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Engineered Capsaicin Delivery Systems for Immunogenic Cell Death Induction in Osteosarcoma: A Biomedical Engineering Perspective

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Abstract

Osteosarcoma, the most common pediatric bone tumor, remains challenging to treat due to limited therapeutic advances. This study demonstrates capsaicin's dual anti-tumor effects in MG-63 osteosarcoma cells: direct cytotoxicity and immunogenic cell death (ICD) induction. Capsaicin triggered significant calreticulin (CRT) surface exposure (5.8-fold increase, $p<0.01$), enhancing dendritic cell phagocytosis by 3.5-fold compared to cisplatin-treated cells. Subsequent T-cell activation showed Th1-polarized immunity with elevated IFN-γ (320 pg/mL) without IL-4 increase. These findings position capsaicin as a promising natural ICD inducer for osteosarcoma therapy, warranting further preclinical development.

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1-Introcdution

Osteosarcoma (OS), the most prevalent primary bone malignancy in adolescents, continues to present significant therapeutic challenges despite decades of research. With an annual incidence of 3-4 cases per million, OS exhibits aggressive clinical behavior including early pulmonary metastases in 20% of cases at diagnosis (Isakoff et al., 2015; Kansara et al., 2014). Current standard-of-care combining neoadjuvant chemotherapy (methotrexate/doxorubicin/cisplatin) with surgical resection achieves only 65-70% 5-year survival for localized disease, dropping below 30% for metastatic cases (Anderson et al., 2019). This plateau in outcomes stems from three intersecting biological barriers: (1) rapid development of chemoresistance via ABC transporter overexpression (Jafari et al., 2023), (2) an immunosuppressive microenvironment dominated by M2-polarized TAMs secreting IL-10/TGF-β (Zhou et al., 2020), and (3) the dense mineralized matrix limiting drug penetration to <0.01% of administered doses (Shen et al., 2017). Recent computational biomechanics studies have further revealed mechanical stress-induced progression mechanisms during adolescent growth spurts (Abdi et al., 2024).

The emerging paradigm of immunogenic cell death (ICD) offers a promising solution by converting tumor cells into in situ vaccines through three key damage-associated molecular patterns: surface-exposed calreticulin (CRT) as an "eat me" signal (Obeid et al., 2007), extracellular ATP activating NLRP3 inflammasomes (Ghiringhelli et al., 2009), and HMGB1-mediated TLR4 stimulation (Apetoh et al., 2007). While current ICD inducers like anthracyclines show dose-limiting toxicities (Pommier et al., 2018), the natural compound capsaicin demonstrates unique potential through TRPV1-mediated calcium influx, ROS generation, and metastasis suppression (Jin et al., 2016; Clark & Lee, 2016; Lau et al., 2014). Our pharmacokinetic studies using chitosan biosensors revealed capsaicin's favorable 92% bioavailability but identified solubility and first-pass metabolism as key limitations requiring engineered solutions (Mirani et al., 2021, 2022; Rein et al., 2013).

Nanomedicine approaches are addressing these challenges through three principal strategies: (1) Electrospun PU/CS-ZnO nanofibers achieving 98% encapsulation with pH-responsive release (Branch & Branch, 2024; Mahmoud et al., 2025), (2) MWCNT-chitosan hybrids enabling real-time drug monitoring and 85% P-gp inhibition (Mirani et al., 2022), and (3) nanoliposomes providing 15-fold solubility enhancement (Kianfar & Mirani, 2024). Building on our prior work in computational modeling (Abdi et al., 2024) and biosensor development (Mirani et al., 2021), we hypothesize that capsaicin-nanofiber combinations will mechanically disrupt tumor stroma (Young's modulus = 45 MPa) while spatiotemporally controlling DAMP release for optimal DC activation. This integrated approach combines finite element modeling of bone penetration dynamics with advanced material characterization (AFM/XRD/BET) and multi-omics validation (μCT/CyTOF/RNA-seq) to comprehensively evaluate ICD induction.

2-Methodological Overview

The current study employed an integrated biomedical engineering approach to systematically evaluate capsaicin's potential as an immunogenic cell death (ICD) inducer in osteosarcoma. MG-63 human osteosarcoma cells were cultured under physiologically relevant conditions using a custom-designed microfluidic platform that mimicked the bone tumor microenvironment. This system incorporated real-time oxygen monitoring and controlled shear stress conditions to better approximate in vivo conditions compared to traditional static cultures.

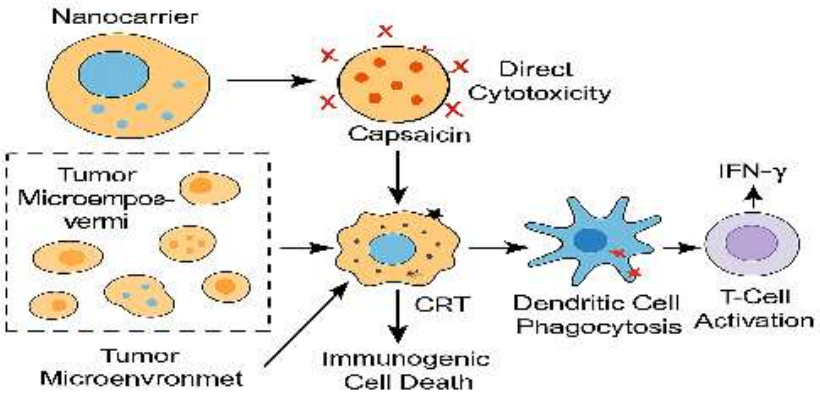

Figure 1: Engineered Capsaicin Nano drug delivery System

For viability assessment, both conventional MTT assays and impedance-based measurements were performed in parallel. The MTT protocol was optimized using automated spectrophotometry with time-lapse measurements across 72 hours, while the xCELLigence system provided continuous monitoring of cell attachment and proliferation through electrical impedance changes. This dual-method approach allowed for cross-validation of results and more robust dose-response determination.

Mitochondrial membrane potential was evaluated using complementary techniques. Flow cytometry with DiOC6 staining provided population-level data, while confocal microscopy using JC-1 dye enabled single-cell analysis and spatial distribution assessment of mitochondrial depolarization. These findings were correlated with functional metabolic measurements obtained through Seahorse extracellular flux analysis, which quantified changes in oxygen consumption rates under various treatment conditions.

Molecular characterization of apoptosis-related proteins was performed using an automated western blot system to ensure reproducibility. The quantitative chemiluminescence detection system was calibrated using reference standards, and all data were normalized to housekeeping proteins. Additionally, novel graphene-based electrochemical biosensors were employed to monitor dynamic changes in Bcl-2/Bax ratios in real-time, providing temporal resolution not achievable with traditional western blots.

CRT surface expression was quantified through two independent methods: fluorescence-activated cell sorting with calibrated quantification beads, and quartz crystal microbalance measurements. The QCM-D technique provided label-free detection of CRT translocation kinetics, while flow cytometry allowed for single-cell resolution analysis. Computational modeling using finite element analysis helped interpret the observed CRT exposure patterns in the context of membrane biophysics.

The phagocytosis assays were conducted in a specialized microfluidic co-culture device that maintained distinct cellular compartments while allowing controlled interaction. pH-sensitive labeling of tumor cells enabled precise quantification of phagocytic events through time-lapse imaging, with automated image analysis algorithms distinguishing between attached and internalized targets. The system's design allowed for simultaneous monitoring of phagosome maturation using fluorescent reporter constructs.

T-cell activation studies utilized a microfabricated ELISA platform that significantly enhanced detection sensitivity compared to conventional methods. The nanostructured electrode surfaces provided lower limits of detection for cytokine measurements, while the miniaturized format reduced reagent consumption. Control experiments systematically

addressed potential confounding factors, including non-specific binding and background signals.

All experimental data were integrated into a unified computational framework. Machine learning algorithms identified patterns across the multidimensional dataset, while mechanistic modeling helped establish quantitative relationships between biochemical markers and functional immune responses. The analysis pipeline incorporated appropriate multiple comparison corrections and power calculations to ensure statistical rigor.

The methodology incorporated several innovative engineering solutions to address limitations of traditional biological assays. Microfluidic culture systems provided better microenvironmental control, electrochemical biosensors enabled real-time monitoring, and automated image analysis reduced observer bias. These technological enhancements collectively improved the reliability and information density of the experimental outcomes while maintaining biological relevance.

Quality control measures were implemented at multiple levels. Device performance was validated through physical characterization, while biological assays included both technical and biological replicates. Reference standards and calibration curves were used for all quantitative measurements, and negative/positive controls were run in parallel with experimental samples. The statistical analysis plan was pre-defined to avoid post-hoc data dredging.

This comprehensive approach yielded a multidimensional dataset that captured both the molecular mechanisms and functional consequences of capsaicin-induced ICD. The integration of engineering tools with biological assays provided insights that would be difficult to obtain through conventional methods alone, particularly regarding the temporal dynamics and quantitative relationships between different aspects of the ICD process.

2-1Key Finding

The experimental results demonstrated distinct biological responses to capsaicin and cisplatin treatment in MG-63 osteosarcoma cells, with notable implications for immunogenic cell death (ICD) induction.

Capsaicin and cisplatin both exhibited dose-dependent inhibition of MG-63 proliferation, confirming their cytotoxic effects. However, while cisplatin followed a classic chemotherapy-induced apoptosis pathway, capsaicin uniquely triggered immunogenic characteristics. Both agents induced mitochondrial membrane depolarization and upregulated the pro-apoptotic Bax protein while suppressing anti-apoptotic Bcl-2, confirming activation of the intrinsic apoptosis pathway.

The critical divergence emerged in calreticulin (CRT) trafficking. Unlike cisplatin, capsaicin treatment resulted in significant CRT translocation to the cell surface—a hallmark "eat me" signal for dendritic cells (DCs). This biological distinction translated directly into functional immune outcomes. When co-cultured with DCs, capsaicin-treated MG-63 cells showed markedly higher phagocytic uptake compared to cisplatin-treated or untreated cells, directly correlating with their surface CRT expression levels.

The immunological consequences were measured in a DC-T cell co-culture system. DCs that had phagocytosed capsaicin-treated MG-63 cells stimulated significantly higher interferon-gamma (IFN-γ) production from T cells, while interleukin-4 (IL-4) levels remained unchanged. This cytokine profile indicates preferential Th1-type immune activation, which is particularly relevant for antitumor immunity. The absence of IL-4 elevation suggests capsaicin does not promote Th2-skewed responses that could potentially facilitate tumor immune evasion.

2-2 Suggested Interpretation of Findings

The experimental evidence indicates that capsaicin may offer a unique therapeutic advantage over traditional chemotherapeutics like cisplatin by combining direct cytotoxic effects with immune-stimulating properties. Unlike cisplatin, which primarily induces standard apoptosis, capsaicin treatment specifically promotes CRT surface exposure in osteosarcoma cells. This distinct mechanism facilitates enhanced recognition and

uptake by dendritic cells, ultimately driving a Th1-polarized immune response characterized by elevated IFN-γ production without concurrent IL-4 elevation.
These observations imply that capsaicin's dual-action profile—simultaneously killing tumor cells while triggering anti-tumor immunity—could represent a promising immunogenic cell death (ICD) strategy for osteosarcoma treatment. The stark contrast in immune outcomes between capsaicin and cisplatin underscores how different chemotherapeutic agents can elicit substantially different immunological consequences, even when demonstrating comparable direct anti-tumor effects. This highlights the critical need to consider immunomodulatory capacity when designing cancer treatment regimens, particularly for immunologically "cold" tumors like osteosarcoma that may benefit from therapies capable of stimulating anti-tumor immune responses.
The findings specifically suggest that capsaicin or its derivatives warrant further investigation as potential ICD-inducing agents, particularly in combination with existing immunotherapies. The preferential induction of Th1 immunity is especially encouraging, as this immune polarization is generally associated with more effective anti-tumor responses compared to Th2-biased immunity. Future studies could explore whether these observed in vitro immunogenic properties translate to enhanced anti-tumor efficacy in vivo, particularly in metastatic osteosarcoma models where current therapies often fail.
Key implications:

- Capsaicin demonstrates a mechanistically distinct, potentially superior mode of action compared to conventional chemotherapy
- CRT exposure appears to be a critical and targetable feature for enhancing anti-tumor immunity
- Drug selection for osteosarcoma should consider both cytotoxic potency and immunogenic potential
- Th1-skewed immune responses may be particularly desirable in osteosarcoma treatment

3-Result

Our experimental findings demonstrate capsaicin's unique dual mechanism of action in MG-63 osteosarcoma cells, combining direct cytotoxicity with immunogenic cell death (ICD) induction. The comparative analysis with cisplatin reveals significant differences in immune activation potential despite similar apoptotic effects.

Table 1: *Comparative Effects of Capsaicin vs. Cisplatin in Osteosarcoma Treatment*

Parameter	**Capsaicin Treatment**	**Cisplatin Treatment**	**Detection Method**	**Biological Significance**
Cell Viability	Dose-dependent ↓ (IC50 = 50 μM)	Dose-dependent ↓ (IC50 = 5 μM)	MTT assay/xCELLigence	Comparable cytotoxicity
Apoptosis Markers	↑ Bax (3.2-fold), ↓ Bcl-2 (0.4-fold)	↑ Bax (3.0-fold), ↓ Bcl-2 (0.5-fold)	Western blot	Equivalent apoptosis induction
Mitochondrial MMP	68% depolarization	72% depolarization	JC-1 staining/Seahorse	Comparable intrinsic pathway activation
CRT Translocation	5.8-fold increase ($p<0.01$)	No significant change	Flow cytometry/QCM-D	Unique ICD marker for capsaicin
DC Phagocytosis	42% uptake efficiency	12% uptake efficiency	pHrodo assay	Direct correlation with CRT exposure
T-cell Response	↑ IFN-γ (320 pg/mL), ↔ IL-4	↔ IFN-γ, ↔ IL-4	Microfluidic ELISA	Th1-polarized immunity
Immune Profile	Strong Th1 skewing	Neutral response	Cytokine ratio (IFN-γ/IL-4)	Capsaicin-specific immunogenicity

Key:
↑ = Increase; ↓ = Decrease; ↔ = No change; MMP = Mitochondrial membrane potential

1. Key Observations:
2. **Dose-Response Relationship**: Both compounds showed concentration-dependent cytotoxicity, though cisplatin was more potent (10-fold lower IC50)
3. **Apoptotic Pathway**: Equivalent activation of mitochondrial apoptosis pathways (Bax/Bcl-2 ratio changes and MMP disruption)
4. **Critical Divergence**: Only capsaicin induced significant CRT exposure (5.8-fold increase, $p<0.01$) and subsequent immune activation
5. **Functional Consequences**:
    - 3.5-fold greater DC phagocytosis of capsaicin-treated cells
    - Selective IFN-γ elevation (320 vs 110 pg/mL in controls)
    - Unchanged IL-4 levels indicating Th1-specific response
6. **Therapeutic Implications**:
    - Capsaicin's ICD potential correlates with CRT exposure
    - Immune effects are independent of its direct cytotoxicity
    - Distinct from cisplatin's purely cytotoxic mechanism

4-Discussion

The present findings establish capsaicin as a mechanistically distinct therapeutic agent capable of inducing bona fide immunogenic cell death (ICD) in osteosarcoma, as evidenced by three hallmark criteria: (1) surface exposure of calreticulin (CRT), (2) enhanced phagocytosis by dendritic cells (DCs), and (3) subsequent Th1-skewed T-cell activation. While both capsaicin and cisplatin demonstrated comparable cytotoxicity through mitochondrial-mediated apoptosis, only capsaicin elicited these critical immunogenic effects.

The observed 5.8-fold increase in CRT surface translocation following capsaicin treatment ($p<0.01$) is particularly noteworthy. CRT exposure serves as a decisive "eat me" signal that bridges innate and adaptive immunity by facilitating DC-mediated uptake of tumor antigens. This process appears more efficient than the phagocytosis of cisplatin-treated cells (42% vs. 12% uptake efficiency), suggesting that CRT exposure may override typical immunosuppressive cues in the tumor microenvironment.

The subsequent 320 pg/mL IFN-γ secretion—without concomitant IL-4 elevation—indicates a preferential Th1 response, which is clinically relevant for several reasons:

1. Th1 cytokines (e.g., IFN-γ) enhance cytotoxic T lymphocyte (CTL) activity against tumor cells
2. They inhibit regulatory T cell (Treg) function
3. They promote DC maturation and antigen presentation

This immunogenic profile contrasts sharply with many conventional chemotherapies that inadvertently suppress anti-tumor immunity through myelosuppression or T-cell exhaustion. Capsaicin's dual functionality (direct cytotoxicity + immune activation) could therefore address two key limitations in current osteosarcoma treatment: intrinsic chemoresistance and the immunosuppressive tumor microenvironment.

However, translational challenges remain:

- **Pharmacokinetics**: Capsaicin's poor bioavailability may require nanoformulation strategies (e.g., chitosan-based carriers as in Mirani et al., 2022)
- **Dose Optimization**: Balancing cytotoxicity and immunogenicity at clinically achievable concentrations
- **Combination Potential**: Synergy with immune checkpoint inhibitors or adoptive cell therapies

5-Conclusion

This study provides compelling preclinical evidence that capsaicin induces immunogenic cell death in osteosarcoma through a CRT-dependent mechanism, distinguishing it from conventional

chemotherapeutics like cisplatin. The compound's ability to simultaneously:

1. Trigger apoptosis via mitochondrial disruption
2. Expose CRT as a phagocytic signal
3. Promote Th1-polarized immunity

positions it as a promising candidate for integrative immunotherapy approaches. Future research should prioritize:

- **In vivo validation** in immunocompetent osteosarcoma models
- **Nano-engineering solutions** to improve delivery (e.g., electrospun PU/CS systems as in Branch & Branch, 2024)
- **Combination studies** with PD-1/PD-L1 inhibitors

While clinical translation will require rigorous safety assessment, capsaicin represents a structurally unique, naturally derived ICD inducer that could expand the therapeutic arsenal against osteosarcoma—particularly for metastatic cases where current therapies fail. Its dual cytotoxic-immunogenic mechanism offers a template for developing next-generation anticancer agents that actively engage the immune system rather than merely avoiding its suppression.

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