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IN VIVO IMAGING OF ADENOMAS OF THE COLON

Background and Significance. Approaches for the molecular imaging of disease-associated biomarkers of the colon that take advantage of emerging high-resolution technologies have not yet been fully realized. Currently, most efforts to develop imaging agents for visualization of colorectal cancer (CRC) have focused on peptides or antibodies targeting surface receptors (*e.g.*, the epidermal growth factor receptor) or enzymes in the extracellular milieu (*e.g.*, matrix metalloproteinases).[1-3] These have shown promise but are still struggling at the pre-clinical stages, possibly due to various disadvantages associated with the compounds such as metabolic instability, poor tissue penetration, poor pharmacokinetics, and immunogenicity. To address this challenge, we have developed a nanotechnology-based strategy for *in vivo* colonoscopic imaging of tumors and evaluation of tumor margins in early stages of colorectal carcinogenesis. The strategy is founded on the observation that the enzyme cyclooxygenase-2 (COX-2) is upregulated in malignant tissue from CRC patients but not in surrounding normal tissue.[4, 5] The COX-2 enzyme has both an oxygenase activity that makes the PGG_2 hydroperoxide product and a peroxidase activity that reduces the hydroperoxide of PGG_2 while concomitantly generating an oxidant. Thus, COX-2 activity is associated with the production of reactive oxygen species (ROS) leading to oxidative stress, as indicated by the generation of DNA damage.[6] Furthermore, oxidative stress enhances the expression of nuclear factor κ-B (NF-κB), which, in turn induces increased COX-2 expression in pathological tissues.[7, 8] Consequently, COX-2 and ROS co-localize in tumor cells, and targeted agents capable of dual COX-2 and ROS detection might enhance early detection of premalignant and malignant lesions of the colon.

We ***hypothesize*** *that nanoparticles encapsulating a redox-sensing probe specific for COX-2 would selectively accumulate and be activated in colorectal adenomas, resulting in specific and sensitive early detection of CRC while reducing the background noise.* To test this hypothesis, we have developed fluorocoxib Q (FQ), a redox-sensing optical imaging agent capable of targeting dual COX-2 and ROS biomarkers in an animal model of colorectal adenomas. The packaging of FQ into redox-sensitive and clinically compatible nanoparticles (NPs), improves its deliverability to the tumor site. The prospect of combining structural and functional imaging readouts obtained with fluorescence-aided colonoscopy measurements would provide powerful diagnostic information for management of colorectal carcinogenesis.

Innovation. In clinical practice, technical aspects of endoscopic imaging have a major role in determining polyp detection rates, together with human factors such as the quality of bowel preparation and the skill of the endoscopist. Colonoscopy is currently performed using white light, and polyps are detected by operators who are trained to discriminate polyps from normal colon by recognizing characteristics, such as protrusion into the lumen and mucosal color changes. However, these features are less discriminatory in smaller and non-polypoid lesions, leading to miss rates of up to 25%. Combining targeted molecular probes and advanced imaging technology could improve polyp detection. Several biomarkers and detection systems have shown promise in preclinical trials, but only topically applied agents have thus far been tested in humans. These agents suffer from the major disadvantage that application to the whole surface area of the colon is seldom achievable. If successful, the proposed studies will lead to the development of a systemically administered highly targeted imaging agent that will markedly improve the visualization of early-stage CRC. The specificity of approach relies on the highly innovative application of simultaneous COX-2-targeting and ROS-dependent NP release and FQ activation, both of which are favored in the tumor microenvironment. Considering the prevalence of CRC and the importance of colonoscopy as the primary method for the detection and treatment of early-stage lesions, this approach has a significant potential for future clinical development to reduce morbidity and save lives.

Specific Aims. To further the development of FQ-NPs, we propose the following aims: *Aim 1. Optimize and evaluate FQ-NPs in colonoscopic imaging of adenomas in vivo; Aim 2. Validate the COX-2- and ROS-specificity of FQ-NP-dependent fluorescent signals in colorectal adenomas in vivo and ex vivo.* The long-term goal of this proposal – to establish a nanotechnology for the molecular endoscopic imaging of CRC in animal models and patients – is one the major focus of the Phi Beta Psi Award.

Preliminary Results. We developed FQ by conjugating fluorocoxib A (FA),[9-13] a COX-2-selective fluorescent imaging agent, with the 2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO) radical. FQ is pro-fluorescent due to quenching of the excited electronic state of its carboxy-X-rhodamine moiety by the nitroxide radical within the molecule.[14] FQ inhibits COX-2 selectively in purified enzyme (COX-2 IC_{50} = 0.33 μM, COX-1 IC_{50} > 4 μM) and cell-based (COX-2 IC_{50} = 0.28 μM) assays. When treated with ROS generated by addition of sodium ascorbate or Fenton's reagent, FQ is converted into a highly fluorescent compound (FQ-H). In cell culture, FQ enters cancer cells freely and binds to COX-2, where in response to ROS, it becomes fluorescently activated. FQ

exhibits a high half-life in vivo (plasma $t_{1/2}$ > 24 h) and metabolic stability long enough for target site accumulation. Our initial solubility measurements have shown that FQ is poorly soluble in water. Therefore, for intravenous injection, we developed a copolymeric micellar formulation for FQ. We synthesized the novel di-block poly(propylene sulfide)-b-poly[oligo(ethylene glycol) methyl ether acrylate] (PPS_{106}-*b*-$POEGA_{17}$) copolymer via a combination of anionic and reversible addition-fragmentation chain-transfer (RAFT) polymerization using a scalable procedure that we reported previously.[15] We nano-formulated FQ using the PPS_{106}-*b*-$POEGA_{17}$ copolymer by our bulk solvent evaporation method. The physicochemical characteristics of FQ-NPs (i.e., hydrodynamic diameter, D_h = 80.4 ± 6.3 nm; zeta potential, ζ = -1.2 ± 0.5 mV; or critical micelle concentration, CMC = 0.072 mg/mL) were rigorously evaluated and confirmed that FQ-NPs constitute a fully aqueous formulation ready for intravenous injection. A particular advantage to the selected copolymer is the fact that FQ-NP degradation and subsequent release of FQ are facilitated in the presence of ROS.

Scientific Approach. To further the development of FQ-NPs, and their ultimate translation to the clinic, we must first more fully characterize their practicality and efficacy as a COX-2- and ROS-targeted imaging agent in an in vivo model of CRC. The general strategy of the proposed program is to first optimize the conditions for the use of FQ-NPs to facilitate the detection and surgical margin delineation of azoxymethane (AOM)/ dextran sodium sulfate (DSS)-induced colorectal adenomas in mice. We will then fully evaluate the COX-2- and ROS-specificity of the NP-FQ-generated fluorescence signals in this model.

Aim 1. We will first use our procedures for scale-up synthesis of FQ and PPS_{106}-*b*-$POEGA_{17}$ and then utilize the copolymer for micellar formulation of FQ as described in the preliminary data. We will then optimize conditions for colonoscopic visualization of colorectal adenomas in B6;129 mice bearing AOS/DMM-induced colon adenomas. After administration (by tail vein injection) of NPs to mice (n=6 animals/group) at doses of 1, 5, 10, and 20 mg/kg, isoflurane vapor will be used to induce anesthesia, and colonoscopy will be performed using a Karl Storz endoscopy system at 1, 3, 6 12, 24, 48 and 72 h post-NP-FQ injection. Immunohistochemistry and immunofluorescence analysis will be conducted using formalin-fixed and paraffin-embedded tissue samples obtained from biopsy to correlate FQ uptake with COX-2 expression in samples of normal and neoplastic colonic tissue. These concentration- and time-dependent *in vivo* endoscopic imaging assays will result in an optimal FQ-NP dosing regimen and imaging time for fluorescence-based colonoscopic tumor imaging. Next, we will treat a group of 6 tumor-bearing animals with the best performing FQ-NP dose level from the previous experiments. After colonoscopy at the optimized time point, animals will be euthanized, and adenomatous and normal colon tissues will be excised and evaluated by a tandem liquid chromatography and mass spectrometry (for FQ concentration) and histopathological analysis. Data will be statistically evaluated using an ANOVA with post Hoc Tukey test.

Aim 2. We will verify the specificity of COX-2 binding and the role of ROS in FQ-mediated fluorescence by the means of pre-injecting mice with vehicle alone, NPs containing the COX-2-sective inhibitor celecoxib to block binding of FQ to the enzyme's active site, or TEMPOL, to serve as a ROS scavenger. Mice will then receive FQ-NPs, and colonoscopy will be conducted using the optimized conditions. Substantially reduced signal in mice pretreated with celecoxib or TEMPOL-containing NPs will verify COX-2- and ROS-specificity. Finally, we will validate NPs in surgical margin visualization of colorectal adenomas *ex vivo* by image-guided removal followed by histopathological analysis.

Biological Variables. We will use both female and male mice because CRC is common to both sexes. We will use 6 mice per group; to detect early CRC tumors with 15% standard deviation, 80% power, $P < 0.05$, as determined by consultation with the Vanderbilt Biostatistics Shared Resource Core. The proposed experimental samples and analytes will be blinded to experimentalists by coding them during resection, histopathology, and LC-MS/MS analysis.

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