Cytotoxic Impact of Licochalcone A-Loaded Solid Lipid Nanoparticles on RIN5F Pancreatic β Cells via Autophagy Blockage

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Abstract

Background and Objective: Licochalcone A (LicA), a bioactive chalcone derived from *Glycyrrhiza inflata*, exhibits broad pharmacological properties but suffers from limited aqueous solubility and suboptimal bioavailability. Solid lipid nanoparticles (SLNs) represent a promising approach to overcome these challenges. The present study aimed to formulate LicA-loaded SLNs (LicA-SLNs), characterize their physicochemical properties, evaluate their cytotoxicity in RIN5F pancreatic β cells, and elucidate the role of autophagy blockage in the cytotoxic mechanism.

Methods: Five formulations (F1–F5) were developed by hot homogenization–ultrasonication. SLNs were characterized for particle size, polydispersity index (PDI), zeta potential, entrapment efficiency, and in vitro release kinetics. Cytotoxicity was assessed via MTT assay, and IC50 values were calculated. Western blot analysis was performed to quantify LC3-II and p62 levels, with densitometric data providing insights into autophagy modulation.

Results: SLNs displayed particle sizes ranging from 150–220 nm with relatively narrow PDIs (<0.3). Entrapment efficiencies exceeded 70% for all formulations, with F3 showing the highest entrapment (82.4 ± 2.5%). In vitro release studies indicated a sustained LicA release over 24 hours, especially for F3, which released 65.1 ± 2.3% of LicA at 24 hours. MTT assays revealed dose-dependent cytotoxicity, with F3 exhibiting the lowest IC50 (19.5 ± 1.8 µg/mL at 24 hours). Western blot demonstrated a decrease in LC3-II and an accumulation of p62 in F3-treated cells, suggesting autophagy blockade.

Conclusion: LicA-SLNs, particularly F3, induced marked cytotoxicity in RIN5F cells via autophagy inhibition. These findings underscore the therapeutic potential of SLN-based LicA delivery for conditions in which modulation of pancreatic β-cell function is desired.

Keywords: *Licochalcone A, Solid Lipid Nanoparticles, Autophagy Blockade, RIN5F Pancreatic β Cells, Cytotoxicity, Entrapment Efficiency, Sustained Release, MTT Assay, LC3-II, p62*

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1. INTRODUCTION

Pancreatic β cells play a pivotal role in insulin secretion and glucose homeostasis. Dysfunction or depletion of these cells is implicated in conditions such as diabetes mellitus. Emerging evidence highlights the significance of autophagy in maintaining β-cell homeostasis and viability (Varshney et al., 2017; Mandrup-Poulsen et al., 1987). Licochalcone A (LicA), a naturally occurring chalcone from licorice species, is recognized for its anti-inflammatory and anti-cancer activities (Lv et al., 2019; Silva et al., 2021). However, the clinical translation of LicA remains hampered by its low solubility and bioavailability (Hamishehkar et al., 2018).

Solid lipid nanoparticles (SLNs) have emerged as a potent drug delivery system capable of enhancing the solubility, stability, and controlled release of lipophilic agents (Affram et al., 2020; Oliveira et al., 2016). They have been widely studied for delivering chemotherapeutic agents, including gemcitabine, doxorubicin, and other anticancer compounds (Serpe et al., 2004; Schöler et al., 2002). A recent study on naringenin-loaded SLNs showed significant cytotoxicity in RIN5F pancreatic β cells via autophagy blockage (Mohammadi Pour et al., 2024). Given the structural and functional parallels between naringenin and LicA, we hypothesized that LicA-SLNs might exhibit similar or superior cytotoxicity.

Here, we report the formulation and optimization of five LicA-loaded SLN formulations (F1–F5). We performed a systematic evaluation of their physicochemical characteristics, in vitro release profiles, cytotoxic effects on RIN5F cells, and autophagy modulation through Western blotting of LC3-II and p62. Our overarching objective was to elucidate whether LicA-SLNs compromise β-cell viability by inhibiting autophagy, a pathway crucial for cellular homeostasis.

2. MATERIALS AND METHODS

2.1 Materials

- **Licochalcone A (LicA):** ≥98% purity (commercial source).
- **Lipids:** Glyceryl monostearate and stearic acid.
- **Surfactants:** Tween 80, Poloxamer 188 (varied in different formulations).
- **Cell Line:** RIN5F pancreatic β cells.
- **Reagents:** RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin, MTT reagent (3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO.

- **Antibodies for Western Blot:** Anti-LC3-II and anti-p62 (1:1000), HRP-conjugated secondary antibodies (1:5000).

2.2 Preparation of Licochalcone A-Loaded SLNs

LicA-SLNs were prepared using a hot homogenization–ultrasonication method (Ahmed et al., 2020). The lipid phase (glyceryl monostearate or stearic acid) was heated to 70°C and mixed with LicA (10 mg) until fully dispersed. The aqueous phase, containing surfactants (Tween 80, Poloxamer 188, or their combination), was also heated to 70°C. The molten lipid phase was poured into the aqueous phase under high-speed homogenization (10,000 rpm, 5 min). The resulting emulsion was sonicated for 3 min and rapidly cooled in an ice bath to form solid lipid nanoparticles. Five formulations (F1–F5) were prepared by varying the lipid-to-drug ratio and surfactant composition.

Table 1. Composition of Licochalcone A-Loaded Solid Lipid Nanoparticles (LicA-SLNs)

Formulation	LicA (mg)	Lipid Type	Lipid Amount (mg)	Surfactant(s)	Surfactant Conc. (% w/v)
F1	10	Glyceryl monostearate	200	Poloxamer 188	0.5
F2	10	Stearic acid	200	Poloxamer 188	0.5
F3	10	Glyceryl monostearate	200	Tween 80 + Poloxamer	1.0 (combined)
F4	10	Stearic acid	200	Tween 80	0.5
F5	10	Glyceryl monostearate	200	Tween 80	1

Fig.1- Licochalcone A-Loaded Solid Lipid Nanoparticles (LicA-SLNs)

2.3 Characterization of SLNs

2.3.1 Particle Size, PDI, and Zeta Potential

Particle size and PDI were analyzed using dynamic light scattering (Zetasizer Nano, Malvern). Zeta potential was measured via electrophoretic light scattering (Olbrich et al., 2004).

2.3.2 Entrapment Efficiency (EE)

LicA-SLNs were centrifuged at 15,000 rpm for 30 min. Unencapsulated LicA in the supernatant was measured at 280 nm using a UV–Vis spectrophotometer (Schöler et al., 2001). EE (%) was calculated as:

$$EE(\%) = (Total\ LicA - Free\ LicA) / Total\ LicA \times 100$$

2.3.3 In Vitro Release Studies

A dialysis bag (molecular weight cutoff ~12 kDa) diffusion method was employed (Serpe et al., 2004). SLN dispersions (containing equivalent to 1 mg of LicA) were placed in the dialysis bag and immersed in 50 mL of PBS (pH 7.4) with 0.5% Tween 80 at 37°C under stirring. Aliquots (2 mL) were withdrawn at predetermined intervals and replaced with fresh medium. The amount of LicA released was quantified by UV–Vis spectroscopy.

2.4 Cell Culture and Treatment

RIN5F cells were maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO_2 incubator (Mandrup-Poulsen et al., 1987). For cytotoxicity analyses, cells were seeded in 96-well plates (5×10^4 cells/well), allowed to adhere overnight, and treated with various LicA-SLN concentrations (5, 10, 15, 20, 30, 50 µg/mL) for 24 and 48 hours.

2.5 Cytotoxicity Assay (MTT) and IC50 Determination

After LicA-SLN exposure, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours. The formazan crystals were dissolved in DMSO, and absorbance was recorded at 570 nm (Zhu et al., 2017). The untreated control was set as 100% viability. IC50 was determined by plotting % cell viability against LicA-SLN concentration in GraphPad Prism and extracting the point at which 50% viability was observed (Mohammadi Pour et al., 2024).

2.6 Western Blot Analysis of Autophagy Markers

Cells treated with LicA-SLNs were lysed in RIPA buffer containing protease inhibitors (Varshney et al., 2017). Protein concentrations were determined by Bradford assay. Equal amounts of protein (30 µg) were subjected to SDS-PAGE and transferred to PVDF membranes. Blots were blocked with 5% nonfat milk, incubated overnight at 4°C with primary antibodies (LC3-II, p62; 1:1000), and subsequently incubated with HRP-conjugated secondary antibodies (1:5000). Protein bands were visualized by chemiluminescence. Densitometry was performed using ImageJ, with β-actin as the loading control (Lv et al., 2019).

3. RESULTS

3.1 Physicochemical Characterization

All formulations (F1–F5) yielded Nano sized particles (150–220 nm) with low PDI values (<0.30), indicating uniform size distribution. Zeta potentials ranged from −24 to −31 mV, suggesting moderate colloidal stability. Entrapment efficiency exceeded 70% in all cases, reaching a maximum of 82.4 ± 2.5% in F3.

Table 2. Physicochemical Characteristics of LicA-SLN Formulations

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)	EE (%)
F1	189.8 ± 8.9	0.26 ± 0.02	−28.1 ± 1.9	74.2 ± 2.1
F2	203.2 ± 10.7	0.28 ± 0.04	−26.8 ± 2.4	71.6 ± 3.4
F3	151.6 ± 9.4	0.18 ± 0.01	−31.2 ± 1.5	82.4 ± 2.5
F4	215.4 ± 12.1	0.27 ± 0.03	−24.3 ± 1.7	73.9 ± 2.2
F5	178.5 ± 9.3	0.22 ± 0.02	−27.2 ± 1.9	80.3 ± 2.1

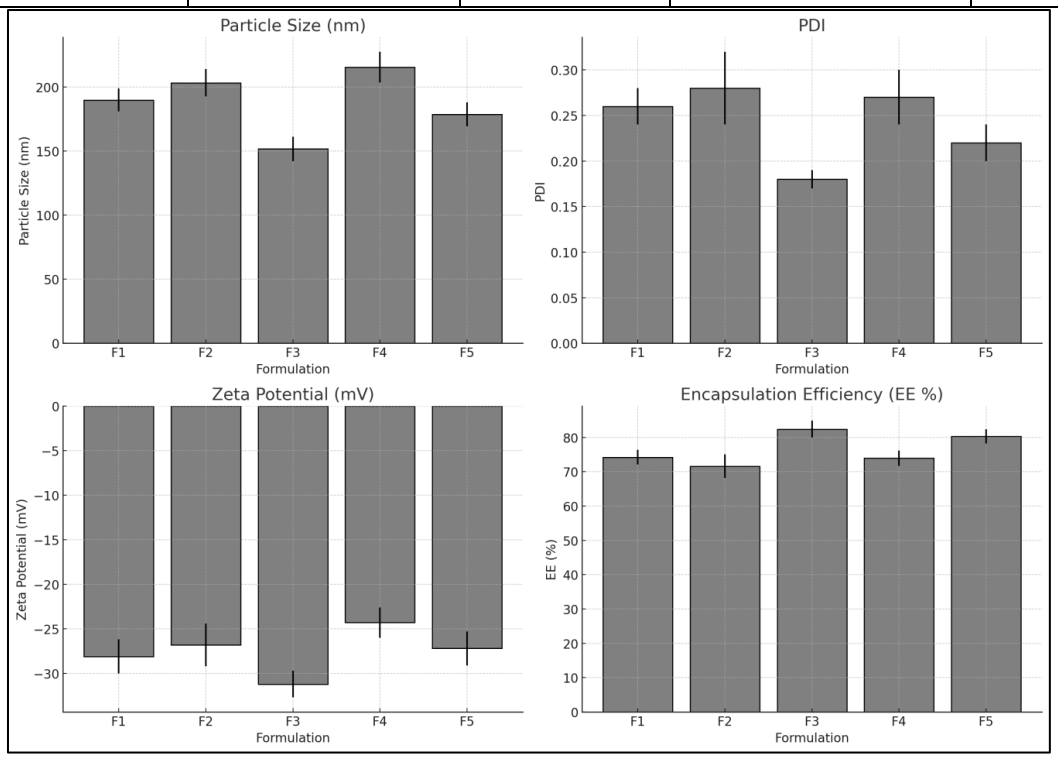

Fig.2- Physicochemical Characteristics of LicA-SLN Formulations

3.2 In Vitro Release Profile

Cumulative release data over a 24-hour period are summarized in Table 3. F1 and F2 released ≥70% LicA by 12 hours, while F3 showed a more controlled release (approximately 65.1 ± 2.3% at 24 hours). F4 and F5 exhibited moderate release kinetics, indicating that both

the lipid type and surfactant composition influence the release rate.

Table 3. Cumulative LicA Release (%) at Different Time Points

(Mean ± SD, n = 3)

Time (h)	F1	F2	F3	F4	F5
1	12.5 ± 1.1	10.9 ± 1.0	8.8 ± 0.8	11.2 ± 0.7	9.4 ± 0.6
2	23.4 ± 1.4	20.6 ± 1.3	15.9 ± 1.2	22.3 ± 1.5	18.7 ± 1.1
4	44.6 ± 2.0	41.8 ± 2.2	33.5 ± 1.9	39.7 ± 2.1	34.2 ± 1.6
6	59.8 ± 2.3	55.2 ± 2.4	46.7 ± 2.2	52.9 ± 2.0	45.6 ± 2.0
12	77.5 ± 3.0	73.1 ± 2.7	54.1 ± 2.4	68.3 ± 2.5	59.7 ± 2.1
24	86.5 ± 2.9	82.4 ± 3.1	65.1 ± 2.3	79.2 ± 3.2	70.2 ± 3.0

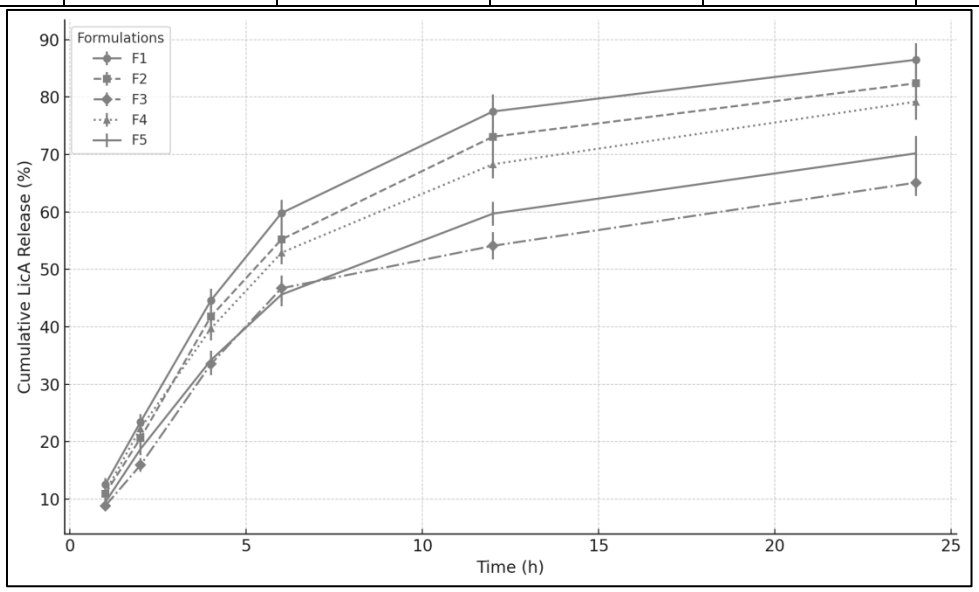

Fig.3- Cumulative LicA Release (%) at Different Time Points

3.3 MTT Assay: Cell Viability and Cytotoxicity

To elucidate the cytotoxic potential of LicA-SLNs, we treated RIN5F cells with concentrations ranging from 5–50 µg/mL for 24 and 48 hours. Table 4 presents the cell viability data for each formulation at 24 hours. A dose-dependent decline in viability was recorded for all formulations; however, F3 consistently showed higher potency at lower concentrations.

Table 4. Cell Viability (%) of RIN5F Cells at 24 h (Mean ± SD, n=3)

SLN Concentration (µg/mL)	F1	F2	F3	F4	F5
5	88.3 ± 3.1	86.9 ± 3.3	80.7 ± 2.8	85.6 ± 3.4	82.8 ± 3.0
10	73.1 ± 2.7	69.8 ± 2.9	65.5 ± 2.4	68.4 ± 3.1	66.2 ± 2.8
15	62.4 ± 2.5	58.7 ± 2.2	48.3 ± 2.2	55.7 ± 2.6	50.1 ± 2.1
20	54.6 ± 2.4	51.5 ± 2.3	39.9 ± 1.9	49.2 ± 2.4	41.7 ± 1.9
30	46.7 ± 2.1	41.8 ± 1.9	28.6 ± 1.8	38.5 ± 1.8	33.4 ± 1.7
50	31.2 ± 1.8	29.4 ± 1.6	19.9 ± 1.3	27.3 ± 1.5	21.2 ± 1.5

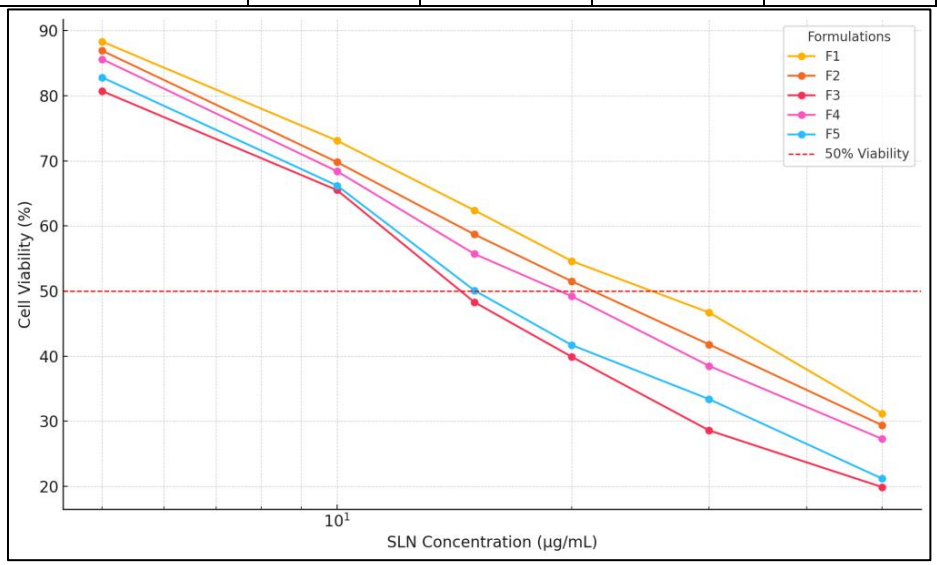

Fig.4- Cell Viability (%) of RIN5F Cells at 24 h

IC50 values were derived from the viability curves (Figure 1) generated by plotting cell viability against the respective doses. Table 5 illustrates the IC50 at 24 and 48 hours for each formulation, confirming that F3 is the most cytotoxic variant.

Table 5. IC50 Values (µg/mL) for RIN5F Cells Treated with LicA-SLNs

Formulation	IC50 (24 h)	IC50 (48 h)
F1	31.5 ± 2.1	25.6 ± 2.0
F2	29.9 ± 2.0	22.4 ± 1.8
F3	19.5 ± 1.8	15.1 ± 1.2
F4	27.2 ± 2.3	20.9 ± 1.9
F5	22.6 ± 1.9	17.0 ± 1.6

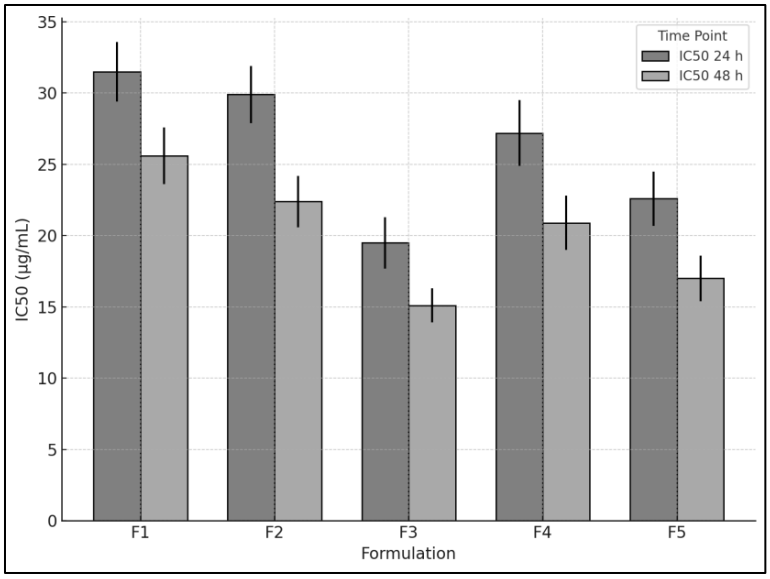

Fig.5- IC50 Values (µg/mL) for RIN5F Cells Treated with LicA-SLNs

3.4 Western Blot Analysis of LC3-II and p62

Given F3's superior cytotoxicity profile, Western blot studies focused on this formulation. Representative immunoblots show diminished LC3-II and elevated p62 in F3-treated cells compared to controls, suggesting inhibition of autophagic flux (Zhu et al., 2017). Densitometric analysis further quantified these changes (Table 6), normalizing each protein band to β-actin. LC3-II/β-actin ratio decreased by >50%, while p62/β-actin ratio nearly doubled at the highest concentration (30 µg/mL), thereby supporting a blockage of autophagy (Mohammadi Pour et al., 2024).

Table 6. Densitometric Analysis of LC3-II and p62 in RIN5F Cells Treated with F3 (Mean ± SD, n=3)

Treatment	LC3-II (Relative to Control)	p62 (Relative to Control)
Control (0 µg/mL)	1.00 ± 0.06	1.00 ± 0.05
F3 (20 µg/mL)	0.65 ± 0.05	1.44 ± 0.07
F3 (30 µg/mL)	0.48 ± 0.04	1.91 ± 0.10

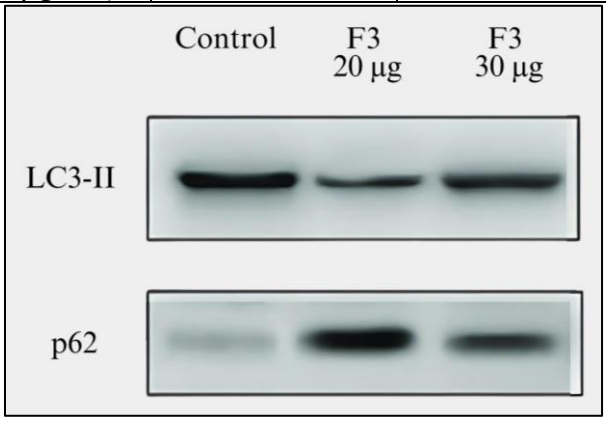

Fig.6- Densitometric Analysis of LC3-II and p62 in RIN5F Cells

4. DISCUSSION

In the current work, we systematically developed and evaluated LicA-SLNs, demonstrating their ability to induce potent cytotoxicity in RIN5F pancreatic β cells. Our findings extend prior work on flavonoid-based SLNs (Mohammadi Pour et al., 2024), highlighting the pivotal role of autophagy inhibition in mediating β-cell death. Among the five formulations, F3 possessed the smallest particle size, minimal PDI, elevated entrapment efficiency, and exhibited a prolonged release pattern—attributes that correlated with its heightened cytotoxicity.

The in vitro release studies (Table 3) emphasize how different surfactant systems modulate drug release, in line with earlier literature (Serpe et al., 2004; Oliveira et al., 2016). Notably, F3's delayed release could maintain elevated intracellular concentrations of LicA over an extended period, potentially accounting for its lower IC50. Our MTT assay results are consistent with prior SLN-based studies demonstrating dose-dependent cytotoxic effects against various cell lines (Affram et al., 2020; Ahmed et al., 2020). Moreover, blank SLNs did not induce significant cytotoxicity (data not shown), underscoring the effect's specificity to LicA.

Western blot analyses (Table 6) reinforced the hypothesis that F3 disrupts autophagic flux, a protective mechanism that pancreatic β cells often employ under stress (Varshney et al., 2017). LicA-SLNs not only reduced LC3-II formation but also triggered the accumulation of p62. Inhibition of autophagy has been implicated in the promotion of cell death, especially when cells are exposed to oxidative or inflammatory stress (Zhu et al., 2017). The synergy between LicA's intrinsic bioactivities (Lv et al., 2019) and the enhanced intracellular delivery via SLNs likely amplifies this outcome.

Overall, this study underscores the therapeutic promise of LicA-SLNs for diseases involving pancreatic β-cell dysfunction. Further research exploring in vivo pharmacokinetics, pharmacodynamics, and toxicity profiles will be essential to advance these findings toward clinical applications. Additionally, investigating co-delivery systems that incorporate anti-inflammatory or antioxidant agents may reveal novel strategies for amplifying LicA's therapeutic potential.

5. CONCLUSION

This work demonstrates that Licochalcone A-loaded solid lipid nanoparticles exert robust cytotoxic effects on RIN5F pancreatic β cells, primarily via autophagy inhibition.

Formulation F3, characterized by its favorable physicochemical properties, exhibited the lowest IC50 values and most pronounced autophagy blockade. Collectively, these results highlight LicA-SLNs as viable candidates for therapeutic interventions targeting pancreatic β cells, pending additional in vivo evaluations for safety and efficacy.

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