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NQO1-mediated tumor-selective lethality and radiosensitization for head and neck cancer

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Abstract

Ionizing radiation (IR) is a key therapeutic regimen for many head and neck cancers (HNCs). However, the 5-year overall survival rate for locally-advanced HNCs is ~50% and better therapeutic efficacy is needed. NAD(P)H:quinone oxidoreductase 1 (NQO1) is over-expressed in many cancers, and β -lapachone (β -lap), an unique NQO1 bioactivatable drug, exploits this enzyme to release massive reactive oxygen species (ROS) levels that synergizes with IR to kill by programmed necrosis. β -Lap represents a novel therapeutic opportunity in HNC leading to tumor-selective lethality that will enhance the efficacy of ionizing radiation. Immunohistochemical staining and western blot assays were used to assess the expression levels of NQO1 in HNC cells and tumors. Forty-five percent of endogenous HNCs express elevated NQO1 levels. In addition,

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multiple HNC cell lines and tumors demonstrated elevated levels of NQO1 expression and activity and were tested for anticancer lethality and radiosensitization by β -lap using long-term survival assays. The combination of nontoxic β -lap doses and IR significantly enhanced NQO1-dependent tumor cell lethality, increased ROS, TUNEL positive cells, DNA damage, NAD⁺ and adenosine triphosphate (ATP) consumption, and resulted in significant antitumor efficacy and prolonged survival in two xenograft murine HNC models, demonstrating β -Lap radiosensitization of HNCs through a NQO1-dependent mechanism. This translational study offers a potential biomarker-driven strategy using NQO1 expression to select tumors susceptible to β -lap-induced radiosensitization.

Keywords

NQO1; β -Lapachone; Head and Neck Cancer; Ionizing Radiation

Introduction

Head and neck cancer (HNC) is a major cause of morbidity and mortality in the United States with over 40,000 new cases and 8,000 deaths estimated in 2015 (1). If detected early, the majority of HNCs are highly curable using combined modality therapy, where systemic chemotherapy or the antibody cetuximab is administered concurrently with radiation. However, many patients present with high-risk local or regionally advanced disease, and many of these patients will develop either locally recurrent or metastatic disease, conditions for which there are no optimal therapeutic regimens. Moreover, patients that develop loco-regional cancer recurrence after receiving standard combined modality therapy have dismal prognoses, even with re-irradiation and concurrent chemotherapy (2). Despite the increasing accuracy of radiation dose delivery, it is clear that biological factors intrinsic to certain tumors confer resistance to therapy (3). Thus, there is great interest in integrating a molecular diagnostic-based approach with targeted therapies for HNC to improve therapeutic efficacy by exploiting tumor-selective targets in combination with radiotherapy.

In multiple tumor histologies, the NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase, xip3; E.C.1.6.5.2), is constitutively over-expressed at levels 5- to 200-fold greater in breast, non-small cell lung, pancreas, and prostate compared to associated normal tissues (4-7), and represents a potential therapeutic target. NQO1 is targeted by the unique quinone, β -lapachone (β -lap, 3,4-Dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione) has anti-tumor effects in multiple tumor histologies through a NQO1-dependent mechanism (8) involving a futile partial redox cycling of β -lap by NQO1 between the parental quinone and a reduced hydroquinone, leading to the generation of significant levels of cytotoxic free radical reactive oxygen species (ROS). These ROS in turn induce massive DNA base damage, as well as DNA strand breaks, manifesting as predominately single strand breaks (SSBs) as well as the much more lethal double strand breaks (DSBs) leading to cancer-specific cytotoxicity.

The tumor-selective cytotoxic effects of β -lap occur via a NQO1-dependent μ -calpain-mediated, caspase-independent programmed necrosis pathway that selectively kills cancer

cells containing high levels of NQO1 compared to adjacent normal tissue (8) resulting in PARP-1 hyperactivation and subsequent cell death (7). These effects occur regardless of cell cycle status, caspase activity, or functional status of Rb and p53 (9, 10). Of the thousands of quinones known, only β -lap, deoxynyboquinone (DNQ), and their derivatives, exploit tumor NQO1 expression to cause decreased survival in a wide range of solid cancers (11, 12), resulting in desirable tumor-selective efficacy.

Sublethal doses of β -lap can be used with IR to elicit radiosensitization (13, 14) through PARP-1 hyperactivation-induced cytotoxicity in prostate cancer (6). Moreover, β -lap treatment also elicits the release of a large amount of calcium from the endoplasmic reticulum (ER) and pre-loading cells with BAPTA-AM, a Ca^{2+} chelator, or forced ectopic over-expression of Catalase, partially protects NQO1+ lung, breast, and pancreatic cancer cell lines from β -lap-induced cell death (11, 15, 16).

The clinically available version of β -lap, ARQ761, is currently in a phase I clinical trial for patients with metastatic solid malignant tumors. Pre-clinical studies in mice with a similar formulation (β -lap-HP β -CD) demonstrated dose-limiting toxicity due to hemolysis attributed to the carrier, hydroxylpropyl- β -cyclodextran (HP β -CD), and that β -lap-induced methemoglobinemia contributed to the hemolysis noted *in vivo* (17). Given the ability of low-dose β -lap to radiosensitize NQO1+ cancer cells (13, 14), combined modality radiotherapy and low-dose β -lap has the potential to further enhance and extend the tumor-selective cytotoxicity of this agent. These rationale led to our hypothesis that β -lap has the potential to enhance the efficacy of radiotherapy in HNC in a NQO1-dependent fashion.

Materials and Methods

Reagents and chemicals

β -Lap was synthesized and purified by Dr. William Bornmann (7). Dicoumarol (DIC), hydrogen peroxide (H_2O_2), Hoechst 33342, bovine serum albumin, cytochrome *c*, and Catalase, were purchased from Sigma-Aldrich (St. Louis, MO). 5-(and-6) chloromethyl-2,7-dichlorodihydrofluorescein diacetate (DCFDA) was purchased from Invitrogen Life Technologies (Eugene, OR). BAPTA-AM was purchased from Abcam (Cambridge, MA). HP β -CD (>98% purity) was purchased from Cyclodextrin Technologies Development, Inc. (High Springs, FL). β -Lap-HP β -CD was prepared as described (18).

Cell culture and transfections

All cell lines were obtained as follows: FaDu, Detroit 562, and CAL-27 cell lines, purchased from American Type Culture Collection; JHU029 and JHU022, Dr. David Sidransky (Johns Hopkins University); SqCC/Y1, Tu-138, MSK-922, TR146, 183, 1483, OSC-19, OSC-19LN1, OSC-19LN2, OSC-19LN3, OSC-19LN4, OSC-19LN5, MDA686TU, MDA686LN, MDA1386TU, MDA1386LN, MDA886LN, MDA1686, HN5, HN30, and HN 31, Dr. Jeffrey Myers, MD Anderson Cancer Center; PCI-13, PCI-15A, and PCI-15B, Dr. Theresa Whiteside (University of Pittsburgh Medical Center); UM-SCC-1, UM-SCC-10A, UM-SCC-11A, UM-SCC-11B, UM-SCC-14A, UM-SCC-14B, UM-SCC-17A, UM-SCC-17B, UM-SCC-22A, UM-SCC-22B, UM-SCC-25, and UM-SCC-47, Dr. Thomas

Carey (University of Michigan). Upon arrival, all cell lines were expanded and aliquots were frozen. Genomic DNA was extracted from one of the aliquots using the DNeasy Blood & Tissue Kit (Qiagen) and sent for genotyping (Powerplex 1.2 [Progenia]). Genotypes were compared against a master list of published HNC genotypes for verification (19), or confirmed as unique against currently available public cell line genotypes. All cell line experiments were conducted using cells with less than 10 passages from the time they were expanded aliquoted and thus all cells used were continuously grown for less than 6 months from the time of genotyping. All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum as described (5), and were verified to be free of mycoplasma. The human shRNA-NQO1 (clone ID: V3LHS_331228) lentivirus vector was purchased from Thermo Scientific (Pittsburgh, PA). Stable shRNA knock down clones and scrambled controls were generated as described (20). All experiments and cell culture were performed without antibiotics.

NQO1 enzyme assays

NQO1 enzyme levels (Supplemental Table 1) were determined as previously described (11). Enzyme activities of NQO1 (U) were calculated as $\eta\text{mol cytochrome } c \text{ reduced/min}/\mu\text{g}$ protein, based on the initial rate of change in NADH optical density (OD) at 550 nm.

Relative survival assays

Relative cell survival levels of HNC cells *in vitro* were determined by 7-day nuclei counting assay. Briefly, $1\sim2 \times 10^3$ cells were pre-plated into 96 well plates 16 h before treatment and exposed to β -lap for 2h or β -lap combined with Catalase (2000 U) or DIC (50 μM) for 2h, or pretreated with the calcium chelator BAPTA-AM (5 μM) for 1h and then with β -lap for 2h. For combined treatment, cells were exposed to IR followed immediately with sub-lethal β -lap doses as determined by prior dose titration. After the appropriate incubation times as outlined above, the medium containing the drugs was removed and drug-free medium was then added and cells incubated for an additional 7 days, fixed, and stained with Hoechst 33342. Images of stained nuclei from each treatment were captured using the IN Cell Analyzer 2000 (GE healthcare, Piscataway, NJ) and relative survival was calculated based on cell number. Relative survival based on DNA assays correlates well with colony forming assays (8). Results were reported as means $\pm$ standard errors ($\pm\text{SE}$) from at least three independent experiments performed in triplicate. All cell irradiation was done using the Mark 1 irradiator with a $^{137}\text{Cesium}$ source delivering a dose rate of 3.49 Gy per minute (JL Shepherd & Associates, San Fernando, CA).

ROS analyses

ROS formation was quantified after β -lap, or β -lap + IR treatment by staining cells with 5 μM DCFDA as described (16), except that analyses were performed using the IN Cell Analyzer 2000. Data were graphed as means $\pm$ standard error (SE) for experiments performed three times, each in triplicate.

Alkaline comet assays

DNA strand breaks were assessed using alkaline comet assays as described (6, 11) (TREVIGEN, Gaithersburg, MD). Comet tail lengths were quantified in >100 cells after β -lap exposure using Komet 5.5 software (Andor Technology USA, South Windsor, CT). Experiments were performed three or more times, each in duplicate (7).

Nucleotide analyses

Changes in intracellular NAD⁺ levels were measured from whole-cell extracts before or after treatment with β -lap, or β -lap + IR using Fluorescent NAD/NADH Detection Kit (Cell Technology, Fremont, CA). ATP levels were analyzed from whole-cell extracts before or after treatment with β -lap, or β -lap + IR using CellTiter-Glo® Luminescent Cell Viability Assays (Promega, Madison, WI). ATP levels in mice xenograft tissues were examined using a colorimetric/fluorometric assay according to the manufacturer's recommended protocol (BioVision, Milpitas, CA). Data were graphed as means $\pm$ SE of experiments performed three times in triplicate.

Flow cytometry

TUNEL positive cells were quantified using APO-DIRECT™ assay [terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)] from BD Pharmingen (BD Biosciences, San Jose, CA). Samples were analyzed by using a FC-500 flow cytometer (Beckman Coulter, Inc., Irving, TX) as previously described (20, 21). All experiments were repeated at least three times with similar results each time.

Immunoblot analyses

Whole cell extracts and immunoblots were prepared and developed as described (20). For immunoblots, primary antibodies (anti-human) and dilutions were: NQO1 (Santa Cruz), 1:5000; Catalase (Santa Cruz), 1:1000; PAR (BD Pharmingen, San Jose, CA), 1:1000; GAPDH (Santa Cruz), 1:20,000; and β -actin (Santa Cruz), 1:20,000. HRP-conjugated secondary antibodies were purchased from Santa Cruz, 1:2000. Results shown are representative of experiments performed at least three times with similar results each time.

Immunohistochemistry (IHC) assays

HNC tissue microarray (TMA) slides were purchased from China (Alenabio, Xian City, Shanxi Province, The People's Republic of China, 710075). IHC staining was performed on 5 μ m thick HNC TMA slides via two-step assays as previously described (22). Cytoplasmic and nuclear staining was independently evaluated by two Pathologists (Drs. Lin and Liu) in a blinded manner. Adjacent sections were stained in the tissue blocks, and many of the tumors did not have adjacent normal tissue companions. Scoring for NQO1 and Catalase staining was graded as follows: no staining or staining observed in less than 10% of tumor cells was given a score 0; faint/barely perceptible staining detected in at least 10% of tumor cells was scored as 1; moderate staining observed in at least 50% of tumor cells were scored as 2; strong staining observed in at least 50% of tumor cells was scored as 3. For two-by-two contingency table analysis, 0 and 1 expression levels were grouped as negative and compared to 2 and 3 expression levels which were grouped as positive. In most cases,

discrepancies were resolved by re-examination and consensus. DNA DSBs was determined by immunofluorescence of γ H2AX foci formation (23). Images were acquired using an Axio Imager 2 microscope (Carl Zeiss) and analyzed using IN Cell Developer Toolbox.

Anti-tumor efficacy

Head and neck tumors were generated by injecting 1×10^6 SqCC/Y1 cells into the left neck area of female severe combined immunodeficiency-non-obese diabetic (SCID-NOD) mice weighing 20-25 grams obtained from the UT Southwestern institutional breeding core. Tumor masses were measured twice per week and mice were stratified so that tumor volumes in each group (5 mice/group) were not statistically different. Four weeks after implantation, mice were treated with HP β -CD or β -lap-HP β -CD through tail vein injection as described (17) as well as sub-lethal doses of β -lap-HP β -CD with or without IR at various doses of Gy, every other day for five treatments. Animals were irradiated using the X-RAD 320 small animal irradiator (Precision X-Ray, North Branford, CT). For combination β -lap-HP β -CD and IR treatments, β -lap-HP β -CD was administered within 30 min after IR. Mice were sacrificed when tumor burdens reached ~ 500 mm³. Survival and tumor volume data were graphed from two separate studies, with 10 combined mice per group. Similar experiments were also repeated using 18-20 grams female athymic nu/nu mice (5 mice/group) purchased from Taconic Bioscience (Hudson, NY). Separately, for pharmacodynamic analyses, SqCC/Y1 tumor-bearing mice were sacrificed 4 h after various treatments. Tumor tissues were removed and snap-frozen for ATP levels, γ H2AX foci formation, and histological examination. All animal studies were carried out under a University of Texas at Southwestern Medical Center Institutional Animal Care and Use Committee approved protocol and in accordance with the guidelines for ethical conduct in the care and use of animals in research.

Statistical analyses

Log-rank tests were applied to Kaplan-Meier survival analyses censored for death. All *in vivo* statistical analyses were performed using Graph Pad Prism software. Statistical analyses of survival data *in vitro* were performed using linear regression analyses whereby analyses of covariance and non-linear regression models and overall *p*-values were simultaneously calculated, comparing intercepts and slopes for different treatments. For equitoxic dose calculations, experimental data was fitted using the Carter's model with algorithms written in R code and the equitoxic doses listed in Supplemental Table 2 were calculated using the parameters of the Carter's model (24). Two-by-two contingency tables using Fisher's exact test with a two-tailed *p*-value were used to determine statistical significance in differential expression between negative and positive expression of NQO1 or Catalase in tumor versus adjacent normal tissue using GraphPad QuickCalcs (<http://graphpad.com/quickcalcs/contingency1.cfm>). Bar graphs were tested for statistical significance using a two-tailed Student T-test with SAS (Service Pack 4) for Windows and SigmaPlot. All results were considered to be statistically significant for *p* values of less than 0.05.

Results

NQO1 is expressed in HNC tissues and cell lines

NQO1 expression and treatment response has been characterized for breast, pancreas, prostate and lung cancer (4-7). This result prompted the examination of NQO1 from HNC patients using a TMA arrayed with 79 patients (NQO1) and 67 patients (Catalase) from all subsites of the head and neck (Supplemental Table 3). Representative IHC micrographs (Fig. 1A) and the cumulative scoring for each patient is summarized (Fig. 1B). A two-by-two contingency table comparing NQO1 and Catalase expression levels for all patients is shown for NQO1 (Fig. 1C) and Catalase (Fig. 1D). There was a statistically significant difference in the elevated expression levels of NQO1 in tumor compared to adjacent normal tissue and Catalase in adjacent normal tissue compared to tumor. Furthermore, in a separate cohort, increased NQO1 expression correlated with poor overall survival in HNC patients (Supplemental Fig. S1).

Since this clinical expression pattern suggests response to β -lapin HNC, we further explored the expression pattern of these two proteins in a large number of HNC cell lines. Representative Western blots demonstrated that NQO1 was highly expressed in many of the HNC cell lines (Fig. 2A). The LD₅₀ of β -lap and NQO1 enzyme activity from IMR90 (closed square) and 41 HNC lines (open squares) were graphed against NQO1 enzyme activities (U), demonstrating a lethality inflection response to β -lap at around 100 NQO1 U (Fig. 2B). There was no correlation between β -lap LD₅₀ and NQO1/Catalase ratios (Fig. 2C). These data strongly suggested that NQO1 levels are critical for β -lap lethality.

HNC cell lines that express NQO1 demonstrate NQO1 activity and response to β -lap

Cell survival was determined after treating with increasing concentrations of β -lapin four representative cell lines: FaDu, SqCC/Y1 and Detroit 562 (NQO1 positive) and UM-SCC-10A (NQO1 negative). Cell death dramatically increased with increasing concentrations of β -lap through a very sharp inflection point in dose response (Fig. 3A) in FaDu, SqCC/Y1, and Detroit 562, but not in UM-SCC-10A. Co-treatment with the specific NQO1 inhibitor DIC (Fig. 3A) resulted in complete abrogation of β -lap-induced cell death in NQO1 expressing HNC cell lines. Furthermore, cell survival was partially rescued, requiring higher concentrations of β -lap for the same effect, in the presence of one hour pretreatment with BAPTA-AM (Supplemental Table 1) as well as exogenous Catalase (Fig. 3A), supporting a role for the generation of ROS and the release of calcium from the ER in β -lap-induced NQO1-dependent cell death in HNC. None of these treatments had a significant effect on UM-SCC-10A cell survival (Fig. 3A, lower right panel).

Units of NQO1 activity were determined for a panel of 41 HNC cell lines and IMR90 primary fibroblasts. If NQO1 is expressed, the enzymatic activity (Supplemental Table 1, column 2) and 50% lethal dose of β -lap (LD₅₀, column 3) is largely a function of NQO1 expression and activity, which can be blocked by DIC (column 4) and above a certain threshold cannot be rescued by catalase. Co-treatment with BAPTA-AM or exogenous Catalase resulted in an increase in the β -lap LD₅₀ for a subset of HNC cell lines and IMR90 primary fibroblasts (compare columns 3 to 5 and 6, respectively).

To further clarify the role of NQO1:Catalase ratio in normal cells, we measured NQO1 enzyme expression and activity as well as Catalase expression in IMR90 primary fibroblast cells and calculated NQO1:Catalase ratios (Fig 3B). NQO1 enzyme activity was extremely low (~11 U), and only ~20% cell death was noted at 5-fold higher β -lap doses compared to LD₅₀ doses of β -lap required to kill NQO1-expressing HNC lines (Fig. 3A-B), and was significantly rescued by exogenous catalase or DIC. These data support cell death through a NQO1-dependent β -lap redox cycling in NQO1+ HNC cells due to overwhelming NQO1 activity, which cannot be overcome by tumor cell catalase, and that endogenous catalase activity in normal tissue protects from low levels of NQO1 activity.

Functional consequences of reduced NQO1 expression and mechanisms of β -lap-induced NQO1-dependent cell death

To further clarify the role of the NQO1 enzyme in β -lap-induced NQO1-dependent cell death, shRNA directed against NQO1 was used to stably knock down NQO1 protein expression (Fig. 4A). Relative survival was then determined for the shRNA scrambled control in the absence or the presence of DIC, as well as for the shRNA NQO1 knockdown clones #7 and #11. Increasing concentrations of β -lap resulted in much less cell death in the NQO1 knockdown clones compared to the NQO1-intact parental cell line (Fig. 4B and Supplemental Fig. S2).

Since β -lap-induced NQO1-dependent cell death has been shown in other cell types to result from the production of ROS that are ultimately converted to H₂O₂, β -lap-induced oxidative stress in the form of ROS was monitored by DCFDA staining. β -Lap induced a three-fold increase in ROS formation by 20 min, and this effect was completely blocked by DIC in FaDu and SqCC/Y1. H₂O₂ (500 μ M, 15 min) served as the positive control and the formation of ROS was unaffected by the presence of DIC (Fig. 4C).

A consequence of increased ROS is localized DNA strand damage, resulting in predominately SSBs as seen by alkaline comet assay. Representative images demonstrated time-dependent increases in the levels of DNA strand breaks with β -lap treatment alone, which was blocked by co-treatment with DIC in SqCC/Y1 and FaDu (Fig. 4D, left panel, Supplemental Fig. S3A). Comet tail lengths represent the means $\pm$ SE from three independent experiments, showing statistically significant differences between β -lap alone or with DIC co-treatment. H₂O₂ (1 mM, 30 min) was used as a positive control and the length of the comet tail was unaffected by the presence of DIC (Fig. 4D, right panel, Supplemental Fig. S3B).

Dramatic increases in ROS and single-strand DNA breaks can lead to PARP1 up-regulation and hyperactivation and high global levels of proteins with poly ADP-ribosylation (PAR) post-translational modifications. Therefore, cellular PAR levels were determined for total PAR in the presence of 5 μ M β -lap without or with DIC co-treatment in FaDu and SqCC/Y1 (Fig. 4E). Representative western blots demonstrated dramatic increases in PARylation in the presence of β -lap that was significantly blocked by DIC. H₂O₂ served as a positive control to generate PARylated proteins to confirm the validity of this assay.

Since PARP1 hyperactivation results in dramatic losses of ATP and NAD^+ in other cell lines (7, 12), ATP and NAD^+ levels were examined in response to β -lapin FaDu and SqCC/Y1. Increasing concentrations of β -lap resulted in dramatic reductions in intracellular ATP and NAD^+ levels that were completely blocked by co-treatment with DIC (Fig. 4F and Supplemental Fig. S3C).

The mechanism of cell death was further explored using TUNEL to quantify the number of cells with DNA DSBs. The percent of TUNEL positive FaDu and SqCC/Y1 was elevated with β -lap exposure at the indicated doses and exposure times and abrogated with the co-treatment of DIC (Fig. 4G).

Sublethal doses of β -lap radiosensitize HNC cell lines and result in enhanced ROS formation, DNA double strand breaks and loss of ATP when combined with IR

Current administration of β -lapin the clinic requires the carrier HP β -CD for solubilization and delivery through the blood. However, this combination has the potential to cause methemoglobinemia at high concentrations, which is dose-limiting in preclinical and clinical studies (17, 25). Since radiotherapy is used to treat a majority of HNC, radiosensitization using sub-lethal doses of β -lap was explored using relative cell survival assays. Consistent with an NQO1-dependent mechanism, the three cell lines FaDu, SqCC/Y1 and Detroit 562 that express NQO1 were radiosensitized by β -lap at concentrations of 1.5 and 2.5 μM , while there was no radiosensitization observed in UM-SCC-10A (Fig. 5A). Equitoxic doses comparing single to combined treatment of β -lap and IR in HNC cells are listed in values of equivalent doses for FaDu, SqCC/Y1 and Detroit 562 (Supplemental Table 2). Furthermore, increasing doses of β -lap resulted in decreased relative survival in the setting of constant 2 Gy, which was completely blocked by the addition of DIC (Supplemental Fig S4).

To further explore the mechanistic basis of β -lap-induced radiosensitization, ROS levels at 20 min (Fig. 5B) and TUNEL+ formation (Fig. 5C) were assessed in SQCC/Y1 after IR in the presence or absence of β -lap. The combination of sublethal doses of β -Lap (2.5 μM) + 2 Gy induced significantly increased levels of ROS and TUNEL+ cells compared to either single treatment alone (* $p < 0.05$; ** $p < 0.01$). β -Lap (5 μM , 20 min or 2 h) served as a positive control and ROS and TUNEL+ cell formation was abrogated by DIC (Figs. 5B-C). Since γH2AX foci, NAD^+ and ATP depletion play key roles in NQO1-dependent β -lap lethality, we also determined γH2AX foci (Fig. 5 D), NAD^+ and ATP depletion (Fig. 5E) 2 h after IR in the presence or absence of β -lap. Shown are representative images of γH2AX formation in the presence of increasing concentrations of β -lap, with or without 2 Gy (Fig. 5D) and measured results (Fig. 5D, bar graphs), demonstrating increased numbers of γH2AX foci/nucleus at 2 h in the presence of β -lap + 2 Gy, compared to mock-irradiated or 2 Gy alone (** $p < 0.001$). A lethal dose of β -lap with or without DIC was used as a positive control for NQO1-dependent formation of γH2AX induction. The loss of NAD^+ and ATP following sublethal doses of β -lap in combination with IR was also assessed in SQCC/Y1. IR enhanced loss of NAD^+ and ATP in the presence of sublethal doses of β -lap in a dose dependent manner for both β -lap and IR (Fig. 5E) with the most pronounced effect at 2.5 μM β -lap and 2 or 3 Gy. Given this unique feature of NAD^+ loss, our data clearly

demonstrate the combination of sublethal doses of β -lap and IR kills cancer cells through programmed necrosis.

Cooperative antitumor efficacy using a combination of β -lap and IR to treat SqCC/Y1 HNC xenograft models

To further evaluate the role of this combination in a preclinical model, xenografts of HNC were employed. SCID-NOD Mice bearing 30 mm³ SqCC/Y1 tumor xenografts were treated every other day for five treatments. Tumor volume measurements (Fig. 6A) and Kaplan-Meier overall survival (Fig. 6B) is graphed for vehicle (HP β -CD, control cohort), β -lap-HP β -CD 10 mg/kg alone, 10 Gy (2 Gy $\times$ 5 fractions) alone and a combination of 10 Gy (2 Gy $\times$ 5 fractions) plus β -lap-HP β -CD 10 mg/kg immediately following IR. There was no difference in TV or OS between the HP β -CD and β -lap-HP β -CD 10 mg/kg cohorts. Treatment with 10 Gy alone resulted in a significant decrease in tumor growth and tumor volume (Fig. 6A) and an increase in overall survival (Fig. 6B), all of which were further enhanced by the co-treatment of β -lap-HP β -CD 10 mg/kg. There was no statistical difference between HP β -CD (20 mg/kg), β -lap-HP β -CD (10 mg/kg), or β -lap-HP β -CD (20 mg/kg) for tumor volume or overall survival in either SCID-NOD or Athymic Nu/Nu mice SqCC/Y1 xenograft models (Supplemental Fig. S5). However, there was a statistically significant difference in tumor volume between β -lap-HP β -CD (10 mg/kg) + 10 or 20 Gy compared to 10 Gy or 20 Gy alone in the SCID-NOD xenograft model (Supplemental Fig. S5).

Short-term *in vivo* pharmacodynamic studies were also performed to assess the effects of the different treatments on γ H2AX foci formation and ATP consumption in the tumors grown in mice. Xenograft SqCC/Y1 tumors were grown in the necks of mice to 30 mm³ and treated as above. After treatment (4 h), the tumors were extracted, flash frozen and assessed for γ H2AX foci formation. Representative images are shown (Fig. 6C) and identified a significant increase in γ H2AX foci formation in the combination treatment β -lap-HP β -CD 10 mg/kg and 2 Gy compared to β -lap-HP β -CD 10 mg/kg alone or 2 Gy alone (Fig. 6D). We also stained Ki-67 in consecutive sections and did not see any significant difference (Supplemental Fig. S6). Furthermore, ATP levels were significantly reduced in the combination arm compared to each single modality treatment or vehicle control (Fig. 6E).

Discussion

In this translational study, we present novel data to support a biomarker-driven clinical trial using ARQ761 (β -lap) concurrently with radiotherapy for HNC based upon tumor expression of NQO1 (Fig. 1). NQO1 was significantly elevated in ~45% of HNC tissues compared to associated normal tissues with the inverse being true for Catalase. This inverse expression ratio in normal cells provides a favorable microenvironment to exploit the therapeutic window of NQO1 activity in the treatment of HNCs given that Catalase in normal tissue protects against NQO1-dependent β -lap lethality by eliminating bystander ROS (H₂O₂) generated by the β -lap futile cycle (26). In addition, increased NQO1 expression correlated with poor overall survival in HNC patients (Supplemental Fig. S1). Therefore, attacking cancers that express NQO1 utilizing a NQO1 bioactivatable drug that

kills through a NQO1-dependent futile redox cycle mechanism presents a theoretically attractive therapeutic target that may overcome previously acquired therapeutic resistance. Since NQO1 is also expressed in HNC cell lines (Fig. 2), these data supported further preclinical evaluation of β -lap as a treatment strategy in HNC.

Radiotherapy, often with concurrent chemotherapy, is a central therapeutic modality for the treatment of locally-advanced and local-regionally recurrent HNC (27). IR treatment creates a variety of DNA lesions, and if unrepaired, DSBs ultimately result in cell death primarily through mitotic catastrophe. Currently, cisplatin-based chemotherapy is used as standard of care to radiosensitize HNC tumors, but the use of chemotherapy also causes non-specific normal tissue cytotoxicity, and only cures ~50% of patients with locally advanced disease. Therefore, there is a need to identify better tumor radiosensitizers in order to increase the effectiveness of IR.

The pharmaceutical version of β -lap, ARQ761, is currently in a phase I clinical trial for patients with metastatic solid malignancies (www.clinicaltrials.gov), and data generated from this trial will further clarify the optimal dosing for patients, which will be incorporated into the design of a phase I clinical trial to assess the safety and toxicity of concurrent β -lap with radiotherapy. Preclinical studies in mice with a similar formulation β -lap-HP β -CD demonstrated that the maximum tolerated dose (MTD) results from methemoglobinemia toxicity resulting from the carrier agent HP β -CD, raising concerns that this same phenomenon may also limit human MTD rather than β -lap itself. Therefore, the development of a therapeutic strategy to leverage the tumor-selective cytotoxicity as well as the radiosensitizing capacity of β -lap (13, 14), while reducing exposure to β -lap-HP β -CD induced side-effects through the use of sublethal doses of β -lap, would be highly desirable, and could be done using a combined modality treatment platform with IR.

To test this hypothesis, we combined sublethal doses of β -lapin combination with IR in both cell lines and mouse xenograft studies. For the first time, our data clearly shows the radiosensitizing effects of sublethal doses of β -lap combined with IR in HNC, enhancing the efficacy of IR through the potentiation and accumulation of DNA damage and DSBs as represented by the formation and prolongation of γ H2AX foci, as well as the increased consumption of intracellular NAD⁺ and ATP *in vitro* and *in vivo* (Figs. 5-6). Combined treatment also resulted in the prolongation of survival and reduced tumor growth without any additional observed cytotoxic effects. The statistically significant difference in tumor growth between 10 or 20 Gy alone and combination therapy β -lap-HP β -CD 10 mg/kg plus 10 or 20 Gy demonstrated the lack of a durable treatment response with IR alone compared to the combination treatments which is not yet evident in the timeframe of the 20 Gy alone survival data since survival lags behind tumor growth (Fig. 6 and Supplemental Fig. S5). This data also support ATP depletion as an important metabolic contributor to the anti-cancer effects of β -lap combined with IR (11, 12, 28).

Recently, it has been shown that endoplasmic reticulum (ER) stress and calcium flux suppresses DNA DSB repair and sensitizes tumor cells to IR by stimulating proteasomal degradation of Rad51 (29). Other reports suggest that ER stress-induced JNK activation is a critical event to cause mitochondrial apoptosis with β -lap alone or in combination with IR;

however, these mechanisms were established with treatment using very high doses of IR (10 Gy) in combination with supra-therapeutic doses of β -lap (30) which is not physiologically feasible for current conventional radiotherapy regimens. Thus, the impact of ER stress on the initiation and amplification of DNA damage, as well as DSB repair, after treatment with β -lap and/or β -lap derivatives alone or in combination with IR exposure is still not well understood and we are actively investigating this in HNC models.

In summary, we show for the first time that 1) HNC tumors express NQO1, 2) normal tissues next to head and neck tumors express catalase, 3) the cell killing effect of β -lap is dependent on a threshold level of expression and activity of NQO1 in HNC, and 4) combination sub-lethal doses of β -lap and IR demonstrate increased efficacy over IR alone in cell lines and in xenograft studies. These data provide compelling evidence to strongly support reduced doses of β -lap as a promising therapeutic partner with IR for the clinical treatment of HNC and extend the application and utility of β -lap-HP β -CD (ARQ761). This study suggests that HNC samples should be screened for NQO1 expression to identify patients that will likely benefit from combined therapy with β -lap and IR. As such, this study supports the translation of concurrent β -lap and IR treatment into a molecular NQO1 expression biomarker driven human phase I clinical trial for high risk HNC to assess the feasibility of combining radiotherapy with ARQ761.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of NQO1 and Catalase in HNC and adjacent normal tissues

(A) Representative images from tissue microarray IHC staining from HNC patients. (B) A statistically significant inverse expression ratio exists between the relative intensity of NQO1 and Catalase staining in the tumor and adjacent normal tissue ($*p < 0.05$). See Supplemental Table 1 for the scoring for each individual patient. A two-by-two contingency table comparing NQO1 and Catalase expression levels for all patients (0 and 1 expression levels were grouped as negative and compared to 2 and 3 expression levels which were grouped as positive [see materials and methods]) is shown for (C) NQO1 and (D) Catalase, demonstrating a statistically significant difference in the elevated expression levels of NQO1 in tumor compared to adjacent normal tissue and Catalase in adjacent normal tissue compared to tumor ($***p < 0.001$; $**p < 0.01$). n, number of patients.

Figure 2. Expression of NQO1 and Catalase in HNC cell lines

(A) Forty-two independent HNC cell lines were probed for NQO1, Catalase and GAPDH expression, demonstrating significant inverse expression in the protein abundance between NQO1 and Catalase, (B) LD₅₀ of β -lap in human primary IMR90 fibroblasts (passage 12, closed square) and in 41 HNC cell lines varying in NQO1 enzyme activity (open squares). (C) LD₅₀ of β -lap in human primary IMR90 fibroblasts (P12, passage 12) and in 41 HNC cell lines varying in NQO1/Catalase ratios (closed circle is LD₅₀ of IMR90 cell line). NQO1:Catalase expression ratios were calculated from relative radiographic intensities on Western blots using NIH Image J. MDA-MB-231 NQO1+ (231NQO1+) and NQO1- (231NQO1-) cells served as positive and negative controls for NQO1 expression. GAPDH served as the loading control.

Figure 3. Functional implications of NQO1 expression in response to β -lap

(A) Shown are four representative cell lines, including FaDu, SqCC/Y1 and Detroit 562 that express NQO1 and the *2 polymorphic UM-SCC-10A that lacks expression (refer to Fig 2A). Several approaches were taken to demonstrate the role of β -lap-induced NQO1-dependent cell death, including adding the specific NQO1 inhibitor, dicoumarol (DIC, 50 μM) or exogenous Catalase (2000 U). β -Lap alone induced significant cell death in a concentration-dependent manner in the three cell lines that expressed NQO1 (β -lap dose indicated on the x-axis) and co-treatment with DIC completely abrogated cell killing by β -lap. No cell death was seen in UM-SCC-10A cells after β -lap exposure. Likewise, co-treatment with exogenous Catalase partially blocked β -lap-induced NQO1-dependent cell death and had no effect when NQO1 was not expressed (UM-SCC-10A, bottom row). Shown are means $\pm$ SE (*** p < 0.001). (B), Western blot analyses for NQO1 and Catalase expression in IMR90 primary fibroblasts (left panel). SqCC/Y1 cells served as positive controls for NQO1 and Catalase expression. GAPDH levels served as a loading control. Relative survival assays in IMR90 primary fibroblasts (right panel) were performed after treatment with β -lap alone for 2 h, with or without DIC (50 μM) or Catalase (2000 U). β -Lap did not induce any significant cell death at concentrations that produced complete cell death in the cell lines that expressed NQO1 (panel A). Supra-high dose β -lap-induced cell

death was abrogated by co-treatment with DIC or Catalase. Shown are means $\pm$ SE ($**p < 0.01$). All experiments were performed three times in triplicate.

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Figure 4. Functional consequences of reduced NQO1 expression and mechanisms of β -lap-induced NQO1-dependent cell death

(A) Lentiviral shRNA against NQO1 was used to stably reduce the protein abundance of NQO1 and a representative western blot for several different independent NQO1 knockdown clones is presented. A non-targeting scrambled shRNA (shSCR) was used to control for the lentiviral vector and GAPDH served as the loading control. (B) β -Lap-induced lethality is rescued in SqCC/Y1 shNQO1 knockdown clones. shSCR and shRNA-NQO1 knockdown cells #7 and #11 were evaluated by relative survival assay after various doses of β -lap exposure (μ M, 2 h) as depicted on the x-axis demonstrating a β -lap induced NQO1 dependent cell death ($***p < 0.001$). (C) β -Lap-induced oxidative stress (reactive oxygen species [ROS], predominately H_2O_2) was monitored by DCFDA staining and was analyzed using high-throughput imaging analysis technology in FaDu and SqCC/Y1 cells after β -lap treatment (5μ M), with or without DIC (50μ M) at the indicated times in minutes across the x-axis. Hydrogen peroxide (500μ M, 15 min) served as the positive control. (D) Time-dependent increase in DNA strand breaks was demonstrated by the alkaline comet assay in the presence of β -lap alone (5μ M, 2 h) or co-treatment with β -lap and DIC (50μ M) in SqCC/Y1 cells, with the graphed comet tail lengths measured using Komet 5.5 software. Shown are representative micrographs of studies performed at least three times and the graphed comet tail lengths are means $\pm$ SE from three experiments. Student's t tests were

performed to assess significance ($***p < 0.001$). (E) Poly ADP-ribosylation (PAR) levels were determined by western blot for total PAR in the presence of 5 μM β -lap without or with co-treatment with DIC (50 μM) in FaDu and SqCC/Y1 cells. GAPDH served as loading control. (F) ATP levels are dramatically reduced by exposure to β -lap at the indicated doses and this response is blocked by co-treatment with DIC (50 μM). Student's t tests were performed to assess for significance ($***p < 0.001$). (G) The percent TUNEL positive FaDu and SqCC/Y1 cells is elevated with β -lap exposure at the indicated doses and abrogated with the co-treatment of DIC (50 μM). Results are means $\pm$ SE for studies performed three times. Student's t tests were performed to assess for significance ($***p < 0.001$).

Figure 5.

Combination low dose radiation and sublethal doses of β-lap result in decreased cell survival, and increased ROS, TUNEL positivity, γH2AX foci formation and ATP consumption. (A) Relative cell survival assays were performed by treating with β-lap at the specified concentration immediately after IR at the indicated doses for cell lines, FaDu, SqCC/Y1, Detroit 562 and UM-SCC-10A. Data represent relative cell survival assays as described in 'Materials and Methods'. Triple asterisks represent statistically significant differences between β-lap (1.5 μM and 2.5 μM) and DMSO (2.5 μM) treatments (****p* < 0.001). (B) Oxidative stress (reactive oxygen species [ROS], predominately H₂O₂) was monitored by DCFDA staining and analyzed using high-throughput imaging analysis technology in SqCC/Y1 HNC cells after combined treatment of low dose IR (Gy) and β-lap (μM) at 20 min time points. β-Lap (5 μM) with or without DIC (50 μM) served as positive and negative controls, respectively. The asterisk represents statistically significant differences between β-lap (2.5 μM) + 2 Gy vs. β-lap (2.5 μM) or 2 Gy alone (**p* < 0.05). (C) Percent TUNEL+ SqCC/Y1 cells were significantly increased with the combination of low dose β-lap (2.5 μM) + 2 Gy or 3 Gy compared to single agents alone. Co-treatment with DIC (50 μM) rescued the combined treatment induced cell death. Results are means ±SE for studies performed three times. Student's *t* tests were performed to assess for significance (**p*

< 0.05; ** p < 0.01). (D) Representative images of γ H2AX foci formation in SqCC/Y1 HNC cells in the presence of increasing doses of β -lap, without or with 2 Gy. Quantitation of means $\pm$ SE of these foci from 100 cells across treatment group combinations demonstrated an increased number of foci at 2 h in the presence of the indicated concentration of β -lap with 2 Gy versus controls (bar chart). Student's t-tests were performed to assess statistical significance between identical β -lap concentrations, without or with IR (** p < 0.001). A lethal dose of β -lap (5 μ M), with or without DIC (50 μ M) was used as controls. (E) IR enhanced NAD⁺ and ATP loss in SqCC/Y1 HNC cells in the presence of sublethal doses of β -lap in a dose-dependent manner for both β -lap with or without IR as indicated. Student's t-tests were performed comparing β -lap alone and β -lap + IR (** p < 0.001).

Figure 6. Cooperative antitumor efficacy using a combination of β-lap and IR to treat HNC xenograft models

Mice bearing 30 mm³ SqCC/Y1 HNC xenografts with high levels of NQO1 expression were treated with 2 Gy every other day for five treatments (10 Gy total dose). β-lap-HPβ-CD 10 mg/kg was intravenously administered by tail-vein injection immediately following 2 Gy treatment. Vehicle alone (HPβ-CD) served as a control cohort (Materials and Methods). Results (means ± SE) are representative of repeated similar experiments (n=10 for each group). Student's t-tests (***) $p < 0.001$ were performed comparing treated vs control groups. (A) Tumor volume measurements and (B) Kaplan-Meier overall survival over the indicated number of days is graphed for control (HPβ-CD), β-lap-HPβ-CD 10 mg/kg alone, 2 Gy alone and a combination of 2 Gy plus β-lap-HPβ-CD 10 mg/kg. Log-rank analyses were performed comparing survival curves using various IR + β-lap-HPβ-CD regimens (***) $p < 0.001$ for the combined treatment compared to each single treatment). Survival curves show equivalency between HPβ-CD and β-lap-HPβ-CD 10 mg/kg. (C-E) Short-term *in vivo* pharmacodynamic studies to assess γH2AX foci formation and ATP levels in SqCC/Y1 HNC cells 4 h after combined treatment with 2 Gy followed by administration of β-lap-HPβ-CD as indicated. (C) Representative micrographs of γH2AX foci formation demonstrating (D) a significant increase in foci formation in the combined treatment group

compared to all other treatments. (E) ATP levels are significantly reduced in the combined treatment and not in the control or single treatments ($**p < 0.01$).

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