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Identification and characterization of new chemical entities targeting APE1 for the prevention of
chemotherapy-induced peripheral neuropathy (CIPN)

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Abstract

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Chemotherapy-induced peripheral neuropathy (CIPN) is a potentially debilitating side effect of a number of chemotherapeutic agents that does not have any FDA-approved interventions or prevention strategies. Although the cellular mechanisms mediating CIPN remain to be determined, several lines of evidence support the notion that DNA damage caused by anticancer therapies could contribute to the neuropathy. DNA damage in sensory neurons following chemotherapy correlates with symptoms of CIPN. Augmenting APE1 function in the base excision repair (BER) pathway reverses this damage and the neurotoxicity caused by anticancer therapies. This neuronal protection is accomplished by either overexpressing APE1 or by using a first generation targeted APE1 small molecule E3330 (also called APX3330). While E3330 has been approved for Phase 1 clinical trials (IND125360), we synthesized and determined whether novel, second-generation APE1 targeted molecules would be protective against neurotoxicity-induced by cisplatin or oxaliplatin while not diminishing the anti-tumor effect of the platins. Measuring various endpoints of neurotoxicity using our *ex vivo* model of sensory neurons in culture, we determined that APX2009 is an effective small molecule that is neuroprotective against cisplatin and oxaliplatin-induced toxicity. APX2009 also demonstrated a strong tumor cell killing effect in tumor cells and the enhanced tumor cell killing was further substantiated in a more robust 3D pancreatic tumor model. Together, these data suggest that the second generation compound, APX2009 is effective in preventing or reversing platinum-induced CIPN, while not affecting the anti-cancer activity of platins.

Introduction

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Chemotherapy-induced peripheral neuropathy (CIPN) is a potentially debilitating side effect of a number of chemotherapeutic agents. The major symptoms of these neuropathies are largely characterized by alterations in peripheral sensory function, suggesting that sensory neurons are a major target of the toxicity. Symptoms can include allodynia, increased sensitivity to cold, loss of proprioception, loss of touch, reduced tendon reflexes and pain. Unlike other major side effects of chemotherapy (e.g. nausea, hair loss, bone marrow failure) (Zafar et al., 2010), there are currently no FDA-approved interventions or prevention strategies for CIPN (Hershman et al., 2014; Stone and DeAngelis, 2016).

Although the cellular mechanisms that cause CIPN are not known, our previous work strongly supports that idea that DNA damage in neurons, which can occur with any cancer therapies contributes to the neuropathy. Therapies including platinum agents and ionizing radiation cause DNA damage in sensory neurons and augmenting the base excision repair (BER) pathway reverses the toxic effects of these chemotherapies (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014; Kim et al., 2015). Reducing the expression of a critical enzyme in the BER pathway, APE1 (also called Ref-1), in sensory neurons amplifies the toxicity to sensory neurons exposed to anticancer therapies, whereas overexpression of APE1 and an APE1 DNA repair proficient-redox deficient mutant protein attenuates the neurotoxicity (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014). The neuronal protection afforded by overexpressing APE1 is mimicked by a first generation APE1 redox-signaling small molecule inhibitor E3330 (also called APX3330) (Vasko et al., 2011; Kelley et al., 2014). This compound is well tolerated in mice and displays suitable pharmacokinetic characteristics (half-life, AUC, bioavailability) with no evidence of acute drug-related severe toxicity or lethality observed in our *in vivo* studies (Fishel et al., 2011). E3330 was investigated by Eisai (Japan) for the potential treatment for chronic hepatitis C. We have developed the drug for cancer therapeutics and subsequently discovered its neuronal protective effects. We have published that E3330 increases the DNA repair activity of APE1 in rat *ex vivo*

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sensory neuronal cultures without decreasing its cancer efficacy (Kelley et al., 2014). Consequently, augmenting APE1 DNA repair activity diminishes the neurotoxic effects of anticancer drugs on sensory neurons, thereby providing an opportunity to intervene with a small molecule that could prevent or reverse CIPN.

Our work with the first generation small molecule, E3330, which will soon enter a phase 1 clinical trial (IND125360), provides a rationale for examining the effects of new second-generation small molecules (Luo et al., 2008; Nyland et al., 2010; Kelley et al., 2011). Consequently, we determined whether novel second-generation APE1 targeted molecules would be protective against neurotoxicity-induced by cisplatin or oxaliplatin while not diminishing the anti-tumor effect of the platinum. To test this, we performed a series of experiments using our *ex vivo* model of sensory neurons in culture measuring various endpoints of neurotoxicity including cell survival, DNA damage and transmitter release. We also assessed the anti-tumor effects of one of these novel small molecules, APX2009, in neuroblastoma cell lines as well as a 3D spheroid pancreatic tumor model and assessed the pharmacokinetics and P450 metabolism of this compound.

Materials and Methods

Materials

General tissue culture supplies were obtained from Invitrogen (Carlsbad, CA), whereas routine chemicals were purchased from Sigma-Aldrich (St. Louis, MO). For sensory neuronal cultures, poly-D-lysine and laminin were purchased from Sigma-Aldrich (St. Louis, MO), nerve growth factor from Harlan Bioproducts for Science (Indianapolis, IN), and normocin from Invivogen (San Diego, CA). Mouse monoclonal antihuman APE1 antibodies were raised in our laboratory and are available from Novus Biologicals (Littleton, CO). Mouse monoclonal anti-phospho-H2AX antibodies were from EMD Millipore (Billerica, MA) and β -Actin monoclonal

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antibody from Thermo Fisher Scientific (Fremont, CA). Chemiluminescence secondary antibodies were from Roche Diagnostics Corp. (Indianapolis, IN).

Cisplatin was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and Oxaliplatin was purchased from LKT Laboratories, Inc. Cisplatin was initially dissolved in N,N-dimethylformamide (Sigma-Aldrich) and stored as a 40mM solution at -80°C and oxaliplatin dissolved in PBS and stored as a 5mM stock at -80°C . Before drug treatment, the stocks were diluted in F-12 growth medium and added to cultures and exposed for 24-72 hours. The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies.

Synthesis of new chemical entities

Complete details of synthesis of the new, second-generation compounds will be provided in a manuscript in preparation. The compounds were synthesized at by Cascade Custom Chemistry, Eugene, OR 97401 USA. In summary, iodolawsone, 2-iodo-3-hydroxy-1,4-naphthoquinone a common intermediate, is available from Cascade Custom Chemistry. As described iodolawsone in subsequent reaction is treated with methacrylic acid or 2-propylacrylic acid, with oxalyl chloride and the corresponding amine, and with sodium methoxide in methanol to yield (2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-dimethylpentanamide (APX2007), (2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-diethylpentanamide (APX2009), and (2E)-2-(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-N,N,2-trimethylprop-2-enamide (APX2032). Further information can be found in the issued patent "Quinone Compounds for Treating Ape1 Mediated Diseases" (Mark R. Kelley and James H. Wikel), issued patent number 9,193,700, issued on 11.24.15.

Chemical structure presentation

Marvin was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 15.8.24.0, 2015, ChemAxon (<http://www.chemaxon.com>). Calculator Plugins were used for structure property prediction, Marvin 15.8.24.0, 2015,

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ChemAxon (<http://www.chemaxon.com>). Molecular modeling was performed using the Open Eye Scientific software OMEGA (OMEGA [2.5.1.4](#)) (Hawkins et al., 2010) and ROCS (ROCS [3.2.1.4](#): OpenEye Scientific Software, Santa Fe, NM. <http://www.eyesopen.com>) (Hawkins et al., 2007). Molecular visualization was performed using the Open Eye Scientific software VIDA (OpenEye Scientific Software, Santa Fe, NM. <http://www.eyesopen.com>).

Sensory neuronal cultures

Primary cultures of sensory neurons were harvested and maintained as previously described (Vasko et al., 2005). Briefly, adult male Sprague-Dawley rats (150–175 g; Harlan, Indianapolis, IN) were euthanized by CO₂ asphyxiation and dorsal root ganglia (DRG) dissected from all spinal levels, transferred to into a collagenase solution (1 mg/ml), incubated for 1hr at 37°C, then dissociated by mechanical agitation. Approximately 30,000 cells or 60,000 cells were plated into each well of 12-well or 6-well culture plates, respectively. All culture dishes were precoated with poly-D-lysine and laminin. Cells were maintained in F-12 media supplemented with 10% horse serum, 2 mM glutamine, 100 µg/ml normocin™, 50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µM 5-fluoro-2'-deoxyuridine (Invitrogen), 150 µM uridine, and 30 ng/ml of NGF in 3% CO₂ at 37°C. Growth medium was changed every other day. Experiments were performed after cells were maintained in culture for 12-14 days.

Neuronal cell viability

Sensory neuronal culture trypan blue exclusion analysis was performed as previously described (Vasko et al., 2011). Cells were detached by adding a 0.05% trypsin-EDTA solution and media to each well. An equal volume of 0.4% (w/v) trypan blue in PBS was added to the cell suspension and the numbers of living cells (i.e. those that exclude the dye) were counted under a phase contrast microscope using a hemacytometer. Percent survival was calculated as the percent of live cells divided by the total cell number (including dead and live cells).

Cell line authentication and characterization

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The IMR32 and SK-N-SH cell lines were obtained from the American Type Culture Collection and grown in RPMI-1640 supplemented with 10% FBS. Cell line identity was confirmed by DNA finger print analysis (IDEXX BioResearch) for species and base-line short-tandem repeat analysis testing. All cell lines were 100% human and a 9-marker short-tandem repeat analysis is on file.

Cell proliferation assay

Cells were seeded in 96-well plates (IMR32: 1000 cells/well; SK-N-SH: 3000 cells/well) and treated for 5 days with APX2007, APX2009, APX2032, or E3330. Final DMSO concentration was $\leq 0.1\%$. Cell viability was determined using the methylene blue assay as previously described (Tonsing-Carter et al., 2015). Each experiment was performed in triplicate and repeated three times. The percent viabilities, normalized to the control, were graphed and ED₅₀ values determined using the Chou-Talalay method (Chou and Talalay, 1984).

Immunoblotting

Immunoblotting was performed as previously described (Kelley et al., 2014). Briefly, cells were lysed in RIPA buffer (Santa Cruz Biotechnology; Santa Cruz, CA, USA) and protein was quantified using the Lowry assay. Proteins were separated by electrophoresis on a 4-12% SDS-polyacrylamide gel. The gel was transferred to a PVDF membrane and incubated overnight at 4° C in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat dry milk while gently agitating. Mouse monoclonal antihuman Ape1 antibodies (1:500), mouse monoclonal anti-phospho H2AX antibodies (1:1000), or β -Actin monoclonal antibody (1:1000) were added to the blocking solution and incubated overnight at room temperature while gently agitating. Antibody binding was detected following appropriate secondary antibody methods using chemiluminescence. The density of the bands was measured using QualityOne® software from Bio-Rad (Hercules, CA) and data expressed as density normalized to actin.

Measurement of calcitonin-gene related peptide release

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For release experiments, cell cultures were washed with HEPES buffer consisting of (in mM) 25 HEPES, 135 NaCl, 3.5 KCl, 2.5 CaCl₂, 1 MgCl₂, 3.3 D-glucose, and 0.1% bovine serum albumin, pH 7.4 and maintained at 37°C. They then were incubated for successive 10 min intervals with 0.4 ml of HEPES buffer alone (basal release), with buffer containing 30 nM capsaicin, then with buffer alone (to assess return to basal release). After each incubation, the buffer was removed and the amount of immunoreactive calcitonin-gene related peptide (CGRP) in each sample was measured using radioimmunoassay (RIA) as previously described (Chen et al., 1996). At the end of the release protocol, CGRP is extracted from the cultures and total content measured using RIA. Since treatments did not significantly alter total content, release data are presented as as fmol of peptide released /well/10 min.

AP endonuclease DNA repair assay

Inhibition or enhancement of APE1 DNA repair endonuclease activity was performed as previously described (Bapat et al., 2010). The APE1 repair activity assay was performed in a plate assay using two annealed oligonucleotides (5'-6-FAM-GCCCC*GGGGACGTACGATATCCCGCTCC-3' and 3'-Q-CGGGGGCCCCCTGCATGCTATAGGGCGAGG-5') containing a quencher on one strand and a fluorescent 6-FAM label with tetrahydrofuran as an AP site mimic. Oligo cleavage at the AP mimic site results in 6-FAM release and detection. The fluorescence was read at five, one-minute intervals using a Tecan Ultra plate reader (Chemical Genomics Core, Indiana University School of Medicine). The rate of the reaction was used to determine the change in APE1 repair activity as compared to the vehicle control.

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described (Luo et al., 2012). Purified APE1 was reduced with 1.0 mM DTT for 10 min and diluted to a final concentration of 0.006 mM with 0.02 mM DTT in PBS. Reduced APE1 was added to EMSA reaction buffer (10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 5% [vol/vol] glycerol) with 2 mL 0.007 mM protein mixture (1:1) of

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purified truncated c-Jun and c-Fos proteins containing DNA-binding domain and leucine zipper and incubated for 30 min. at room temperature. The EMSA assay was performed as previously described (Luo et al., 2008; Nyland et al., 2010; Kelley et al., 2011; Luo et al., 2012).

Transient luciferase reporter assays

Reporter assays were performed as previously described (Georgiadis et al., 2008; Kelley et al., 2011; Cardoso et al., 2012b; Luo et al., 2012). Cells were transfected with NF- κ B–Luciferase construct containing an NF- κ B–response promoter and driving the expression of a luciferase gene and a *Renilla* luciferase control reporter vector pRL-CMV. After a 24-h transfection period, cells were lysed, and *Firefly* and *Renilla* luciferase activities were assayed using *Renilla* luciferase activity for normalization. All of the transfection experiments were performed in triplicate and repeated at least three times in independent experiments. Data are expressed as mean \pm standard error from a representative experiment, and Student's *t* tests were performed.

Tumor and cancer associated fibroblast (CAF) 3D co-cultures

Patient-derived tumor cells and CAF19 cells were a kind gift from Dr. Anirban Maitra (M.D. Anderson Cancer Center)(Jones et al., 2008). All cell lines were authenticated via STR analysis (IDEXX BioResearch) and checked routinely for mycoplasma contamination. Ultra low attachment 96-well plates (Corning Inc., Life Sciences) were used to generate 3-dimensional tumor spheroids in the presence and absence of CAFs as described previously (Sempere et al., 2011; Arpin, 2015). TdTomato-labeled PDAC cells and EGFP-labeled CAFs are resuspended in colorless DMEM media containing 3% Reduced Growth Factor Matrigel (BD Biosciences) and 5% FBS at a cell ratio of 1:4 (tumor:CAF) and fed on days 4 and 8 following plating. Both cell populations are quantitated for intensity and area via Thermo ArrayScan at day 12 of co-culture.

Pharmacokinetics (PK) and P450 metabolism analysis

PK studies were performed in the IU Simon Cancer Center Clinical Pharmacology Analytical Core (CPAC) as previously described for E3330 (Fishel et al., 2011) and standards

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for the novel compounds used. P450 metabolism studies using human microsomes were also performed in CPAC directed by Dr. David Jones.

Statistical analysis

Data is expressed as the mean \pm SEM from a minimum of three independent harvests or experiments. Statistically significant differences between controls and various treatments were assessed using Student t-tests. Differences in cell survival using trypan blue exclusion, gamma-H2AX (pH2AX), and CGRP release were determined using two-way analysis of variance (ANOVA) and Tukey's post hoc test.

Results

Chemical synthesis of E3330 analogs, validation of redox inhibition and pharmacokinetics

We synthesized a number of novel analogs of E3330 and have been able to replace the core dimethoxybenzoquinone (A) with a naphthoquinone ring, the methyl group (B) on the ring structure has been replaced with various halogens or hydrogen, and the carbon chain (C) on the double bond can be shortened to modulate activity (Figure 1A) (Luo et al., 2008; Nyland et al., 2010; Kelley et al., 2011). In our continuing efforts, we modified the carboxylic acid moiety (D) in concert with shortening the carbon chain (C) on the double bond. These changes modified two physical properties of the structure. E3330 exists as a charged molecule at physiological pH. We prepared amide derivatives of the carboxylic acid (D), which are not charged supporting chemical feature. In addition, E3330 has a very lipophilic carbon chain which we believed to be a modifiable feature. The new structures have significantly shorter carbon chains (C) on the double bond and are therefore less lipophilic. Detailed synthesis data can be found in patent 9,089,605. Three new structures from the compounds made (Figure 1B) were analyzed in our redox APE1 electrophoretic mobility shift assay (EMSA) studies to determine which compounds affect the redox function of APE1 as previously described (Georgiadis et al.,

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2008; Kelley et al., 2011; Su et al., 2011; Luo et al., 2012; Zhang et al., 2013). The compounds had redox inhibition IC_{50} s of: APX2007 2 μ M, APX2009 1 μ M, and APX2032 1 μ M (Supplemental Figure 1A). E3330 has been previously presented and has an IC_{50} of 25 μ M in similar assays (Su et al., 2011; Luo et al., 2012; Zhang et al., 2013)

We performed reporter transactivation assays to verify the new compounds were effective in cells and hit their target APE1 which, in this assay, regulates NF κ B function as previously described (Luo et al., 2008; Nyland et al., 2010). In these assays, all three compounds, APX2007, APX2009 and APX2032 demonstrated similar inhibition of NF κ B binding to the reporter construct with an IC_{50} of 7 μ M, while E3330 has an activity of 45 μ M (Supplemental Figure 1B) (Luo et al., 2008; Nyland et al., 2010). Additionally, we determined the ED_{50} for tumor cell killing in two neuroblastoma cell lines, IMR32 (p53wt, MYCN amplified) and SK-N-SH (p53wt, MYCN non-amplified) (Supplemental Figure 1C and 1D and Supplemental Figure 3). All three compounds had a reduced ED_{50} compared to E3330; 7-10 fold greater in IMR32 cells and 4-6 fold greater in SK-N-SH cells (Supplemental Figure 1C and 1D and Supplemental Figure 3). The enhanced tumor cell killing data is consistent with the increased efficacy of the compounds on APE1 function as demonstrated by EMSA and transactivation data in Supplemental Figure 1. We also assessed the pharmacokinetic profile of APX2009. As shown in Supplemental Figure 2, the half-life of APX2009 is 25.8 hours compared to 3.6 hours for E3330 or an approximate 7-fold half-life increase. Additionally, using human microsomes in a P450 metabolism analysis, APX2009 had a 173 vs 20-minute half-life or an 8.7-fold increase (Supplemental Figure 2).

When the sensory neuronal cultures were exposed to E3330 at 10, 20 or 40 μ M for 24 hours, there was no significant cell death as measured by trypan blue exclusion (Figure 2A) confirming our previous work (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014). In a similar manner, exposing cultures to various concentrations of APX2009 did not result in a significant reduction in cell viability (Figure 2A). In contrast, treating cells with 40 μ M

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APX2007 for 24 hours or with 20 μ M or 40 μ M APX2032 for 24 hours resulted in a significant reduction in cell viability (Figure 2A). In a similar manner exposing cultures to 20 or 40 μ M APX2007, or APX2032 for 72 hours caused a significant increase in cell death (data not shown).

DNA repair activity assays were performed as previously described (Bapat et al., 2010). As shown in Supplemental Figure 4, only APX2009 demonstrated a stimulation of APE1 repair activity in this assay and in the nanomolar range, a significant increase in activity compared to E3330 (Supplemental Figure 2). APX2007 and APX2032 had no effect, either for stimulation or inhibition of APE1 endonuclease activity.

E3330 and APX2009, but not APX2007 or APX2032 attenuate cisplatin-induced cell death in sensory neuronal cultures

Previous studies in our laboratory have shown that augmenting APE1 repair activity attenuates the ability of cisplatin to cause cell death in sensory neuronal cultures (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014). Since exposing neuronal cultures to E3330 is neuroprotective (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014), we determined whether it and other analogs would affect cisplatin-induced cell death in our cultures. As we have previously shown (Jiang et al., 2008; Kelley et al., 2014), exposing neuronal cultures to increasing concentrations of cisplatin for 24 hours causes a concentration-dependent reduction in cell viability to 66 ± 5 % and 50 ± 7 % for 30 and 100 μ M, respectively (Figure 2B). This cisplatin-induced cell death was blocked by exposing neuronal cultures to E3330 (20 μ M) or to APX2009 (20 μ M) for 48 hours prior to and throughout the cisplatin treatment (Figure 2B). In contrast, pretreatment with 20 μ M of APX2007 or APX2032 did not attenuate the cisplatin-induced cell death, with the combination of APX2032 and cisplatin (100 μ M) reducing cell viability to 9 ± 9 % (Figure 2B). Therefore, APX2009 protects sensory neuronal cultures against cisplatin-induced cell death at all dose levels used whereas APX2007 and APX2032 caused cell killing at high dose (100 μ M).

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E3330 and APX2009, but not APX2007 or APX2032 attenuate cisplatin-induced decrease in transmitter release from sensory neurons

Although relatively high concentrations of cisplatin are necessary to cause cell death in sensory neuronal cultures, lower concentrations reduce transmitter release from sensory neurons (Jiang et al., 2008, Kelley et al., 2014). Thus, we determined whether E3330 analogs could attenuate a functional endpoint of cisplatin-induced neurotoxicity, i.e. the decrease in capsaicin-evoked release of CGRP. When sensory neurons in culture were exposed to E3330 (20 μ M) or APX2009 (10 or 20 μ M) for 72 hours and CGRP release examined, there was no significant change in either basal (resting) release or release stimulated by 30 nM capsaicin when compared untreated cells (Figure 3A). However, pretreatment with APX2007 or APX2032 (10 μ M) for 72 hours also did not affect CGRP release, whereas 20 μ M of each caused a significant increase in capsaicin-stimulated release (Figure 3A). None of the drugs at the concentrations tested altered the total content of CGRP in the cultures (data not shown). Confirming our previous results, neuronal cultures exposed to 10 μ M cisplatin results in a significant reduction in the capsaicin-evoked release of CGRP (Figure 3B) (Jiang et al., 2008). Pretreating cultures with 20 μ M of E3330 or the APX compounds for 48 hours prior to and throughout exposure to cisplatin abolished the reduction in release caused by the anticancer drug (Figure 3B). A 72 hour treatment with 10 μ M APX2007 or APX2009 did not prevent the cisplatin-induced reduction in release, but 10 μ M APX2032 did block the effect of cisplatin. Since APX2007 and APX2032 alone augmented transmitter release, the reversal of the cisplatin effect could be nonspecific. In contrast, both E3330 and APX2009 appear neuroprotective since they do not alter release when given alone.

APX2009 significantly reduces DNA damage induced by cisplatin in sensory neuronal cultures

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As further confirmation of the neuroprotective effects of APX2009 following cisplatin treatment, the levels of phospho-H2AX (pH2AX), a marker of DNA damage (Podhorecka et al., 2010; Redon et al., 2010; Crowe et al., 2011), were measured in sensory neuronal cultures in the absence or presence of various E3330 analogs. When cultures were exposed to 10 μ M cisplatin for 24 or 48 hours, there is a significant increase in the levels of pH2AX as measured using Western blotting confirming DNA damage by the platinum compound (Figure 4). Pretreating cultures with APX2009 (20 μ M) for 48 hours prior to and throughout exposure to cisplatin significantly reduces the levels of pH2AX. In contrast, neither APX2007 nor APX2032 (20 μ M) altered the ability of cisplatin to produce DNA damage (Figure 4).

APX2009 is neuroprotective against oxaliplatin-induced neurotoxicity

Based on the findings that APX2009, but not APX2007 and APX2032 protected against cisplatin-induced DNA damage and decreased CGRP release, we prioritized APX2009 for use in subsequent studies with another platinum agent, oxaliplatin. Cisplatin and oxaliplatin both produce significant levels of ROS in cells, with cisplatin producing higher levels (Preston et al., 2009; Kelley et al., 2014; Kim et al., 2015). However, the DNA cross-links produced by these two agent differ: with cisplatin producing Pt-1-2-d(GpG) intrastrand DNA crosslinks while oxaliplatin creates predominantly Pt-1-3 d(ApG) interstrand DNA crosslinks (Kelley et al., 2014). We have previously demonstrated that E3330 protects against both cisplatin- and oxaliplatin-induced neurotoxicity (Kelley et al., 2014). Therefore, we wanted to determine if APX2009 has a similar protective effect following oxaliplatin treatment which would also support our hypothesis that it is the repair of oxidative DNA damage participates in the regulation of the platinum cross-link removal as shown with E3330 (Kelley et al., 2014; Kim et al., 2015). As shown in Figure 5A, a 72 hour treatment with 10 or 20 μ M APX2009 protected the sensory neuronal cultures from cell killing caused by a 24 exposure to oxaliplatin. In a similar manner, pretreating neuronal cultures with APX2009 for 48 hours prior to and throughout exposure to

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oxaliplatin for 24 hours, prevented the oxaliplatin-induced decrease in CGRP release from sensory neurons (Figure 5B).

APX2009 also significantly reduced the phosphorylation of H2AX after 24 and 48 hr treatments of oxaliplatin (Figure 5C) indicating that its neuroprotective effects may be due to reduced DNA damage.

Treatment of human PDAC 3D tumor model with APX2009

While the neuroprotective effects of APX2009 are evident, we also wanted to investigate whether these E3330 analogs were capable of tumor cell killing similar to what we have observed with E3330 (Vasko et al., 2011; Kelley et al., 2014; Kim et al., 2015). A three-dimensional co-culture model of pancreatic cancer established in our laboratories was used as an *ex vivo* system that included both low passage patient- derived tumor cells and cancer-associated fibroblasts (Arpin, 2015). We assessed the effects of APX2009-induced cytotoxicity on the area and intensity of both tumor cells alone and in co-culture with CAFs. Spheroids composed of patient-derived PDAC cells (Pa03C – labeled red) and CAF19 cells (labeled green) were treated with APX2009 (Figure 6), and the area and intensity of red and green fluorescence were evaluated separately as markers for each cell type (Figures 6A-C). Interestingly, CAFs were not significantly affected by APX2009 treatment, again suggesting that non-tumorigenic cells can tolerate the effects of APE1 inhibition more than tumor cells. This data is similar to what is observed with E3330 (Logsdon et al., 2015) but being effective at lower dose levels, validating APX2009 as a potential PDAC therapeutic agent while also showing CIPN protective indications.

Additionally, we determined the ED₅₀ for tumor cell killing in two neuroblastoma cell lines, IMR32 (p53wt, MYCN amplified) and SK-N-SH (p53wt, MYCN non-amplified) (Supplemental Figure 1C and 1D and Supplemental Figure 3). All three compounds had a reduced ED₅₀ compared to E3330 of 7-10 fold greater in IMR32 cells and 4-6 fold greater in SK-

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N-SH cells (Supplemental Figure 1C and 1D and Supplemental Figure 3). For APX2009, tumor cell killing was increased 4.3-8.7 fold when compared to E3330 for either the neuroblastoma or 3D pancreatic tumor cell models (Supplemental Figure 2). This tumor cell killing data is consistent with the increased effect on APE1 function demonstrated by the EMSA and transactivation data (Supplemental Figures 1 and 2) .

Discussion

One of the major concerns for patients during and post-cancer treatment and survivorship is the development of chemotherapy-induced peripheral neuropathy (CIPN), which can limit patients from receiving truly therapeutic levels of these drugs and can have a significant impact of quality of life for patients. The platinum agents are still one of the major families of chemotherapy used as standard of care (SOC) in a wide variety of cancer scenarios. However, despite the abundant use of platinum-based chemotherapy, little is known about the mechanisms by which these drugs cause neuropathy or how to prevent or treat this debilitating toxicity (Ness et al., 2013). Furthermore, unlike a number of other major side effects of chemotherapy [e.g. nausea, hair loss, bone marrow failure (Zafar et al., 2010)], there are no standard, effective treatments to prevent or reverse CIPN. The American Society of Clinical Oncology (ASCO) recently determined there are no current clinical agents recommended for the prevention of CIPN (Hershman et al., 2014). Thus, there remains a strong clinical need for developing new drug treatments based on discovery of mechanisms mediating CIPN.

In our previous studies using an experimental model of isolated sensory neurons in culture, we established a causal relationship between cancer-therapy-induced neurotoxicity and DNA damage and repair and more specifically base excision repair (BER) and APE1 (Vasko et al., 2005; Jiang et al., 2008; Vasko et al., 2011; Kelley et al., 2014; Kim et al., 2015; Georgiadis et al., 2016). We demonstrated that reducing the activity of the DNA BER pathway by reducing expression of the APE1 increased the neurotoxicity produced by anticancer treatment, whereas,

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augmenting the activity of APE1 lessened the neurotoxicity (Vasko et al., 2005; Jiang et al., 2008; Jiang et al., 2009; Vasko et al., 2011). Additionally, we demonstrated that APE1's DNA *repair* function, **not** the redox signaling function is crucial for sensory neuron survival and function (Vasko et al., 2011). We also identified a small molecule, E3330 that has IND approval and is slated for clinical trials which has shown protective effects in preclinical models for CIPN. While E3330 is a targeted inhibitor of APE1's redox function, it appears in the setting of sensory neurons that it can also enhance the DNA repair (AP endonuclease) activity of APE1. Although this seems counter-intuitive, on closer inspection it is not so unexpected. E3330, and the new second-generation compounds described in this manuscript, have been shown to attack Cys65, the primary redox Cys in APE1 altering APE1's ability to act as a reducing agent on downstream transcription factors (Su et al., 2011). This activity also disrupts the interaction of Cys65 with Cys93 and Cys99, mainly through inhibition of disulfide bond formation, which causes the APE1 protein to unfold over time (Su et al., 2011; Luo et al., 2012; Zhang et al., 2013). This unfolding primarily alters the amino end of APE1; affecting its interactions with downstream transcription factor targets, by perturbing the equilibrium of the folded/unfolded states of APE1 and facilitating APE1 repair activity (Kelley et al., 2011; Su et al., 2011; Luo et al., 2012). This disengagement of APE1 from its redox activity could enhance APE1 repair endonuclease activity (Kelley et al., 2014) and was observed for APX2009 (Supplemental Figure 4) and previously with E3330 (Kelley et al., 2014). Enhancing repair activity through APE1 redox inhibition is not observed in tumor cells (Luo et al., 2008; Nyland et al., 2010; Kelley et al., 2011). Consequently, APX2009 has a significant *in vitro* endonuclease activity and is neuroprotective, whereas APX2007 or APX2032 do not have enhanced repair stimulation and do not attenuate cisplatin-induced neurotoxicity. This divergence of activity does not occur with the ability of these compounds to inhibit APE1 redox activity since all three molecules have similar IC50s (Supplemental Figures 2 and 3).

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This dual nature of blocking APE1 redox function leading to neuroprotection in isolated sensory neurons and *in vivo* models (Vasko et al., 2005; Kelley et al., 2014), while augmenting cancer therapeutics (Jiang et al., 2010; Fishel et al., 2011; Cardoso et al., 2012a) could offer a “win-win” for preventing or treating CIPN. E3330 has recently been approved for Phase 1 safety trials and dose expansion in pancreatic and solid tumors in the US. Preclinical and clinical PK and toxicity data of E3330 in a non-cancer paradigm demonstrates reasonable and unremarkable toxicity (IND125360). However, we are interested in developing new second-generation compounds of this parent molecule that could have an increased efficacy for both CIPN protection as well as tumor killing without an increase in toxicity. This is the foundation for an extensive structure-activity relationship (SAR) effort based on modifying the various chemical aspects of E3330 as shown in Figure 1 and Supplemental Figure 1. Through these efforts, we identified three compounds, APX2007, APX2009 and APX2032, that demonstrated increased potency for APE1 targeting in our biochemical screening assays (Figure 1) as well as tumor killing (Figure 6 and Supplemental Figures 1 and 3). Out of these three compounds, APX2009 emerged as a leading candidate as it did not show any negative effects on DRG neuronal cultures alone or in combination with cisplatin or oxaliplatin (Figures 2-5) and similar tumor killing as the other two compounds at significantly lower levels than E3330 (Supplemental Figures 1-3). APX2007 and APX2032 did not protect nearly as well against the DNA damage and the cisplatin CGRP release protection is presumably due to the stimulation of CGRP release under basal conditions rather than after platinum challenge (Figures 2-5). Our results demonstrate that APX2009 has a similar neuronal protective activity in the *ex vivo* studies as E3330 against cisplatin and oxaliplatin, but at lower concentrations than E3330. APX2009 also demonstrates an increased antitumor activity (approximately 4-9X) at lower doses than E3330 in our anti-tumor studies using two cancer tumor types and a 3D tumor model (Figure 6 and Supplemental Figures 1 and 3). In addition, APX2009 has a longer half-life than E3330 in mice and a longer half-life in human microsome P450 studies (Supplemental Figure 2), suggesting

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that its pharmacokinetic profile might be beneficial in treating patients. In summary, APX2009 has an increase in tumor cell killing, PK and P450 metabolism half-life compared to E3330 and a more effective APE1 AP endonuclease stimulation than E3330. This supports its potential to have an increase in CIPN protection and increased tumor cell killing (Supplemental Figure 2). Further studies are warranted, however, to compare the potential toxicities of these compounds to ascertain their relative therapeutic indices and to determine whether APX2009 is effective in tumor killing efficacy while protecting or reversing CIPN in tumor-bearing animal studies.

Authorship Contributions:

Participated in research design: Kelley and Vasko

Development of methodology: Kelley, Vasko, Wikel, Pollok, and Fishel

Acquisition of data: Kelley, Wikel, Guo, Pollok, Bailey, Wireman, Fishel, and Vasko

Performed data analysis: Kelley, Wikel, Pollok, Wireman, Fishel and Vasko

Wrote or contributed to the writing of the manuscript: Kelley, Wikel, Pollok, Fishel and Vasko

Administrative, technical, or material support: Kelley, Wikel, and Vasko

Study Supervision: Kelley and Vasko

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References

- Arpin CC, Mac, S., Jiang, Y., Cheng, H., Page, B. D. G., Kamocka, M. M., Saftchenary, S., Su, H., Todic, A., Ball, D., Rosa, D. A., Lai, P.-S., Gomez-Biagi, R. F., Ali, A. M., Kerman, K., McElyea, K. C., Sandusky, G. E., Gunning, P. T. & Fishel, M. L. (2015) Applying Small Molecule STAT3 Protein Inhibitors as Pancreatic Cancer Therapeutics. *Molecular Cancer Therapeutics*.
- Bapat A, Glass LS, Luo M, Fishel ML, Long EC, Georgiadis MM and Kelley MR (2010) Novel small-molecule inhibitor of apurinic/aprimidinic endonuclease 1 blocks proliferation and reduces viability of glioblastoma cells. *J Pharmacol Exp Ther* **334**:988-998.
- Cardoso AA, Jiang Y, Luo M, Reed AM, He Y, Kelley MR and Fishel ML (2012a) APE1/Ref-1 Redox Function Regulates STAT3 Transcriptional Activity and APE1/Ref-1-STAT3 Dual-Targeting Synergize to Effectively Inhibit Pancreatic Cancer Cell Survival. *PLoS ONE* **7**:e47462 (
- Cardoso AA, Jiang Y, Luo M, Reed AM, Shahda S, He Y, Maitra A, Kelley MR and Fishel ML (2012b) APE1/Ref-1 regulates STAT3 transcriptional activity and APE1/Ref-1-STAT3 dual-targeting effectively inhibits pancreatic cancer cell survival. *PLoS One* **7**:e47462
- Chou TC and Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**:27-55.
- Crowe SL, Tsukerman S, Gale K, Jorgensen TJ and Kondratyev AD (2011) Phosphorylation of Histone H2A.X as an Early Marker of Neuronal Endangerment following Seizures in the Adult Rat Brain. *The Journal of Neuroscience* **31**:7648-7656.
- Fishel M, Jiang Y, Rajeshkumar N, Sinn A, He Y, Shen C, Jones D, Pollok K, Maitra A and Kelley M (2011) Impact of APE1/Ref-1 Redox Inhibition on Pancreatic Tumor Growth. *Molecular Cancer Therapeutics* **10**:1698-1708.
- Georgiadis MM, Chen Q, Meng J, Guo C, Wireman R, Reed A, Vasko MR and Kelley MR (2016) Small molecule activation of apurinic/aprimidinic endonuclease 1 reduces DNA damage induced by cisplatin in cultured sensory neurons. *DNA Repair (Amst)* **41**:32-41.
- Georgiadis MM, Luo M, Gaur RK, Delaplane S, Li X and Kelley MR (2008) Evolution of the redox function in mammalian apurinic/aprimidinic endonuclease. *Mutat Res* **643**:54-63.
- Hawkins PCD, Skillman AG and Nicholls A (2007) Comparison of Shape-Matching and Docking as Virtual Screening Tools. *Journal of Medicinal Chemistry* **50**:74-82.
- Hawkins PCD, Skillman AG, Warren GL, Ellingson BA and Stahl MT (2010) Conformer Generation with OMEGA: Algorithm and Validation Using High Quality Structures from

JPET #235283

- the Protein Databank and Cambridge Structural Database. *Journal of Chemical Information and Modeling* **50**:572-584.
- Hershman DL, Lacchetti C, Dworkin RH, Lavoie Smith EM, Bleeker J, Cavaletti G, Chauhan C, Gavin P, Lavino A, Lustberg MB, Paice J, Schneider B, Smith ML, Smith T, Terstriep S, Wagner-Johnston N, Bak K and Loprinzi CL (2014) Prevention and Management of Chemotherapy-Induced Peripheral Neuropathy in Survivors of Adult Cancers: American Society of Clinical Oncology Clinical Practice Guideline. *Journal of Clinical Oncology* **32**:1941-1967.
- Jiang Y, Guo C, Fishel ML, Wang ZY, Vasko MR and Kelley MR (2009) Role of APE1 in differentiated neuroblastoma SH-SY5Y cells in response to oxidative stress: use of APE1 small molecule inhibitors to delineate APE1 functions. *DNA Repair (Amst)* **8**:1273-1282.
- Jiang Y, Guo C, Vasko MR and Kelley MR (2008) Implications of Apurinic/Apyrimidinic Endonuclease in Reactive Oxygen Signaling Response after Cisplatin Treatment of Dorsal Root Ganglion Neurons. *Cancer Res* **68**:6425-6434.
- Jiang Y, Sandusky GE, Kelley MR and Fishel ML (2010) Reduced expression of DNA repair and redox signaling protein APE1/Ref-1 impairs human pancreatic cancer cell survival, proliferation, and cell cycle progression. *Cancer Investigation* **28**:885-895.
- Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE and Kinzler KW (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* **321**:1801-1806.
- Kelley MR, Jiang Y, Guo C, Reed A, Meng H and Vasko MR (2014) Role of the DNA Base Excision Repair Protein, APE1 in Cisplatin, Oxaliplatin, or Carboplatin Induced Sensory Neuropathy. *PLoS One* **9**:e106485.
- Kelley MR, Luo M, Reed A, Su D, Delaplane S, Borch RF, Nyland II RL, Gross ML and Georgiadis M (2011) Functional analysis of new and novel analogs of E3330 that block the redox signaling activity of the multifunctional AP endonuclease/redox signaling enzyme APE1/Ref-1. *Antioxid Redox Signal* **14**:1387-1401.
- Kim H-S, Guo C, Thompson EL, Jiang Y, Kelley MR, Vasko MR and Lee S-H (2015) APE1, the DNA base excision repair protein, regulates the removal of platinum adducts in sensory

JPET #235283

- neuronal cultures by NER. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **779**:96-104.
- Logsdon DP, Cheng H, Luo M, Shahda S, Ivan M, Hao Y, Tong Y, Yu Z, Zyromski N, Schipani E, Liu Y, Supuran CT, Kelley MR and Fishel ML (2015) Targeting APE1/Ref-1 Results in Inhibition of Hypoxia Signaling Genes, in *AACR-NC-EORTC International Conference: Molecular Targets and Cancer Therapeutics*, Mol Cancer Ther December 2015 14; B158, Boston, MA.
- Luo M, Delaplane S, Jiang A, Reed A, He Y, Fishel M, Nyland RL, 2nd, Borch RF, Qiao X, Georgiadis MM and Kelley MR (2008) Role of the multifunctional DNA repair and redox signaling protein Ape1/Ref-1 in cancer and endothelial cells: small-molecule inhibition of the redox function of Ape1. *Antioxid Redox Signal* **10**:1853-1867.
- Luo M, Zhang J, He H, Su D, Chen Q, Gross ML, Kelley MR and Georgiadis MM (2012) Characterization of the redox activity and disulfide bond formation in apurinic/aprimidinic endonuclease. *Biochemistry* **51**:695-705.
- Ness KK, Jones KE, Smith WA, Spunt SL, Wilson CL, Armstrong GT, Srivastava DK, Robison LL, Hudson MM and Gurney JG (2013) Chemotherapy-related neuropathic symptoms and functional impairment in adult survivors of extracranial solid tumors of childhood: results from the St. Jude Lifetime Cohort Study. *Arch Phys Med Rehabil* **94**:1451-1457.
- Nyland RL, Luo M, Kelley MR and Borch RF (2010) Design and Synthesis of Novel Quinone Inhibitors Targeted to the Redox Function of Apurinic/Apyrimidinic Endonuclease 1/Redox Enhancing Factor-1 (Ape1/Ref-1). *J Med Chem* **53**:1200-1210.
- Podhorecka M, Skladanowski A and Bozko P (2010) H2AX Phosphorylation: Its Role in DNA Damage Response and Cancer Therapy. *J Nucleic Acids* **2010**.
- Preston TJ, Henderson JT, McCallum GP and Wells PG (2009) Base excision repair of reactive oxygen species-initiated 7,8-dihydro-8-oxo-2- β -deoxyguanosine inhibits the cytotoxicity of platinum anticancer drugs. *Mol Cancer Ther* **8**:2015-2026.
- Redon CE, Nakamura AJ, Zhang Y-W, Ji J, Bonner WM, Kinders RJ, Parchment RE, Doroshow JH and Pommier Y (2010) Histone γ H2AX and Poly(ADP-Ribose) as Clinical Pharmacodynamic Biomarkers. *Clinical Cancer Research* **16**:4532-4542.
- Sempere LF, Gunn JR and Korc M (2011) A novel 3-dimensional culture system uncovers growth stimulatory actions by TGF β in pancreatic cancer cells. *Cancer Biol Ther* **12**:198-207.
- Stone JB and DeAngelis LM (2016) Cancer-treatment-induced neurotoxicity[mdash]focus on newer treatments. *Nat Rev Clin Oncol* **13**:92-105.

JPET #235283

- Su D, Delaplane S, Luo M, Rempel DL, Vu B, Kelley MR, Gross ML and Georgiadis MM (2011) Interactions of Apurinic/Apyrimidinic Endonuclease with a Redox Inhibitor: Evidence for an Alternate Conformation of the Enzyme. *Biochemistry* **50**:82-92.
- Tonsing-Carter E, Bailey BJ, Saadatzadeh MR, Ding J, Wang H, Sinn AL, Peterman KM, Spragins TK, Silver JM, Sprouse AA, Georgiadis TM, Gunter TZ, Long EC, Minto RE, Marchal CC, Batuello CN, Safa AR, Hanenberg H, Territo PR, Sandusky GE, Mayo LD, Eischen CM, Shannon HE and Pollok KE (2015) Potentiation of Carboplatin-Mediated DNA Damage by the Mdm2 Modulator Nutlin-3a in a Humanized Orthotopic Breast-to-Lung Metastatic Model. *Mol Cancer Ther* **14**:2850-2863.
- Vasko MR, Guo C and Kelley MR (2005) The multifunctional DNA repair/redox enzyme Ape1/Ref-1 promotes survival of neurons after oxidative stress. *DNA Repair (Amst)* **4**:367-379.
- Vasko MR, Guo C, Thompson EL and Kelley MR (2011) The repair function of the multifunctional DNA repair/redox protein APE1 is neuroprotective after ionizing radiation. *DNA Repair (Amst)* **10**:942-952.
- Zafar SY, Marcello JE, Wheeler JL, Rowe KL, Morse MA, Herndon JE, 2nd and Abernethy AP (2010) Treatment-related toxicity and supportive care in metastatic colorectal cancer. *J Support Oncol* **8**:15-20.
- Zhang J, Luo M, Marasco D, Logsdon D, LaFavers KA, Chen Q, Reed A, Kelley MR, Gross ML and Georgiadis MM (2013) Inhibition of apurinic/aprimidinic endonuclease I's redox activity revisited. *Biochemistry* **52**:2955-2966.

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Footnotes

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Disclosure of Potential Conflict of Interest: Mark R. Kelley has licensed E3330 (APX3330) through Indiana University Research and Technology Corporation to ApeX Therapeutics. ApeX Therapeutics had neither control nor oversight of the studies, interpretation, or presentation of the data in this manuscript.

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Figure Legends

Figure 1. New chemical entities (NCE); E3330 analogs. A, Schematic of E3330 and new compounds. Groups that were investigated include the Quinone series (A), 3-Position series (B), Alkyl sidechain series (C), and Carboxylic Acid/Amine series (D). B, Current new molecules with more potent Ref-1 redox inhibition. Drawing, displaying and characterizing chemical structures, substructures and reactions were performed as described in Materials and Methods.

Figure 2. Pretreatment with E3330 and APX2009, but not APX2007 or APX2032 attenuates cisplatin-induced cell death in sensory neuronal cultures. **A:** Each column represents the mean \pm SEM of percent survival of cells from cultures treated with various concentrations of drugs as indicated for 24 hours (A). Cell viability as measured by trypan blue exclusion was determined on day 14 in culture from 3 independent harvests. An asterisk indicates significant difference in survival in after drug treatment compared to no drug treatment using ANOVA and Tukey's post hoc test. **B:** Neuronal cultures were exposed to vehicle (DMSO) or to 20 μ M of E3330, APX2007, APX2009 or APX2032 APX drugs (as indicated) for 72 hours and to various concentrations of cisplatin for 24 hours. Each column represents the mean \pm SEM of the percent survival of cells as measured by trypan blue exclusion. An asterisk indicates significant difference in cultures not treated with cisplatin compared to cultures treated with the drug using ANOVA and Tukey's post hoc test.

Figure 3. E3330 and APX2009 do not alter CGRP release from sensory neurons in culture, but attenuate the cisplatin-induced reduction in capsaicin-evoked release of CGRP. Each column represents the mean \pm SEM of basal release (open columns) or capsaicin-stimulated release (shaded columns) of CGRP in fmol/well/min. A, Cultures were exposed to medium or to 10 or 20 μ M of the various drugs (as indicated) for 72 hours prior to

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release experiments. B, Cultures were exposed to medium or to 10 or 20 μM of the various drugs (as indicated) for 72 hours and to cisplatin for 24 hours prior to release experiments. An asterisk indicates a significant difference in capsaicin-stimulated release compared to untreated cells using ANOVA and Tukey's post hoc test.

Figure 4. APX2009, but not APX2007 or APX2032 attenuate the cisplatin-induced

phosphorylation of H2AX in sensory neuronal cultures.

The top panel shows representative Western blots of phospho-H2AX (pH2AX) and vinculin from cultures prior to and after 24 and 48 hours of exposure to 10 μM cisplatin. Cultures were exposed to DMSO as a vehicle control or to 20 μM APX2007, APX2009 or APX2032 for 72 hours before and during cisplatin treatment as indicated. The bottom panel represents the mean \pm SEM of the densitometry of pH2AX expression normalized to vinculin from 3 independent experiments. An asterisk indicates a statistically significant increase in pH2AX density in cells treated with cisplatin, whereas a cross indicates a significant change by drug compared to DMSO controls at the same time points using ANOVA and Tukey's post hoc test.

Figure 5. APX2009 attenuates the oxaliplatin-induced toxicity of sensory neurons in

culture.

A, Each column represents the mean \pm SEM of percent cells surviving as measured by trypan blue exclusion after a 24 hour exposure to various concentrations of oxaliplatin as indicated. Cultures are treated for 72 hours with DMSO as a vehicle control (left) 10 μM

APX2009 (center) or 20 μM APX2009 (right). B, Columns represent the mean \pm SEM of the

basal release of CGRP (open columns) or release stimulated by 30 nM capsaicin (shaded

columns) in fmol/well/min. The horizontal bar indicates cultures exposed to 30 μM oxaliplatin for

24 hours and 10 or 20 μM APX2009 for 72 hours prior to release experiments. C, The top panel

shows representative Western blots of phospho-H2AX (pH2AX) and vinculin from cultures prior

to and after 24 and 48 hours of exposure to 30 μM oxaliplatin and DMSO or 20 μM APX2009 for

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72 hours before and during cisplatin treatment as indicated. The bottom panel represents the mean \pm SEM of the densitometry of pH2AX expression normalized to vinculin from 3 independent experiments. An asterisk indicates a statistically significant difference on oxaliplatin treated cultures compared to controls using ANOVA and Tukey's post hoc test.

Figure 6. Tumor, but not CAF cell killing by APX2009 in PDAC 3D model. A, Pa03C (tumor cells (transduced with TdTomato) were grown in 3D cultures in the presence and absence of CAFs (transduced with EGFP). Tumor cells alone (left side in panels A and B) and tumor cells with CAFs in spheroids are shown (panels A and B). The middle and right quantitation graphs in A and B show the tumor (middle) vs. CAF (right) intensity (A) and area (B). Spheroids were treated with APX2009 and the area of intensity (panel A) and area (panel B) of tumor (red) and CAF (green) were quantified following 12 days in culture. Representative images are shown in C. Differences were determined using both Student's t test (vehicle control vs drug treatment at each dose) and one-way ANOVA and statistical differences were observed for the tumor alone or tumor co-cultured with CAFs (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). No differences were observed in CAFs treated with APX2009 from control, similar to what has been observed for E3330 (Logsdon et al., 2015).

Figure 1

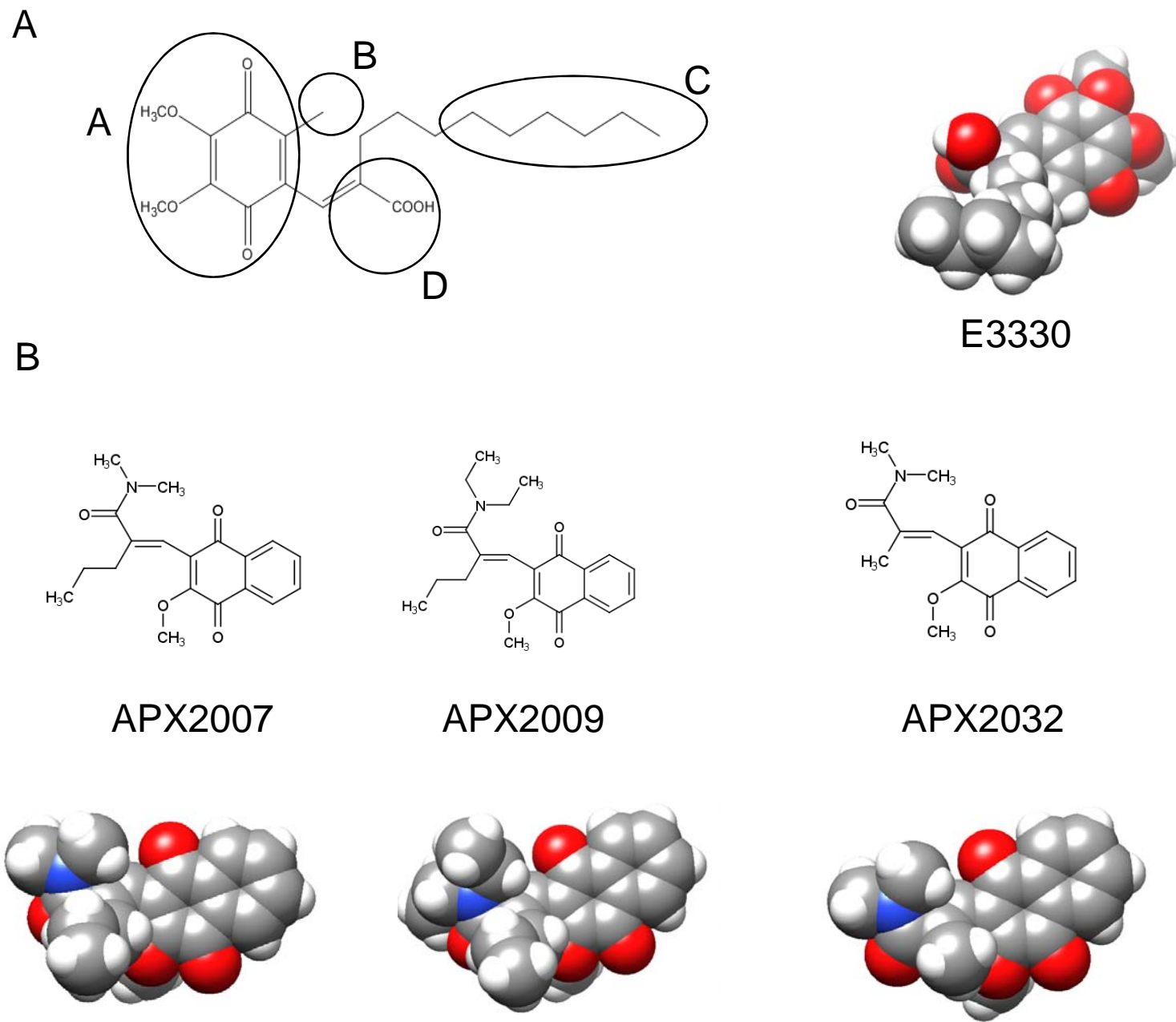
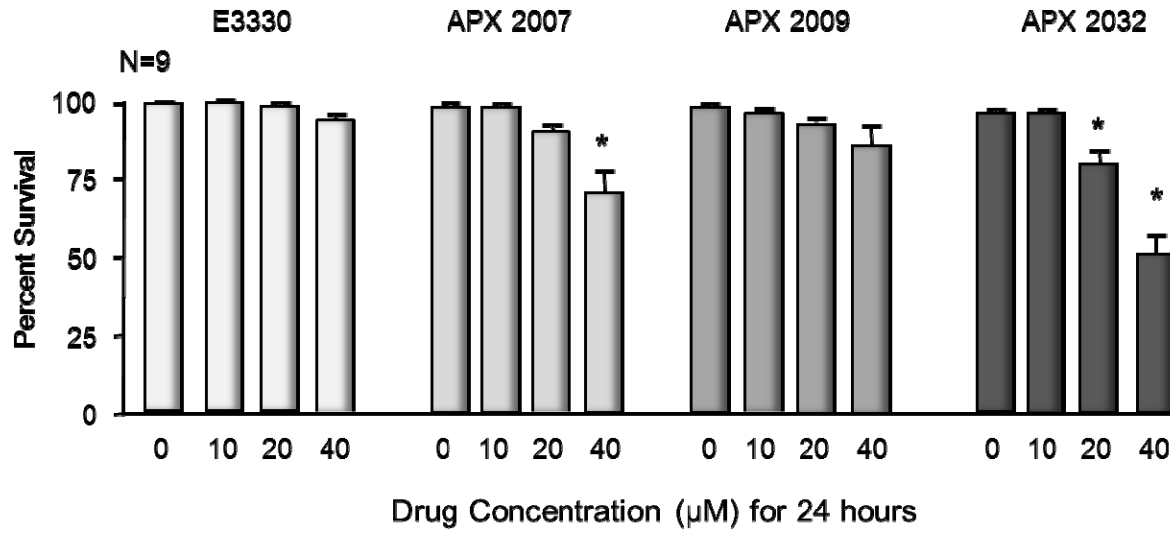


Figure 2

A



B

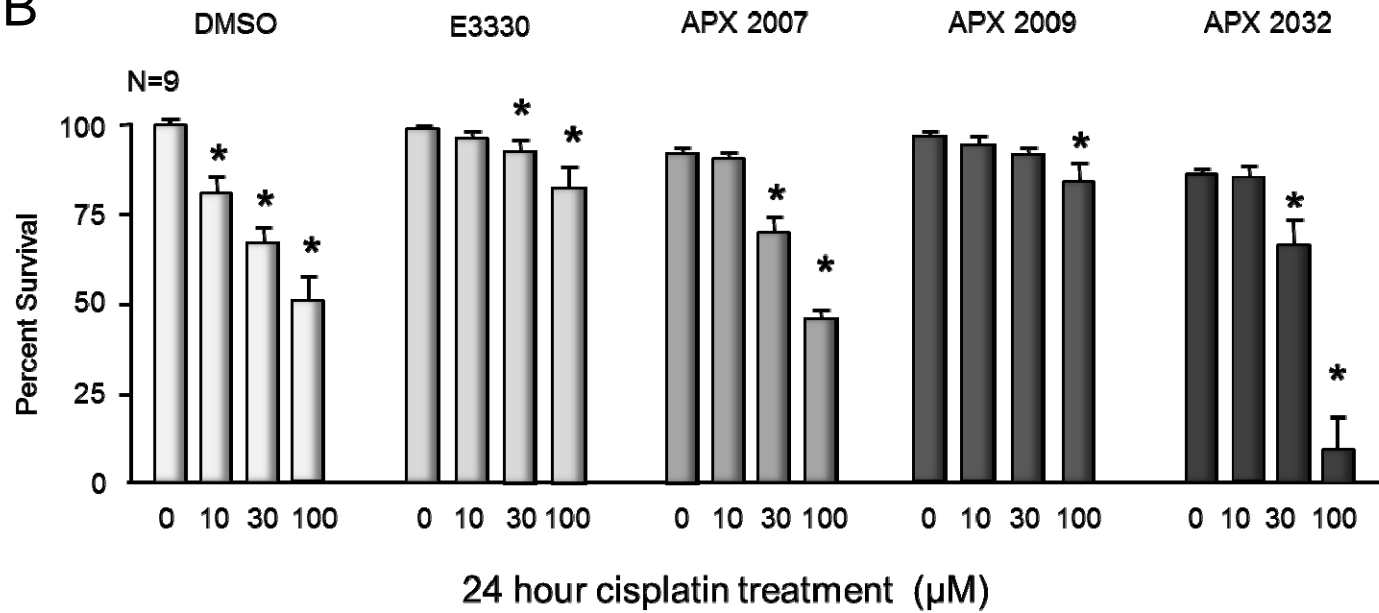
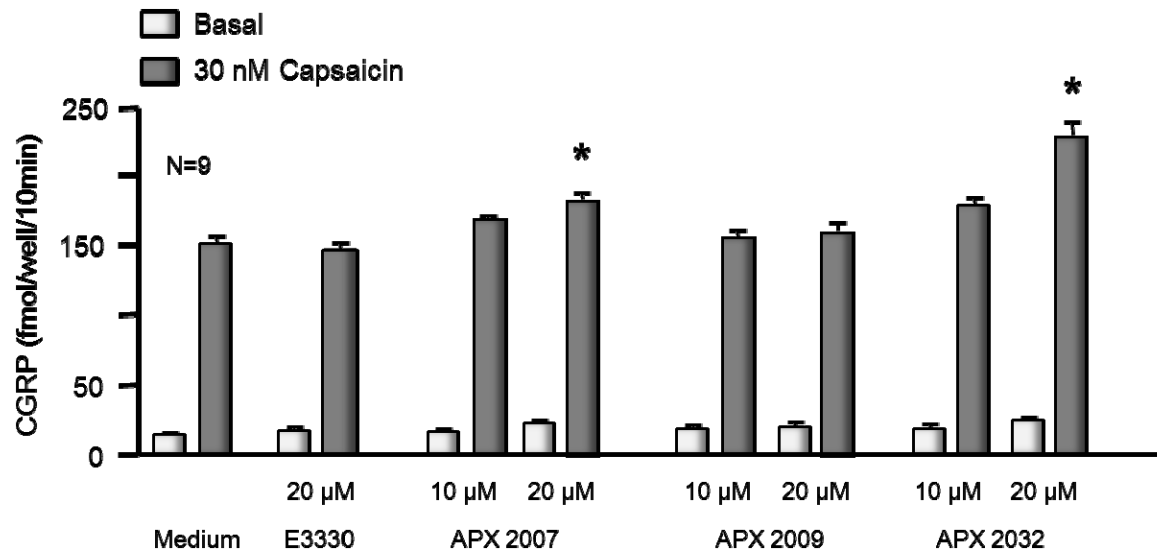


Figure 3

A



B

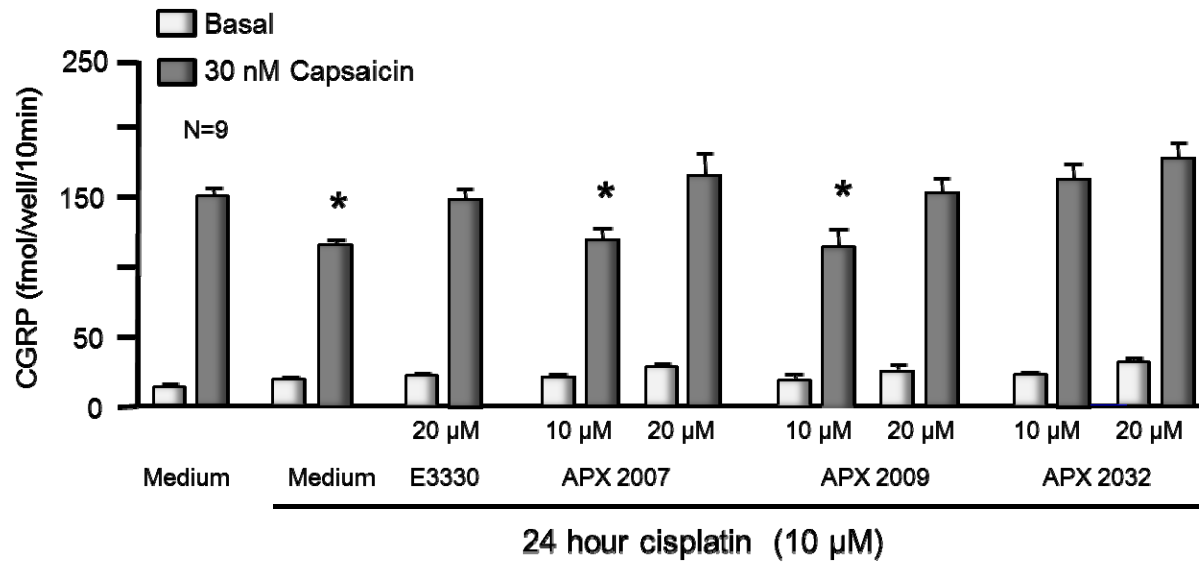


Figure 4

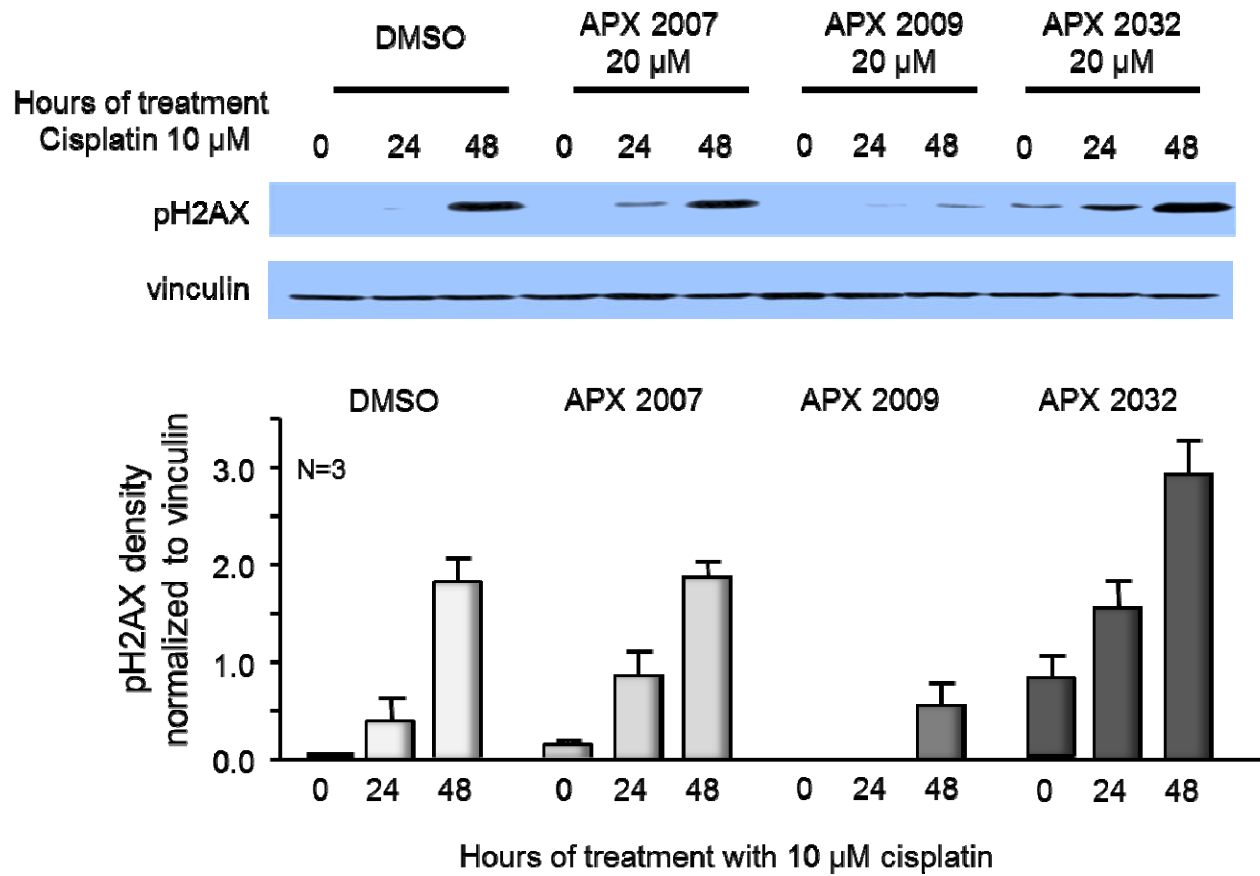


Figure 5

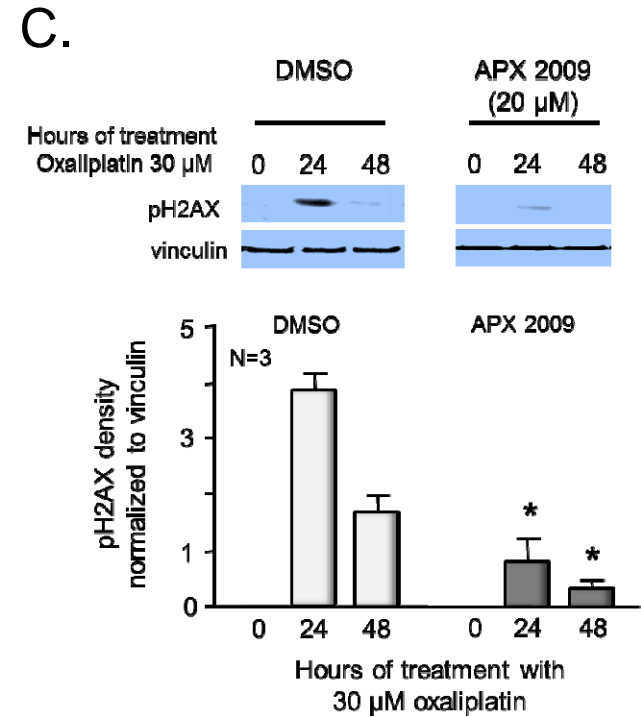
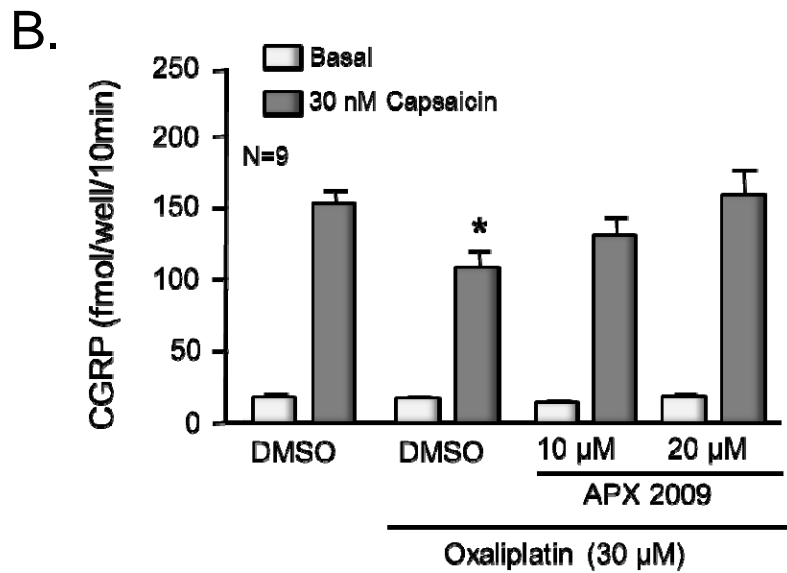
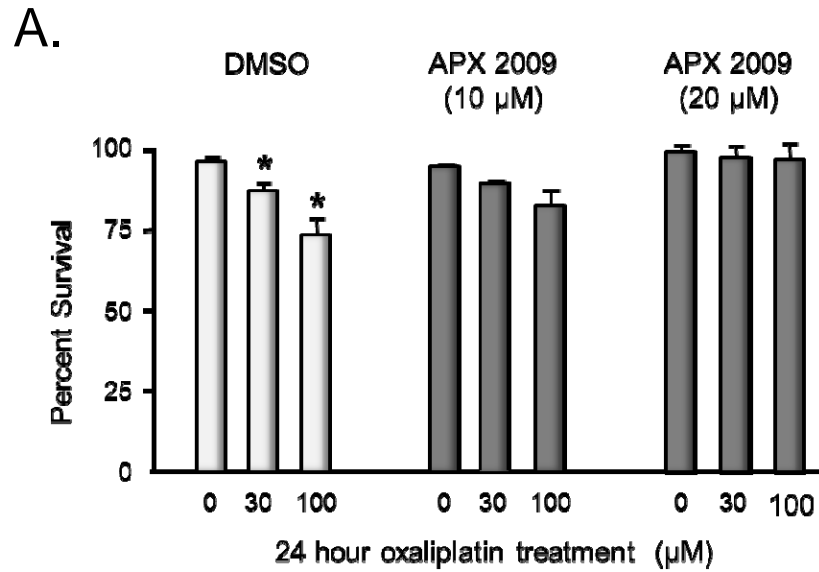


Figure 6

