Following Anticancer Drug Activity in Cell Lysates
with DNA Devices

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Abstract:

There is a great need to track the selectivity of anticancer drug activity and to understand the mechanisms of associated biological activity. Here we focus our studies on the specific NQO1 bioactivatable drug, β-lapachone, which is in several Phase I clinical trials to treat human nonsmall cell lung, pancreatic and breast cancers. Multi-electrode chips with electrochemically-active DNA monolayers are used to track anticancer drug activity in cellular lysates and correlate cell death activity with DNA damage. Cells were prepared from the triple-negative breast cancer (TNBC) cell line, MDA-MB-231 (231) to be proficient or deficient in expression of the NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme, which is overexpressed in most solid

cancers and lacking in control healthy cells. Cells were lysed and added to chips, and the impact of β-lapachone (β-lap), an NQO1-dependent DNA-damaging drug, was tracked with DNA electrochemical signal changes arising from drug-induced DNA damage. Electrochemical DNA devices showed a 3.7-fold difference in the electrochemical responses in NQO1+ over NQO1− cell lysates, as well as 10 to 20-fold selectivity to catalase and dicoumarol controls that deactivate DNA damaging pathways. Concentration-dependence studies revealed that 1.4 µM β-lap correlated with the onset of cell death from viability assays and the midpoint of DNA damage on the chip, and 2.5 µM β-lap correlated with the midpoint of cell death and the saturation of DNA damage on the chip. Results indicate that these devices could inform therapeutic decisions for cancer treatment.

Keywords: DNA damage, DNA repair, electrochemical biosensing, drug assay, drug screening, diagnostic

1. Introduction

There is a great need to not only detect cancer at its onset, but also determine which cancer therapy balances drug effectiveness with minimal side-effects for a particular patient. For these reasons, it would be highly beneficial to develop sensors that can follow drug activity for cancer-selective treatments that can be applied to discover patient-specific responses (Patolsky et al. 2006). In addition, there is a great benefit to produce platforms that can quickly track the underlying biological activity of drugs in order to understand mechanisms of action, particularly
with respect to DNA damage (Carozzi et al. 2015; Roos et al. 2016). Such platforms with modular control of intracellular components would enable the systematic investigation of biological pathways of drug activity for further understanding and refinement of targeted cancer lethality.

A number of cancer treatments cause DNA damage to bring about cell death, such as cisplatin, doxorubicin, and methotrexate (Cheung-Ong et al. 2013). Others are under development for selective targeting of cancer. Among these, we are investigating a drug, β-lapachone (β-lap, ARQ761 in clinical form), due to its great potential to treat a number of aggressive solid tumours, including cancers that overexpress NQO1 and that have no existing effective treatments such as nonsmall cell lung and pancreatic cancers. These cancers are so lethal that five year survival rates are only 15% and 5%, respectively (Huang et al. 2016). β-Lap exploits cancer-specific overexpression of NAD(P)H:quinone oxidoreductase 1 (NQO1). NQO1 metabolizes unique quinones, such as β-lap, to selectively kill cancer cells (Huang et al. 2016). NQO1 attempts to detoxify this drug in a two-electron oxidoreduction, changing it to a highly unstable hydroquinone (HQ) form. This hydroquinone form of β-lap rapidly and spontaneously redox cycles in two steps back to the original drug, allowing NQO1 to work in a futile manner on the drug which causes loss of NAD(P)H and accumulation of NAD(P)+. For each mole of β-lap, ~60 moles of NAD(P)H are used and ~120 moles of hydrogen peroxide (H$_2$O$_2$) are produced in two minutes (Bey et al. 2013). H$_2$O$_2$, a reactive oxygen species (ROS), subsequently causes oxidative DNA base damage, creating massive levels of 8-oxoguanine-triphosphate, and 8-oxoguanine defects incorporated into DNA. High levels of DNA single strand breaks result from repair of this oxidative damage, and DNA double strand breaks are likewise created in a delayed manner.
from accumulation of DNA single strand breaks. This damage triggers specific, but overlapping, DNA repair processes, sequentially generating DNA base damage, abasic sites, DNA single-strand breaks, and then DNA double-strand breaks in a delayed manner. This ultimately leads to selective hyperactivation of PARP1 and cancer programmed necrosis, referred to as NAD+-keresis (Moore et al. 2015). NQO1 is constitutively over-expressed in most solid cancers, particularly in non-small cell lung, pancreatic, prostate, and breast cancers (Bey et al. 2007; Cao et al. 2014; Chakrabarti et al. 2015; Dong et al. 2010; Huang et al. 2012; Moore et al. 2015). Furthermore, β-lap-induced cell death avoids many resistance pathways, and a modified form (ARQ761) of β-lap is currently under several Phase I/Ib clinical trials. Thus, detecting its selective activity and gaining understanding of its functionality, particularly the connection between DNA damage and cell death, have potential health implications for the diagnosis and treatment of most solid cancers due to their NQO1 overexpression.

To follow drug specific activity in cell lysates, we employed multi-electrode chip devices for electrochemical detection with self-assembled DNA monolayers (Slinker et al. 2010; Slinker et al. 2011; Wohlgamuth et al. 2013a). This approach provides a unique means of following DNA repair activity (Boon et al. 2002; DeRosa et al. 2005; Furst et al. 2014; McWilliams et al. 2014) like that induced by DNA damaging drugs, such as β-lap. In these devices, DNA monolayers on electrodes can succumb to damage by reactive oxygen species like H₂O₂. In turn, this damage can be accessed by repair proteins in the surrounding solution (Kahanda et al. 2016). These DNA monolayers thus function as a natural recognition element of biological DNA damage repair. Furthermore, the DNA monolayers on our chips are modified with redox probes that undergo surface-bound electrochemical reactions (Gorodetsky et al. 2008a). These electrochemical
reactions, facilitated by charge transfer reactions through the DNA (Genereux and Barton 2010), are very sensitive to changes in the structure and integrity of the DNA base pairs (Boon et al. 2000; Drummond et al. 2003; Liu and Barton 2005; Wohlgamuth et al. 2013b).

Previously, we demonstrated that these electrochemical DNA devices could be used to track β-lap activity in buffer solutions containing NQO1 and various cofactors for drug activity (Kahanda et al. 2016). However, cellular samples are considerably more complex, with competitive DNA binding proteins, additional protein-protein interactions, and competing biochemical reactions and pathways, all of which are important to consider if these devices are to be used for practical medical applications or biological assays. Furthermore, our recent studies with over 100 cancer cell lines demonstrates that cell lysates properly reproduce the NQO1 enzyme concentration and activity found in cells (Huang et al. 2016). In addition, cell lysates are currently used with clinical laboratory improvement amendment (CLIA) laboratory assessments of NQO1 activities for patient samples as an initial predictor of patient response to β-lap derivatives. However, these assays are limited in that they do not directly measure the drug-induced damage repair response, which can vary significantly from NQO1 levels based on many factors, including the concentrations and activities of inherent repair proteins, antioxidant enzymes, and endogenous calcium inhibitors (e.g., calpastatin).

In this work we followed β-lap activity in cell lysates with electrochemical DNA chip devices to explore the inherent cell biology of this drug, to quantify DNA damage response, to correlate this observed damage with drug-induced cell death, and to evaluate these devices for potential applications. Cells proficient or deficient in NQO1 expression, were lysed and added to multi-electrode chips. The impact of β-lap addition to each lysate was quantified through electrochemical signal changes arising from drug-induced DNA damage. Specificity to NQO1
and DNA damaging pathways were investigated. β-Lap concentration-dependence was followed on chips and quantitatively compared to β-lap cell viability studies.

2. Materials and Methods

2.1 Cell Culture, Chemicals and Reagents

MDA-MB- 231 cells were obtained from American Tissue Culture Collection (ATCC, Manasas, VA). NQO1+ and NQO1- 231 stable cell lines were generated as described elsewhere (Cao et al. 2014). Cells were cultured under 5% CO₂/95% air atmosphere at 37 °C in RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Logan, UT). Cells were tested and were mycoplasma-free. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Scientific Extraction Reagents (Thermo Scientific Cat. No. 78833, Pierce Biotechnology, Rockford, IL) according to the manufacturer’s protocol. β-Lapachone (β-lap) was synthesized as described (Pink et al. 2000) and stock solutions prepared at 50 mM in DMSO. DMSO and Hoechst 33258e were purchased from Sigma-Aldrich (St. Louis, MO). NADH was purchased from Sigma Aldrich (N8129) and used as received.

2.2 Western Blot analysis and NQO1 Enzyme Activity Assays

NQO1+ and NQO1- 231 cells were lysed in ice-cold RIPA with protease and phosphatase inhibitors (Santa Cruz, Dallas, TX) and whole-cell extracts were prepared by centrifugation (14000 rpm, 15 min) to remove insoluble components. Protein concentrations were determined by using a BCA assay (Thermo Scientific, Waltham, MA) to normalize the loading volumes. Proteins were separated by a 4-20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA) and
transferred onto PVDF membranes. Primary antibodies for protein detection included: NQO1 (monoclonal mouse, 3187S, Cell Signaling Technology) and α-tubulin (monoclonal mouse, DM1A, Sigma) and were performed in Sigma 1X casein blocking buffer at 4 °C overnight. Secondary HRP-conjugated antibodies were incubated for 1 h at room temperature, followed by detection with SuperSignal West Pico (Thermo Scientific). Bands were quantified by the mean intensity using NIH Image J and normalized to α-tubulin. NQO1 enzyme activities for the cell lines were measured as dicoumarol-inhibited units as described elsewhere (Pink et al. 2000).

2.3 DNA Survival Assays

NQO1+ and NQO1- 231 cells were seeded at 10,000 cells/well in a 48-well plate (Fisher Scientific, Corning, Catalog No.07-200-86, Pittsburgh, PA) and allowed to attach overnight. Cells were then treated for 2 h with β-lap (0, 0.5, 1, 2, 4, 6 μM) in 6 replicates per dose. The drug was removed after 2 h and replaced with 1 mL of RPMI with 10% FBS media. After ~7 days (or until cells reach 90% confluency for the untreated control), the media was removed and cells were washed with 1X PBS twice. Deionized water (250 μL) was added to each well and cells were lysed by freeze-thaw method and stained with 500 μL Hoechst dye (from stock of 50 μL of Hoechst 33258 (Sigma-Aldrich, Cat. No. 14530) in 50 mL of 1X TNE buffer). Cells were incubated in the dark for 2 h at room temperature and DNA content was quantified by fluorescence (460 nm) in a Victor X3 plate reader (Perkin-Elmer, Waltham, MA). Readings were plotted as treated/control (T/C) ± SEM representing relative survival.

2.4 Synthesis of Oligonucleotides

Thiolated oligonucleotide sequences were obtained from Integrated DNA Technologies (IDT). The thiol linker was incorporated into the DNA with the Glen Research thiol-modifier C6 S-S
phosphoramidite. The DNA containing the Nile blue precursor base, a 5-[3-acrylate NHS ester] deoxyuridine phosphoramidite from Glen Research, was purchased from Trilink BioTechnologies and the dye covalently coupled under ultramild conditions according to established procedures (Gorodetsky et al. 2008b). Approximately 20 mg of Nile Blue A perchlorate (Aldrich, Cat. No 370088) was dissolved one millilitre of 9:1 by volume dichloromethane:N,N-diisopropylethylamine. This solution was mixed with the DNA on solid support bearing the 5-[3-acrylate NHS ester] deoxyuridine phosphoramidite and placed on a shaker overnight (~16 h) to couple the dye. Subsequently, the solid supports were rinsed successively with dichloromethane, methanol, and acetonitrile. The DNA on solid support was dried in a speedvacuum. The DNA was cleaved from solid support and deprotected under ultramild conditions with two successive immersions in 800 μL 50 mM KCO$_3$ in methanol for 4 h, followed by rinsing the supports with this 50 mM KCO$_3$ solution. All of these fractions of KCO$_3$ in methanol were collected, dried by speedvacuum, suspended in 50 mM, pH 8.0 ammonium acetate buffer, and filtered for subsequent high performance liquid chromatography below.

2.5 Purification and Characterization of Oligonucleotides

All oligonucleotides were purified via two rounds of high performance liquid chromatography (HPLC) on a Shimadzu LC-20AD instrument. In the first purification round, DNA oligonucleotides with the 4,4′-dimethoxytrityl group on were eluted, while in the second purification round, DNA oligonucleotides with the 4,4′-dimethoxytrityl (DMT) group off were purified, each according to previous reports (McWilliams et al. 2015; Wohlgamuth et al. 2014).
Thiol disulfide bonds were cleaved (and the DMT group removed) by dissolving the strand in 1 mL with approximately 20 mg of dithiothreitol (Aldrich) in concentrated NH₄OH, allowing the reaction to proceed for 2 h at room temperature, and subsequently drying with a speedvacuum. DMT groups were removed from the probe strands by a reaction in acetic acid. The DNA was suspended in 200 µL of deionized water, and 800 µL of concentrated acetic acid was added to the solution for 20 minutes. The reaction was quenched at this time by adding at least 1 mL of ethanol to this mixture, and dried by speed vacuum.

The identity of the desired products was confirmed by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) on a Shimadzu Axima Confidence mass spectrometer.

2.6 Preparation of Duplex DNA

The oligonucleotides were quantified via UV-visible spectroscopy on a Beckman DU-800 UV-Visible spectrophotometer. Duplex DNA was prepared by mixing equimolar amounts of complementary strands and annealing the solution to 95 °C, followed by slow cooling to room temperature over a period of 90 minutes. The formation of duplex DNA was verified by temperature dependent absorbance measurements and melting temperature analysis.

2.7 Fabrication of Multiplexed Gold Electrode Devices

The chips/substrates featuring multiplexed gold electrodes were prepared as previously described (McWilliams et al. 2015; Slinker et al. 2010). In brief, 1 mm thick Si wafers featuring a 10 000 Å thick oxide layer (Silicon Quest, Inc.) were patterned via a two-layer process. For the first layer, the gold electrodes were deposited by a lift-off technique. Initially, the wafers were
cleaned thoroughly in 1165 Remover (Microchem, Inc.) to remove organic impurities. SPR 220 3.0 photoresist (Microchem, Inc.) was then spin-cast at 2000 rpm onto the wafers and baked. The photoresist was in turn patterned with a Karl Suss MA6 contact aligner and a chrome photomask. Following post-exposure baking, the wafers were developed in AZ 300 MIF developer for 1 min and rinsed thoroughly with deionized water. A 100 Å Ti adhesion layer and a 1000 Å Au layer were deposited onto the wafers via electron beam physical vapor deposition. The wafers were then immersed in Remover PG (Microchem, Inc.) overnight and sonicated to complete metal lift-off. Subsequently, the wafers were baked again and cleaned by UV ozone treatment. For the second layer, SU-8 2002 photoresist was spin-cast onto the wafers at 3000 rpm, baked, and photopatterned as an insulator, thereby isolating the exposed gold working electrode areas from the contact pads. The wafers were then developed in SU-8 Developer (Microchem, Inc.) for 1 min and baked to permanently set the photoresist. Finally, the completed wafers were diced into 1-in. by 1-in. chips by hand with a diamond scribe and stored under vacuum. The resulting multiplexed electrodes allowed for the self-assembly of four distinct DNA monolayers, each with four-fold redundancy, on a single chip/substrate, facilitating direct, unambiguous comparisons between different DNA monolayers.

2.8 Self-Assembly of DNA Monolayers

The DNA monolayers were self-assembled onto gold electrode pads from a 5 mM phosphate, 50 mM sodium chloride, pH = 7 buffer solution over a period of 12 h to 18 h through gold-thiol self-assembly. The substrates were backfilled with mercaptohexanol for 1 h to remove nonspecifically bound DNA and then thoroughly rinsed with buffer to remove residual mercaptohexanol.
2.9 Electrochemistry of DNA Monolayers

The multiplexed substrates were placed in the custom mount, which was connected to electrochemical testing hardware (a CH Instruments CHI750D Electrochemical Analyzer and a CHI 684 Multiplexer). The electrochemical measurements were performed in 5 mM phosphate, 50 mM sodium chloride, pH = 7 buffer with 4 mM spermidine. Square wave voltammetry was recorded with a 4 mV increment, a 25 mV amplitude, and a 60 Hz frequency. The membranes used to separate nuclear lysates from cytoplasmic lysates were the microdialysis strips associated with the Pierce 96-well microdialysis plate, 3.5K molecular weight cutoff (MWCO) from Thermo Scientific.

3. Results and Discussion

3.1 Cell lysate concept
FIGURE 1 (color web only). Concept of cell lysate experiments. Nuclear and cytoplasmic lysates are separately collected from cells. Nuclear lysates are added directly into a well over the chip, while nuclear lysates are added to a dialysis strip filter over the chip that retains large biomolecules but passes small molecules. The β-lapachone (β-lap) drug and NADH are added to the dialysis strip, which form a redox cycle with NQO1 (see Figure 3b) that releases superoxide and eventually hydrogen peroxide. Redox-probe labelled DNA monolayers on the electrodes of chip support electrochemical charge transfer. Hydrogen peroxide from the drug reaction damages the DNA on the chip, and repair proteins from the chip remove the damaged base, causing a loss of the electrochemical signal.
Our general approach for following β-lap activity in cell lysates is shown in Figure 1. Specifically, devices and cells were prepared to carefully reproduce key features of a natural cellular environment to follow drug-induced DNA damage and subsequent damage repair in probe-modified DNA monolayers on the chip. It was necessary to maintain isolation between nuclear and cytoplasmic lysates, maintaining a similar distribution of proteins and cofactors as accomplished by a nuclear membrane in a cell. For this purpose, nuclear and cytoplasmic cell extracts were prepared separately and collected. Cell lysates with high NQO1 concentrations consistent with cancer (NQO1+) and lower concentrations similar to noncancerous cells (NQO1 knockdown cells, NQO1−) were prepared (See Figure 2). Lysates were then pipetted into a well over a chip capable of electrochemical reactions through DNA monolayers on electrode surfaces. The cytoplasmic components, which contained NQO1, were added to a 5 kDa size-exclusion filter suspended in the well over the chip, which retained proteins but permitted the interchange of small molecules. β-Lap and NADH (as NADH was anticipated to be lost in lysis) were also added to the filter, though they could interchange with the solution directly over the chip. Nuclear extracts, containing repair proteins, were added directly to the well solution over the chip. For the NQO1+ cell lysates, NQO1 sequentially uses NADH followed by β-lap to reactively cycle catalytically and make H₂O₂—one mole of β-lap can form 120 moles of H₂O₂ in 2 mins (see Figure 3). This H₂O₂ oxidatively damages the DNA on the electrode surfaces, forming damage products, such as 8-oxoguanine. Repair proteins from the nuclear extracts then remove the damaged bases, causing a lowering of the electrochemical signal on the chip. This approach enables real-time monitoring of electrochemical reactions and control over the specific biological interactions initiated.
3.2 β-Lapachone is Selectively Lethal in 231 NQO1+ Versus 231 NQO1− Cells

FIGURE 2. (A) Western blot depicting NQO1 levels with relative enzymatic activity and β-lap LD50 concentrations. (B) Survival curve of human triple negative breast cancer, 231 NQO1+ and NQO1− cells that were treated for 2 h with various doses of β-lap (n=6) and cultures were allowed to grow for 7-10 days thereafter for colony forming activity measurements. Data plotted as treated/control (T/C) ± standard error of the mean (SEM). ****p<0.0001

The cells used to generate cell lysates for device study of NQO1 dependent lethality of β-lap were prepared from the triple-negative breast cancer (TNBC) cell line, MDA-MB-231 (231). The cell line has a *2 NQO1 polymorphism and lacked expression of NQO1 protein and enzyme activity (Figure 2) (Pink et al. 2000). The cell line was corrected for NQO1 expression by lentiviral infection without affecting growth rates in vitro or in vivo (Pink et al. 2000). NQO1+ MDA-MB-231 (231NQO1+) or NQO1− MDA-MB-231 (231NQO1−) cells were used
to examine the lethality of β-lap treatment and for comparison with subsequent chip experiments. Figure 2A shows the expression of NQO1 in each cell sample. NQO1 expression is clearly visible in NQO1+ cells, while being completely absent in NQO1− cells. The NQO1 enzyme activity in these cell samples were measured as dicoumarol-inhibited units (U). NQO1+ cells showed 350 ±25 (U) and NQO1− cells 10 ±3 (U) NQO1 enzyme activity. Thus, differential activity was observed, consistent with the distinct NQO1 expression levels in each cell sample.

β-Lap-induced lethality in NQO1+ cancer cells occurs through rapid generation of superoxide (~120 moles per mole of β-lap in 2 min) (Bey et al. 2013), which leads to H₂O₂ formation and subsequent large scale DNA damage. The DNA damage hyperactivates PARP1 and depletes NAD+ (together with NADH and ATP) in exposed cells (Ough et al. 2005). NQO1+ and NQO1− 231 cells were treated with various doses of β-lap for 2 h and NQO1+ cells showed lethality at ≥ 2 μM (Figure 2b). Cell death happens in an NQO1-dependent manner as 231 NQO1− cells were spared from lethality, while 231 NQO1+ cells showed a 50% lethal dose (LD₅₀) using only 2 μM β-lap (Figure 2B). Co-administration of the NQO1 inhibitor, dicoumarol, spared lethality in NQO1+ cancer cells (Huang et al. 2016).

3.3 Electrochemical Measurements of β-lap Activity in Cell Lysates.
FIGURE 3 (color web only). Results of cell lysate experiments. a) Square wave voltammetry percent signal loss after addition of β-lap to chips treated with NQO1 proficient cell lysates.
(NQO1+), NQO1 deficient cells (NQO1−), NQO1+ cells with catalase, and NQO1+ cells with dicoumarol. Error bars represent standard error of the mean. Asterisks represent t-test p-values as follows: ***p≤0.001; ****p≤0.0001. b) Example square wave voltammetry before and after β-lap treatment in NQO1+ and NQO1− cell lysates. c) Typical reaction cycle for β-lap in NQO1+ cell lysates, showing the catalytic production of H2O2 reactive oxygen species (ROS). d) Typical reaction cycle for β-lap in NQO1− and dicoumarol-treated NQO1+ cell lysates. Dicoumarol inhibits NQO1 activity. e) Typical reaction cycle for β-lap in NQO1+ cell lysates treated with catalase. Catalase abrogates the effects of H2O2 by degrading it into H2O and O2.

Chip experiments were performed with both NQO1+ and NQO1− cell lysates to determine the selective DNA damaging influence of β-lap activity. Cytoplasmic and nuclear lysates were added to chips as shown in Figure 1, and signals were recorded before and after β-lap addition. The results of following 2.5 µM β-lap activity in cell lysates are shown in Figure 3a, with example raw data curves shown in Figure 3b and in the supplementary material. For 23 chip electrodes treated with NQO1+ cell lysates, β-lap addition lowered the square wave voltammetry (SWV) signal by 45 ± 5%, consistent with significant DNA damage caused by β-lap redox cycling with NQO1, peroxide production, and subsequent DNA damage (see Figure 3c). Electrodes treated with NQO1− cell lysates showed a considerably smaller change, 12 ± 2%, a factor of 3.7 lower than the change found for NQO1+ cells. β-Lap can undergo one-electron oxidoreductions at a much lower rate and cause a low level of ROS in NQO1− cells, which typically does not kill these cells (Huang et al., 2016). This lower signal change is anticipated as the lower NQO1 concentration cannot catalyse the same degree of hydrogen peroxide production from β-lap (see Figure 3d), and hence lower damage is produced. This 370% signal change difference and
statistical confidence is significantly better than the ~70% signal change difference that we previously observed in buffer solution for NQO1 free controls (Kahanda et al. 2016). This is likely owed to the greater diversity of repair proteins and higher enzymatic turnover in the lysates versus the single repair protein and limited enzymes used in buffer solution. The large signal change difference between NQO1+ and NQO1− lysates illustrates the differential activity of this drug with respect to NQO1 levels, a handle for cancer-specific treatment and selective cancer cell death.

Additional experiments were performed in NQO1+ cells to account for NQO1 and hydrogen peroxide dependence on the damage reaction. Catalase is an enzyme that rapidly degrades H$_2$O$_2$ into water and oxygen and plays a dominant role in peroxide regulation. Dramatic forced over-expression of abnormally elevated catalase production was shown to lead to β-lap resistance in NQO1+ cancer cells (Bey et al. 2013). Figure 3e illustrates the change in reaction mechanism when high concentrations of catalase are present. Figure 3a shows the relative signal change of β-lap treatment after adding 3 nM catalase to chips treated with NQO1+ lysate (raw SWV curves are shown in the supporting info). In this case, an average signal change of only 2.2 ± 1.2% was observed, a dramatic reduction in signal change of over 20-fold compared to the NQO1+ lysate treatment without catalase.

NQO1+ chips were also treated with dicoumarol. Dicoumarol is a hallmark inhibitor of NQO1 activity (Ernster 1967; Ernster et al. 1960; Scott et al. 2011), acting through competitive binding with NAD(P)H and inhibiting electron transfer to flavin adenine dinucleotide (Figure 3d). Dicoumarol treatment greatly reduces the signal change produced by β-lap in NQO1+ cells, lowering it about 10-fold to 4.7 ± 0.9% (raw SWV curves are shown in the supporting info). Such a change is consistent with NQO1 deactivation by dicoumarol.
All of these results are supported by low values of the P-values of the t-test. Each negative control (NQO1−, catalase, dicoumarol) showed a P-value of less than 0.001 when compared to the NQO1+ lysate measurements, indicating high statistical independence. P-values in subsequent control experiments were also found to be similarly low.

3.4 Drug Activity with Concentration

FIGURE 4 (color web only). β-Lap concentration-dependence. a) Square wave voltammetry percent signal loss after addition of β-lap to chips treated with NQO1 proficient cell lysates (NQO1+) and NQO1 deficient cells (NQO1−). Asterisks represent t-test p-values as follows: ***p≤0.001; ****p≤0.0001. Cell lines are numerical fits to the Hill equation. b) Cell death
fraction of 231 NQO1+ cells versus device signal loss (% loss SWV peak current) for NQO1+ cell lysates. Error bars represent standard error of the mean.

Subsequently, we investigated the concentration dependence of β-lap on our devices in the presence of NQO1+ or NQO1− cell lysates. This provided a correlation between observed signal changes on the chip and cell viability assays (Figure 2) to track the connection of DNA damage to selective cell death. Furthermore, the sensitivity of the chip to subthreshold levels of cell activity would be important in therapeutic monitoring of drug activity and in following co-therapies involving β-lap. Figure 4a shows the results of adding successive amounts of β-lap to chips in the presence of NQO1+ or NQO1− cell lysates. Example raw data curves are shown in the supplementary material. For NQO1+ cell lysates, a 4.6% SWV signal change is produced at 0.1 µM β-lap, marking an onset of DNA damage, while 1 µM β-lap induces an 18% SWV change. Increasing the concentration to 2.5 µM β-lap in NQO1+ lysates results in a doubling of signal change to nearly 40% SWV peak current loss. This signal change appears to saturate near 50% signal loss at 10 µM β-lap. Very little signal change (< 1%) is seen in NQO1− lysates for β-lap concentrations of 1 µM and below, with an onset of nonspecific activity occurring near 2.5 µM β-lap. This nonspecific activity remains minimal (~5%) for higher concentrations, such that the p value of the student’s T test comparison between NQO1+ versus NQO1− measurements remains lower than 0.001 for all concentrations from 1 to 10 µM β-lap. Thus, a very significant selective difference in drug activity is apparent for β-lap in lysates with differential NQO1 activity.
In Figure 4a, the NQO1+ and NQO1− curves were both fit well with the Hill equation of the form:

\[ S[\beta] = \frac{[\beta]^n}{[\beta_{1/2}]^n + [\beta]^n} \]  \hspace{1cm} (1)

where \( S[\beta] \) is the SWV signal loss at a particular concentration of β-lap, \([\beta]\) is the β-lap concentration, \([\beta_{1/2}]\) is the β-lap concentration at the midpoint of the signal change, and \( n \) is the Hill coefficient. For the NQO1+ lysate signal, \([\beta_{1/2}]\) was found to be 1.4 µM, and \( n \) was 2.0.

Concerning the \([\beta_{1/2}]\) value, interestingly, this is highly correlated with the concentration for onset of cell death activity in 231 NQO1+ cells (Figure 2b). That is, a midpoint concentration for observation of DNA damage on the device lines up with the concentration for onset of cell death from cell viability experiments. Concerning \( n \), a Hill coefficient of 2.0 indicates a cooperative β-lap effect—that is, the affinity for DNA damage increases as β-lap is successively added.

Previous studies of β-lap-induced single- and double-strand breaks in MCF-7 breast cancer cells similarly revealed a super-linear dependence of these defects on β-lap concentration (Bentle et al. 2007).

To further investigate the correlation of device signal changes with survival effects observed in cells, we plotted (Figure 4b) the cell death fraction (viability) versus the SWV percent signal change from the device. It also follows a sigmoidal relationship of the form:

\[ D(S) = \frac{D_{\text{max}}}{1 + e^{-k(S-S_{1/2})}} \]  \hspace{1cm} (2)

where \( D(S) \) is the cell death fraction at a corresponding SWV signal loss of \( S \), \( D_{\text{max}} \) is the maximum cell death fraction observed, \( S \) is the square wave voltammetry signal loss (independent graph variable), \( S_{1/2} \) is the SWV signal loss where half of the maximum cell death
fraction is achieved, and $k^{-1}$ is the rate/slope of the graph near $S_{1/2}$. Notably, $S_{1/2}$ occurs at 39% signal loss, where the concentration curve of Figure 4a begins to saturate. That is, a midpoint concentration of β-lap of cell death/viability (2.5 µM) is connected to the concentration of the onset of saturating DNA damage on the chip. Thus, it appears that an overwhelming amount of β-lap induced DNA damage is necessary to bring about cancer cell death, consistent with proposed mechanistic death pathways (Silvers et al. 2017).

3.5 Additional Device Controls and Considerations
FIGURE 5 (color web only). Various controls of β-lap activity. a) 2.5 µM β-lap activity with NQO1+ cell system before and after nuclear lysate addition. b) 2.5 µM β-lap activity with NQO1+ cell system before and after cytoplasmic lysate addition. c) 2.5 µM β-lap activity with NQO1+ cell system without using the filter. Asterisks represent t-test p-values as follows: **p≤0.01; ***p≤0.001; ****p≤0.0001. For 5c, the t-test statistics were taken against the NQO1+ data of Figure 3a. Error bars represent standard error of the mean.

To confirm our understanding of the roles of each component in following β-lap activity by DNA electrochemistry on chips, we performed additional sequential experiments with NQO1+ lysates. In Figure 5a, we followed the effect of initially withholding nuclear lysates from the chip. Example raw data curves are shown in the supplementary material. Chips were treated with
cytoplasmic lysates in the dialysis strip and NADH, but initially no nuclear lysate. Under these conditions, the signal change upon adding 2.5 µM β-lap was found to be 4.6 ± 1.4%, similar to the signals from various negative controls shown in Figure 3 and Figure 4 (NQO1−, dicoumarol, catalase). The lack of signal change follows from the understanding that without the nuclear lysates, no DNA repair proteins are available to repair DNA damage and disrupt the DNA electrochemistry. When the nuclear lysate was subsequently added, a large signal change followed (71 ± 1%), consistent with the introduction of repair proteins that can remove the damaged base from DNA (Figure 1). This signal change is larger than others due to the signal losses from nonspecific binding of the repair proteins in the nuclear lysate. (These nonspecific effects are accounted for in other experiments by treating with nuclear lysate before β-lap addition.) In Figure 5b, we next observed the effect of withholding the cytoplasmic lysate. With only nuclear lysates and NADH present, addition of 2.5 µM β-lap again produced a minimal (5.0 ± 1.7%) change. Under this condition, no NQO1 is present to catalyse the β-lap reaction cycle (Figure 3b). When the cytoplasmic lysate component is added, a large signal change occurs (42 ± 2%), consistent with the addition of NQO1 and the catalysis of the β-lap reaction. Finally, we checked for evidence that the filter was necessary for clear observation of the activity. We added both the cytoplasmic lysate and nuclear lysate directly to the chip, then observed the signal change upon addition of the β-lap was very small, within zero in consideration of the standard error. The reason for this is not fully clear, but some inferences can be drawn from our previous study in buffer mixtures on chips (Kahanda et al. 2016). In these experiments, only NQO1, NADH, iron, transferrin, formamidopyrimidine DNA glycosylase (FPG, a repair protein), and β-lap were introduced to the chip. Differential β-lap activity was only observed with the filter in place, indicating that either NQO1 interfered with DNA repair activity, or that the repair
glycosylase interfered with the NQO1 redox cycle activity. In either case, in consideration of the underlying biological segregation of these components, a filter was added to serve like a membrane between nuclear and cytoplasmic proteins. Clearly, in our lysate study, this filter is similarly important to maintain biological relevance and observe β-lap activity.

3.6 Concept of Device Application
FIGURE 6 (color web only). Concept of applying technology for anticancer drug screening. Multiple electrodes are established on a chip with separate solution wells to evaluate multiple anticancer drugs from patient cancer and control cell samples. In this example, three drugs are evaluated. The left half of the chip is reserved for cancer cell samples, and the right for control cells. The DNA damaging response of each drug is evaluated by the loss in SWV signal.

Overall, we envision that this means of detection can be extended to the study of multiple anticancer drugs to evaluate drug efficacy for particular cancers and to screen for personalized treatments for improved patient outcomes. The concept for the screening of three independent drugs is illustrated in Figure 6. Multi-electrode chips with separate wells for each experiment are shown. For each of the three drugs, experiments are performed in both cancer cell extracts and control cell extracts. The DNA damaging response of each drug under each condition is recorded by the loss of SWV signal. For this example, Drug 1 does not produce a large drop in SWV
signal, indicative of ineffectual activity. Hence, Drug 1 would be deemed as not ideal for treatment. Drug 2 shows a large signal loss on the cancer side, but also a significant simultaneous loss on the control side. This would indicate significant damage to both control and cancer cells. While potent against cancer, this drug would be deemed unselective due to its toxicity to control cells, and also not ideal for treatment. Drug 3 shows the ideal combination of a large SWV signal drop for the cancer side and little to no loss on the control cell side. Of the treatments screened, Drug 3 would be the recommended drug due to its high activity in cancer cells and low activity in control cells.

Along these lines, this technology is complementary to current nanotechnology efforts with microfluidics to screen for cancer (Kim et al. 2012; Pandya et al. 2017; Sykes et al. 2014) and disease (Besant et al. 2015; Taneja et al. 2017). While most closely related to a viability assay, this approach examines the more fundamental role of DNA damage repair, which equilibrates in seconds to minutes rather than hours. This also goes beyond biomarker assays, such as NQO1 (Tan and Berridge 2010) or even 8-oxoguanine (Kow and Dare 2000), evaluating DNA damage through the first step in base excision repair in real time for kinetics studies. Thus, this technology could complement and advance current efforts in understanding the selective treatment of cancers and diseases.

4. Conclusions

Multi-electrode chips with DNA monolayers were used to track anticancer drug activity in cellular lysates and correlate cell death activity with DNA damage. MDA-MB-231 triple-negative breast cancer cells were deficient in expression of NAD(P)H:quinone oxidoreductase 1 (NQO1) due to a *2 homozygous polymorphism in the gene. We constructed NQO1+ 231 cells
through lentiviral transfection to mimic cancerous (NQO1+) or control (NQO1-) cells. Cells were lysed and added to chips, and the impact of β-lapachone (β-lap), a NQO1-dependent DNA-damaging drug, was followed with DNA electrochemical signal changes arising from drug-induced DNA damage. Devices showed an approximate four-fold difference in electrochemical response to NQO1+ over NQO1− cells, as well as great selectivity to catalase and dicoumarol controls that deactivate DNA damaging pathways. Concentration dependence studies revealed that 1.4 µM β-lap correlates with the midpoint of device signal change and the onset of cell death, and 2.5 µM β-lap correlates with the saturation of DNA damage on the chip and the midpoint of cell death. Concentration dependence was fit with the Hill equation, revealing that β-lap affinity for DNA damage increases as β-lap is successively added. Multiple controls affirm that the mechanism of signal change depends on the details of NQO1 activity and peroxide formation, in that catalase and dicoumarol protected from β-lap DNA damage. Controls also show that it is necessary to use a device filter component that segregates nuclear and cytoplasmic components, a membrane filter that mimics a nuclear membrane. We also conceptually demonstrated how these devices could inform therapeutic decisions through measurement of the potency and selectivity of DNA damaging drugs.

Acknowledgements

D.A.B gratefully acknowledges support from NIH grants 1R01 CA221158-01 and 1R01 R01CA224493-01. The authors acknowledge support from the Office of Naval Research grant number N000141612741. The authors would like to dedicate this paper to the late C. S. Rupert, a UT Dallas Biology professor and a pioneer of DNA repair research.

Disclosures
D.A.B. is developing a commercial form of an NQO1 activatable drug with Systems Oncology, Inc. and Toray, Inc. D.K., N.S., and J.D.S declare no competing financial interests.

Appendix A. Supplementary Material

Supplementary data associated with this article can be found in the online version at:

References


Highlights

- Devices track drug activity in cell lysates by following DNA damage electrochemically
- 3.7-fold differences between cancer and enzymatic knockdown control cell lysates
- 10 & 20-fold differences observed with dicoumarol and catalase abrogation pathways
- Drug-induced DNA damage on chip correlates with key points of cell viability
- DNA devices follow potency and selectivity of drugs, potentially informing therapy