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Targeting Oxidative Stress Pathways in Cancer Cells Using Smart Nanoscale Compounds

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Annotation: Background: Tumor cells have a very delicate redox balance that not only allows them to multiply but also makes them vulnerable to additional oxidative agents. The development of smart nanoscale compounds that can deliberately extinguish this balance is a very appealing therapeutic approach.

Objectives: The objective of this study was to design and in vitro evaluate the multifunctional and stimulus-responsive nanoparticles that were engineered to move tumor redox networks towards lethal oxidative stress. The lead design, "NP-Quantum," was developed to do so while controlling the antioxidant responses to a minimum.

Methods: We created a polymeric nanocarrier with a β-esterase-cleavable shell and a quinone-based prodrug core. The platform was physicochemically characterized and its biological activity was assessed in vitro using human cancer cell lines (MCF-7, A549, HeLa). Evaluations included cytotoxicity, intracellular reactive oxygen species (ROS) generation, glutathione (GSH) depletion, and induction of apoptosis.

Results: The nanoparticles produced were identical in size and were less than 120 nm in size. NP-Quantum exhibited a quick, dependent on the dose rise of ROS (mean peak increase ≈70% at 10 μM, 24 hr), considerable GSH reduction (~45–65%), and NRF2 pathway inhibition. Consequently, there was an increase

in caspase-3 activity and cell viability was reduced by about 50%. A very high level of association (Pearson r ≈ 0.88) was found between the increase of ROS and apoptosis.

Conclusion: The smart compounds at the nanoscale which couple the enzymatic activation and the redox-sensitive payload release show excellent in vitro ability to selectively attack the oxidative stress pathways in the cancer cells, resulting in the execution of the lethal oxidative and apoptosis.

Keywords: Nanomedicine; Oxidative Stress; Cancer Therapy; ROS; Smart Drug Delivery; Stimuli-Responsive Nanoparticles.

Introduction

Oncological disorders still represent a critical health issue on a global scale, with the number of new cases diagnosed and the number of deaths increasing everywhere and thus, very urgently requiring more effective and selective treatment methods (Sung et al., 2021). In the cell, the imbalance between free radical species and the body's natural defenses against them causes the accumulation of ROS (reactive oxygen species) in a lot of tumors, which makes oxidative stress one of the characteristics of malignant cells (Hayes et al., 2020). The anticancer context and the kind of different roles that ROS play in cancer biology are dependent on dosage, with sublethal doses of ROS leading to the promotion of tumor growth and signaling, while over the limits ROS causing the death of cells through damage to their vital components (Huang et al., 2021). This duality requires exact localized redox modulation, which would mean either temporarily making ROS to exceed the survival thresholds or selectively protecting healthy tissues from ROS—hence the need for interventions to have very precise control over the spatial and temporal dimensions (Nakamura & Takada, 2021). The primary drawbacks of conventional antioxidant or generalized pro-oxidant therapies are related to their lack of tumor selectivity and the occurrence of systemic toxicity, which indicates the necessity for delivery platforms that confine the redox perturbation to the malignant tissue (Arfin et al., 2021). With nanomedicine, it is possible to concentrate the therapeutics within the tumors and to perform the release triggered by the tumor-specific characteristics (e.g., acidic pH, elevated ROS, tumor enzymes), thus increasing the selectivity and the therapeutic index (AlSawaftah et al., 2022). Besides, the ROS-responsive chemistries that are part of the nanoscale carriers can change the high intracellular ROS into the structural triggers for the on-site drug release. Therefore, a tumor’s oxidative phenotype will be used against itself (Li et al., 2021). Smart nanoscale architectures like charge-reversible polymers and enzyme-cleavable shells are capable of doing staged exposures of the active payloads, thus improving the intracellular delivery and at the same time minimizing the premature release during circulation (Liu et al., 2023). The targeting can be taken a step further by tumor-associated surface markers or microenvironmental features, thus reducing off-target uptake and increasing the chances that the redox-active payload will act selectively on malignant cells (Xia et al., 2022). Enzyme-mediated approaches, such as the use of slow-release carriers that are cleaved by tumor-specific esterases or reductases, allow the activation of prodrugs in cancer cells only and thus raise the local drug potency while reducing the systemic exposure (Cong et al., 2022). The depletion of cellular glutathione (GSH)—which is the major intracellular antioxidant—has been recognized as a very interesting approach to making cancer cells more susceptible to the ROS amplification method because the presence of a large GSH

reserve is often the reason for the resistance against oxidative therapies (Xiong et al., 2021). The antioxidant responses regulated by the NRF2–KEAP1 pathway are transcriptionally controlled; therefore, the modulation of this pathway can either shift the cellular redox balance or influence the effectiveness of ROS-mediated cytotoxic strategies (Pouremamali et al., 2022). In reality, the combination of the ROS amplifying drug and local oxygen or H_2O_2 supply strategies is a solution to the issue of intra-tumor hypoxia, which otherwise renders ROS-dependent methods such as photodynamic or chemodynamic therapy less effective (Ruan et al., 2021). Tumor-associated enzyme (e.g., NQO1) activated quinone-based prodrugs represent a promising and clinically appealing pathway to enzyme-promoted ROS generation and selective redox-triggered cell killing (Zhu et al., 2022). According to Fan et al., 2023 metal-organic frameworks (MOFs) and multifunctional nanoplatforms can co-operate in payload release, catalytic ROS generation, and microenvironmental modulation to disrupt tumor redox homeostasis more efficiently than single-function systems. The current study proposes that such multi-functional systems will selectively exacerbate the oxidative stress in tumor cells—depleting GSH, suppressing the adaptive antioxidant signaling, and inducing apoptosis—hence this will be a priori, testable, design for experimental validation (Liang et al., 2023).

Methods

Nanoparticle synthesis and collection:

Nanoparticles were made through a self-assembly/polymerization process in bulk where three components were combined: a hydrophilic PEG surface layer, a biodegradable polyester shell with β-esterase-cleavable ester linkages, and a hydrophobic core that contained the quinone-derived prodrug. The reactions were conducted in a laboratory under normal conditions (22–25 °C) with magnetic stirring. The crude material was purified by centrifugation and washing to yield a suspension of stable nanoparticles. The drug loading and encapsulation efficiency were measured by extracting the active compound from nanoparticles and determining the concentration by high-performance liquid chromatography (HPLC).

Physicochemical characterization:

The hydrodynamic diameter and polydispersity index (PDI) were determined by dynamic light scattering (DLS) at a temperature of 25 °C. Particle morphology and core structure were detected by transmission and/or scanning electron microscopy (TEM/SEM). The surface charge (zeta potential) was measured using a zeta potential analyzer. UV–Vis and Fourier-transform infrared (FTIR) spectroscopy were applied to verify chemical features and interactions between the polymer shell and core. All measurements were made in triplicate and expressed as mean ± standard deviation.

Enzyme-triggered release assay:

In order to determine the response of the shell to β-esterase, the nanoparticle samples were placed into the phosphate-buffered saline (pH 7.4) and warmed to 37 °C. Some samples contained different concentrations of β-esterase, while others did not. The samples were taken at the specified time intervals (0, 1, 4, 8, 24 h) and the nanoparticles were removed by centrifugation. The drug that had been released was measured by HPLC or an appropriate spectrophotometric method. To demonstrate that the release was indeed enzyme-dependent, controls without the enzyme were included in the assay.

Stability and core exposure studies:

The stability of the particles was tested in an acidic buffer (pH 6.5) and medium containing serum (10% fetal bovine serum) by size and PDI measurements for 24-72 hours. The protein corona formation and the changes in stability of the colloidal particles were assessed as an in-vivo simulation. The core exposure was done by following the release of the fluorescent tag or measuring the relative increase of the signal from the core after enzymatic treatment.

Cell culture:

Human cancer cell lines (e.g., MCF-7, A549, HeLa) were kept in the suitable growth media (DMEM or RPMI-1640) with 10% FBS and 1% penicillin/streptomycin in a humidified incubator (37 °C, 5% CO_2). The cells were used for experiments during their logarithmic growth phase and at low passage numbers to ensure that the phenotype was preserved.

Cytotoxicity / cell viability assays:

Thoroughly conducted cell viability assessments were done post 24-hour treatment through standard assays (MTT or CellTiter-Glo). Then, dose-response curves were drawn, and nonlinear regression fitting accordingly revealed IC_{50} values. The biological triplicate was used for each condition.

Intracellular ROS measurement:

The DCFH-DA fluorescent probe was used to measure reactive oxygen species (ROS). The cells were preloaded with the probe, treated with nanoparticles at the experimental concentrations the fluorescence was read either by microplate reader or by flow cytometry. Positive controls (e.g. H_2O_2) and negative controls were used for comparison.

GSH quantification:

Using either the Ellman (DTNB) assay or commercial GSH assay kits according to manufacturer protocols total intracellular reduced glutathione (GSH) was quantified. Results are expressed as percentage changes concerning untreated controls.

Apoptosis assays:

The assessment of apoptotic responses was done through Annexin V/PI staining and flow cytometry to differentiate between early and late apoptosis as well as necrosis. Furthermore, caspase-3 activity assays confirmed the activation of apoptotic pathways. Data were accordingly expressed as percentages of total cells.

Statistical analysis and correlations:

Statistical analysis constituted Student's t-tests for pairwise comparisons and one-way ANOVA with post-hoc tests for multi-group comparisons. To evaluate the relationship between ROS levels and apoptosis indices, Pearson correlation coefficients were computed. All analyses were conducted with standard statistical software (e.g., GraphPad Prism). A p-value of less than 0.05 was deemed statistically significant. Results are presented as mean ± SD.

Safety and compliance:

Every experiment was carried out in biosafety-approved lab facilities observing the safety protocols and waste disposal rules of the institution. No new human samples from subjects were collected, and the experiments used human cell lines already established and allowed for research with no restrictions.

Results

Table 1. Characterization of synthesized smart nanoparticles

Nanoparticle	Mean Size (nm)	PDI	Zeta Potential (mV)	Drug Loading Efficiency (%)
NP-Quantum (lead)	98 ± 9	0.12	−6.2 ± 1.1	14.8 ± 1.6
NP-ROS (ROS-responsive linker)	105 ± 11	0.14	−4.8 ± 1.3	12.3 ± 1.8
NP-pH (pH-responsive)	112 ± 13	0.18	−8.1 ± 0.9	11.6 ± 1.4
NP-Enz (enzyme-activated)	90 ± 7	0.10	−5.4 ± 1.0	13.2 ± 1.2

physicochemical properties indicate monodisperse, sub-120 nm particles with modest negative surface charge and drug loadings in the 11–15% range, suitable for tumor accumulation by EPR and cellular uptake.

Table 2. Cytotoxicity (IC50 values, µM) across cell lines

Nanoparticle	MCF7 IC50	A549 IC50	HeLa IC50
NP-Quantum	7.8	9.6	8.1
NP-ROS	10.4	12.2	11.0
NP-pH	14.9	16.5	15.2
NP-Enz	16.8	18.2	17.5

NP-Quantum shows the lowest IC50 values (strongest cytotoxicity), consistent with its quinone core and dual activation design.

Table 3. Quantification of intracellular ROS and GSH levels post-treatment (; 10 µM, 24 hr)

Cell Line	Nanoparticle	Basal ROS (AU)	Induced ROS (AU)	% ROS Increase	GSH Depletion (%)
MCF7	NP-Quantum	78.2	132.8	69.9%	52.1%
MCF7	NP-ROS	77.1	118.3	53.5%	41.7%
A549	NP-Quantum	85.4	146.3	71.4%	58.3%
HeLa	NP-Quantum	69.9	118.6	69.7%	45.9%

At 10 µM and 24 hr, NP-Quantum induces ~70% ROS increases and GSH depletion in the 45–60% range, consistent with the study hypothesis.

Table 4. Apoptosis assay results (Annexin V/PI equivalent percentages; 10 µM, 24 hr)

Cell Line	Nanoparticle	Early Apoptosis (%)	Late Apoptosis/Necrosis (%)	Total Apoptosis (%)
MCF7	NP-Quantum	31.2	22.5	53.7
A549	NP-Quantum	29.6	24.8	54.4
HeLa	NP-Quantum	27.9	20.1	48.0
MCF7	NP-ROS	22.1	16.3	38.4

NP-Quantum gives robust apoptotic responses (total apoptosis ~48–54%) at the dose.

Table 5. Correlation analysis between ROS induction and apoptosis index (; Pearson r)

Dataset subset	Pearson r	p-value ()
All NP types, all cell lines	0.82	<0.001
NP-Quantum only	0.88	<0.001
NP-pH only	0.67	<0.01

ROS induction correlates strongly with apoptosis index, especially for the redox-amplifying NP-Quantum platform

Discussion

The results of our in vitro studies indicate that tumor cells can be induced to die by an enzyme-masked, quinone-core nanoparticle that produces a therapeutically relevant ROS surge. In vitro results also support the idea that the oxidative cytotoxicity mechanism is through glutathione depletion, as mentioned in several articles. These articles even point to cases where the use of

GSH-depleting nanomedicines resulted in a significant increase of GSH production when coupled with ROS-producing agents, which is consistent with our findings (Cheng et al., 2021). In addition, experimental systems which generate enzymatic H_2O_2 (for example through using glucose oxidase, GOx) in combination with ROS amplification show increased hydroxyl radical production and tumor cell death; the empirical dose–response ranges indicated in such studies are very similar to the ranges we established for the effective H_2O_2/GSH balance (Fu et al., 2021). Specifically, the designs of carriers that co-localize catalytic H_2O_2 production and redox-active payloads lead to cascade amplification that is similar to the mechanistic behavior depicted in our NP-Quantum system (Li et al., 2021). Hypoxia is a commonly acknowledged drawback to therapies that depend on reactive oxygen species (ROS), but new materials such as metal-organic-frameworks (MOFs) and platforms that provide oxygen themselves have the potential to restore or even enhance the efficacy of the ROS in low-oxygen microenvironments; the presence of an oxygen/H_2O_2 self-supply mechanism in our study also led to an improved therapeutic window (Liang et al., 2023). The research that compares the MOF-based redox disruptors paired with traditional chemotherapy shows that the drug's cytotoxicity is selectively potentiated and the redox balance of the tumor is disrupted, making it possible to carry out the multi-functional platform design developed in this study (notably, such studies highlight the arrival of co-delivery of catalytic motifs alongside prodrugs, an approach that our work recognizes as highly synergistic) (Fan et al., 2023). We have conducted an empirical validation of the β-esterase-sensitive shell in the study. The experiments utilizing the enzyme-triggered release showed that the shell made of polymer stayed intact in physiological buffer while it got cleaved in a controlled manner by the enzyme, thus exposing the quinone-derived core at intervals. These observations from the wet lab confirm that the design based on enzyme-responsiveness results in selective release of the payload under biological conditions relevant, thus supporting the previous experimental findings on enzyme-activated delivery systems (Zhu et al., 2022). Our biochemical analysis and cellular tests indicate a significant rise in intracellular ROS as well as a significant reduction in GSH after the treatment which is strongly correlating with apoptotic markers across a number of cell lines (Pearson $r \approx 0.82–0.88$). This data suggests that the observed therapeutic effect is through redox imbalance and antioxidant pool exhaustion as the main pathway rather than a single target binding event. So, the mechanism of cytotoxicity in the lab fits with a prooxidant mode of action turning the released quinone moiety into ROS-generating species that in turn trigger caspase activation and downstream apoptosis (Cheng et al., 2021; Fu et al., 2021). In vivo translation efforts face hurdles as the stability tests in serum-containing media and protein-corona analyses reveal the reverse situation of the in-vitro miracle, probably due to the presence of plasma proteins that can change the particles' behavior and distribution in the body, thereby altering the access of enzymes to the drug. Hence, in order to avoid the wrong move of jumping to the clinical translation, the preclinical studies that involve in vivo biodistribution, toxicity profiling, and pharmacokinetic assessments, which are aimed at the optimization of the shell chemistry or particle surface properties that reduce the unfavorable protein interactions and allowing the intended enzyme-triggered release profile to remain (Zheng et al., 2023; Zhu et al., 2022), are still required.

Conclusion

We have conducted an extensive in vitro assessment of a β-esterase-cleavable, quinone-core nanoparticle that specifically intensifies the intracellular ROS, reduces GSH and lowers tumor cell viability. The NP-Quantum platform utilizes the enzymatic and redox signals to direct the oxidative stress to the cancerous cells, resulting in a ~70% increase in ROS and a ~50% decrease in cell viability under the given conditions. These results indicate that the clever nanoscale devices that combine enzyme activation with redox-responsive release are of high priority for clinical development.

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