2020). During the first year, different concentrations of the nanodrug were prepared to assess potential side effects, and clinical and laboratory investigations were conducted over six months in laboratory mice at the laboratory of Zist Faravard Pars Company, located in the industrial city of Rasht. The findings from this phase were subsequently published in *Artificial Cells, Nanomedicine, and Biotechnology: An International Journal* in 2018, and the formulation was subsequently protected by patent.