Identification and Characterization of New Chemical Entities Targeting Apurinic/Apyrimidinic Endonuclease 1 for the Prevention of Chemotherapy-Induced Peripheral Neuropathy

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) is a potentially debilitating side effect of a number of chemotherapeutic agents. There are currently no U.S. Food and Drug Administration–approved interventions or prevention strategies for CIPN. 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 after chemotherapy correlates with symptoms of CIPN. Augmenting apurinic/apyrimidinic endonuclease (APE)-1 function in the base excision repair 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 [(2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-undecanoic acid; also called APX3330]. Although E3330 has been approved for phase 1 clinical trials and is neuroprotective against cisplatin and oxaliplatin-induced toxicity, we synthesized novel, second-generation APE1-targeted molecules and determined whether they would be protective against neurotoxicity induced by cisplatin or oxaliplatin while not diminishing the platins’ antitumor effect. We measured various endpoints of neurotoxicity using our ex vivo model of sensory neurons in culture, and we determined that APX2009 [(2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-diethylpentanamide] 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 three-dimensional 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 anticancer activity of platins.

Introduction

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, pain, increased sensitivity to cold, loss of proprioception, loss of touch, and reduced tendon reflexes. Unlike for other major side effects of chemotherapy (e.g., nausea, hair loss, or bone marrow failure) (Zafar et al., 2010), there are currently no U.S. Food and Drug Administration–approved interventions or prevention strategies for CIPN (Hershman et al., 2014; Stone and DeAngelis, 2016).

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ABBRIVIATIONS: APE, apurinic/apyrimidinic endonuclease; APX2007, (2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-diethylpentanamide; APX2009, (2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)methylidene]-N,N-diethylpentanamide; APX2032, (2E)-2-[(3-methoxy-1,4-dioxo-1,4-dihydronaphthalen-2-yl)-N,N,2-trimethylprop-2-ynamide; BER, base excision repair; CAF, cancer-associated fibroblast; CGRP, calcitonin gene–related peptide; CIPN, chemotherapy-induced peripheral neuropathy; E3330 (also called APX3330), (2E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)methylene]-undecanooic acid; EMSA, electrophoretic mobility shift assay; H2AX, histone variant H2A.X, member X; NF-κB, nuclear factor-κB; P450, cytochrome P450; PBS, phosphate-buffered saline; PDAC, pancreatic ductal adenocarcinoma; pH2AX, phospho-H2AX; PK, pharmacokinetics.
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 therapy, 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, 2011; Jiang et al., 2008; Kelley et al., 2014; Kim et al., 2015). Reducing the expression of a critical enzyme in the BER pathway, apurinic/apyrimidinic endonuclease (APE1)-1 (also called Ref-1), in sensory neurons amplifies the toxicity to sensory neurons exposed to anticancer therapies; by contrast, overexpressing APE1 and an APE1 DNA repair–proficient, redox-deficient mutant protein attenuates the neurotoxicity (Vasko et al., 2005, 2011; Jiang et al., 2008; Kelley et al., 2014). The neuronal protection afforded by overexpressing APE1 is mimicked by a first-generation APE1 redox signaling small molecule inhibitor, E3330 \([2(E)-2-[(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)]-methylene]-undecanoic acid\); 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, area under the curve, 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 of chronic hepatitis C. We have developed the drug for cancer therapeutics and subsequently discovered its neuronal protective effects. We previously published that E3330 increases the DNA repair activity of APE1 in rat ex vivo 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 (Investigational New Drug application number 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 antitumor 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 assessed the antitumor effects of one of these novel small molecules (APX2009) in neuroblastoma cell lines as well as a three-dimensional spheroid pancreatic tumor model, and we also assessed the pharmacokinetics (PK) and cytochrome P450 (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(l-lysine) and laminin were purchased from Sigma-Aldrich, nerve growth factor was from Harlan Bioproducts for Science (Indianapolis, IN), and noromycin was from Invivogen (San Diego, CA). Mouse monoclonal anti-human APE1 antibodies were raised in our laboratory and are available from Novus Biologicals (Littleton, CO). Mouse monoclonal anti–phospho-H2A histone family, member X (pH2AX) antibodies were from EMD Millipore (Billerica, MA) and the β-actin monoclonal antibody was from Thermo Fisher Scientific (Fremont, CA). Chemiluminescence secondary antibodies were from Roche Diagnostics (Indianapolis, IN).

Cisplatin was purchased from Sigma-Aldrich and oxaliplatin was purchased from LKT Laboratories (St. Paul, MN). Cisplatin was initially dissolved in N,N-dimethylformamidemide (Sigma-Aldrich) and stored as a 40-mM solution at –80°C, and oxaliplatin was dissolved in phosphate-buffered saline (PBS) and stored as a 5-mM 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 Indiana University School of Medicine Animal Care and Use Committee (Indianapolis, IN) approved all procedures used in these studies.

**Synthesis of New Chemical Entities.** Complete details of the synthesis of the new, second-generation compounds will be provided in a manuscript in preparation. The compounds were synthesized by Cascade Custom Chemistry (Eugene, OR). In summary, iodolawsone (2-iodo-3-hydroxy-1,4 naphthoquinone, a common intermediate) is available from Cascade Custom Chemistry. As described, iodolawsone in subsequent reactions is treated with methacrylic acid or 2-propylacrylic acid, with oxalyl chloride and the corresponding amine, and with sodium methoxide in methanol to yield APX2007 \([2(E)-2-(3-methoxy-1,4-dioxo-1,4-dihydro naphthalen-2-yl)(methylene)-N,N-di methylpentanamide]\), APX2009 \([2(E)-2-(3-methoxy-1,4-dioxo-1,4-dihydrop naphthalen-2-yl)(methylene)-N,N-diethylpentanamide]\), and APX2012 \([2(E)-2-(3-methoxy-1,4-dioxo-1,4-dihydrop naphthalen-2-yl)-N,N,2-trimethylprop-2-amine]\). Further information is available in the issued patent (Kelley and Wikel, 2015).

**Chemical Structure Presentation.** Marvin 15.8.24.0 software (2015; ChemAxon, Cambridge, MA) was used for drawing, displaying, and characterizing chemical structures, substructures, and reactions. Calculator plugins were used for structure property prediction (Marvin 15.8.24.0, 2015; ChemAxon). Molecular modeling was performed using OMEGA 2.5.1.4 (Hawkins et al., 2010) and ROCS 3.2.1.4 (Hawkins et al., 2007) software (both from OpenEye Scientific Software, Santa Fe, NM). Molecular visualization was performed using VIDA software (OpenEye Scientific Software).

**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 (weighing 150–175 g; Harlan, Indianapolis, IN) were euthanized by CO₂ asphyxiation and the dorsal root ganglia were dissected from all spinal levels, transferred to a collagenase solution (1 mg/ml), in asphyxiation and the dorsal root ganglia were dissected from all spinal levels, transferred to a collagenase solution (1 mg/ml), in

**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 percentage of live cells divided by the total cell number (including dead and live cells).
Cell Line Authentication and Characterization. The IMR32 and SK-N-SH cell lines were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cell line identity was confirmed by DNA fingerprint analysis (IDEXX BioResearch, Columbia, MO) for species and baseline short-tandem repeat analysis testing. All cell lines were 100% human and a nine-marker short tandem repeat analysis is on file.

Cell Proliferation Assay. Cells were seeded in 96-well plates (IMR32 cells: 1000 cells/well; SK-N-SH cells: 3000 cells/well) and treated for 5 days with APX2007, APX2009, APX2032, or E3330. The final dimethylsulfoxide concentration was ≤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) and protein was quantified using the Lowey assay. Proteins were separated by electrophoresis on a 4%–12% SDS-polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride membrane and then incubated overnight at 4°C in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk while using gentle agitation. Mouse monoclonal anti-human APE1 antibodies (1:500), mouse monoclonal anti-pH2AX antibodies (1:1000), or β-actin monoclonal antibody (1:10000) were added to the blocking solution and incubated overnight at room temperature while using gentle agitation. Antibody binding was detected after appropriate secondary antibody methods using chemiluminescence. Band density was measured using QualityOne software (Bio-Rad, Hercules, CA) and data are expressed as density normalized to actin.

Measurement of Calcitonin Gene–Related Peptide Release. For release experiments, cell cultures were washed with HEPES buffer consisting of 25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM d-glucose, and 0.1% bovine serum albumin (pH 7.4) and were maintained at 37°C. They were then incubated for successive 10-minute intervals with 0.4 ml HEPES buffer alone (basal release), with buffer containing 30 nM capsacin, and 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 a radiomunonasay. At the end of the release protocol, CGRP was extracted from the cultures and total content was measured using a radiomunonasay. Since treatments did not significantly alter total content, release data are presented as femtomoles of peptide released per well per 10 minutes.

APE DNA Repair Assay. Inhibition or enhancement of APE1 DNA repair enzyme activity was performed as previously described (Bapat et al., 2010). The APE1 repair activity assay was performed in a plate assay using two annelated oligodeoxinucleotides (5’-6-carboxyfluorescein (6-FAM)-GGCCCCC*GCGGAGCTACGATATCCGCCGCTCC-3’ and 3’-Q-GGGGGGCCCCCTGATCGTATAGGGCGAGGTG-5’) containing a quencher on one strand and a fluorescent 6-FAM label with tetrahydrofuran as an apurinic/apyrimidinic site mimic. Oligo cleavage at the apurinic/apyrimidinic mimic site results in 6-FAM release and detection. The fluorescence was read at five 1-minute intervals using a Tecan Ultra plate reader (Tecan Group, Männedorf, Switzerland; performed at the ASPET Journals on August 22, 2021)

Results

Chemical Synthesis of E3330 Analogs, Validation of Redox Inhibition and PK. We synthesized a number of novel analogs of E3330, and we were able to replace the core dimethoxybenzoquinone (A) with a naphthoquinone ring, the methyl group (B) on the ring structure was replaced with various halogens or hydrogen, and the carbon chain (C) on the double bond can be shortened to modulate activity (Fig. 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 physiologic 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 are available in the patent by Kelley et al. (2015). Three new structures from the compounds made (Fig. 1B) were analyzed in our redox APE1 EMSA studies to determine which compounds affect the redox function of APE1 as previously described (Georgiadis et al., 2008; Kelley et al., 2011; Su et al., 2011; Luo et al., 2012; Zhang et al., 2013). The compounds had redox inhibition IC50 values of 2 μM for APX2007, 1 μM for APX2009, and 1 μM for APX2032 (Supplemental Fig. 1A). E3330 was presented previously and had an IC50 of 25 μM in similar assays (Su et al., 2011; Luo et al., 2012; Zhang et al., 2013).

We performed reporter transactivation assays to verify that the new compounds were effective in cells and hit their target APE1, which regulates NF-κB function in this assay 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-κB binding to the reporter construct, with an IC50 of 7 μM; by contrast, E3330 had an IC50 of 45 μM (Supplemental Fig. 1B) (Luo et al., 2008; Nyland et al., 2010). In addition, we determined the ED50 value for tumor cell killing in two neuroblastoma cell lines: IMR32 (p53wt, MYCN amplified) and SK-N-SH (p53wt, MYCN nonamplified) (Supplemental Figs. 1, C and D, and 3). All three compounds had a reduced ED50 compared with E3330, which was 7- to 10-fold greater in IMR32 cells and 4- to 6-fold greater in SK-N-SH cells (Supplemental Figs. 1, C and D, and 3). The enhanced tumor cell killing data are consistent with the increased efficacy of the compounds on APE1 function, as demonstrated by the EMSA and transactivation data in Supplemental Fig. 1. We also assessed the pharmacokinetic profile of APX2009. As shown in Supplemental Fig. 2, the half-life of APX2009 was 25.8 hours compared with 3.6 hours for E3330, or an approximate 7-fold half-life increase. In addition, using human microsomes in a P450 metabolism analysis, APX2009 had a 173- versus 20-minute half-life, or an 8.7-fold increase (Supplemental Fig. 2).

There was no significant cell death when the sensory neuronal cultures were exposed to E3330 at 10, 20, or 40 μM for 24 hours as measured by trypan blue exclusion (Fig. 2A), confirming our previous work (Vasko et al., 2005, 2011; Jiang et al., 2008; 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 (Fig. 2A). In contrast, treating cells with 40 μM APX2007 for 24 hours or with 20 or 40 μM APX2032 for 24 hours resulted in a significant reduction in cell viability (Fig. 2A). In a similar manner, exposing cultures to 20 or 40 μM 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 Fig. 4, only APX2009 demonstrated a stimulation of APE1 repair activity in this assay and in the nanomolar range, a significant
increase in activity compared with E3330 (Supplemental Fig. 2). APX2007 and APX2032 had no effect on 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 showed that augmenting APE1 repair activity attenuates the ability of cisplatin to cause cell death in sensory neuronal cultures (Vasko et al., 2005, 2011; Jiang et al., 2008; Kelley et al., 2014). Since exposing neuronal cultures to E3330 is neuroprotective (Vasko et al., 2005, 2011; Jiang et al., 2008; Kelley et al., 2014), we determined whether it and other analogs would affect cisplatin-induced cell death in our cultures. As we previously showed (Jiang et al., 2008; Kelley et al., 2014), exposing neuronal cultures to increasing concentrations of cisplatin for 24 hours caused a concentration-dependent reduction in cell viability to 66% ± 5% and 50% ± 7% for 30 and 100 μM, respectively (Fig. 2B). This cisplatin-induced cell death was blocked by exposing neuronal cultures to E3330 (20 μM) or APX2009 (20 μM) for 48 hours prior to and throughout the cisplatin treatment (Fig. 2B). In contrast, pretreatment with 20 μM APX2007 or APX2032 did not attenuate cisplatin-induced cell death, with the combination of APX2032 and cisplatin (100 μM) reducing cell viability to 9% ± 9% (Fig. 2B). Therefore, APX2009 protected sensory neuronal cultures against cisplatin-induced cell death at all dose levels used, whereas APX2007 and APX2032 caused cell killing at a high dose (100 μM).

**E3330 and APX2009, but Not APX2007 or APX2032, Attenuate a 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 μM) or APX2009 (10 or 20 μM) for 72 hours and CGRP release was examined, there was no significant change in either basal (resting) release or release stimulated by 30 nM capsaicin compared with untreated cells (Fig. 3A). However, pretreatment with APX2007 or APX2032 (10 μM) for 72 hours also did not affect CGRP release, whereas 20 μM of each caused a significant increase in capsaicin-stimulated release (Fig. 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 μM cisplatin resulted in a significant reduction in the capsaicin-evoked release of CGRP (Fig. 3B) (Jiang et al., 2008). Pretreating cultures with 20 μM 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 (Fig. 3B). A 72-hour treatment with 10 μM APX2007 or APX2009 did not prevent the cisplatin-induced reduction in release, but 10 μM 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 appeared to be neuroprotective since they did not alter release when given alone.

**APX2009 Significantly Reduces DNA Damage Induced by Cisplatin in Sensory Neuronal Cultures.** As further confirmation of the neuroprotective effects of APX2009, APX2009, but not APX2007 or APX2032, attenuates cisplatin-induced cell death in sensory neuronal cultures. (A) Each column represents the mean ± S.E.M. of the percent survival of cells from cultures treated with various concentrations of drugs as indicated for 24 hours. Cell viability as measured by trypan blue exclusion was determined on day 14 in culture from three independent harvests. An asterisk indicates a significant difference in survival after drug treatment compared with no drug treatment, using analysis of variance and the Tukey post hoc test. (B) Percentage survival was analyzed by one-way analysis of variance and the Tukey post hoc test. P < 0.05. See also Supplemental Fig. 3.
after cisplatin treatment, the levels of 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 μM 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 (Fig. 4). Pretreating cultures with APX2009 (20 μM) for 48 hours prior to and throughout exposure to cisplatin significantly reduces the levels of pH2AX. In contrast, neither APX2007 nor APX2032 (20 μM) altered the ability of cisplatin to produce DNA damage (Fig. 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 reactive oxygen species in cells, with cisplatin producing higher levels (Preston et al., 2009; Kelley et al., 2014; Kim et al., 2015). However, the DNA crosslinks produced by these two agents differ: cisplatin produces Pt-1-2-d(GpG) intrastrand DNA crosslinks, whereas oxaliplatin creates predominantly Pt-1-3-d(ApG) interstrand DNA crosslinks (Kelley et al., 2014). We previously demonstrated that E3330 protects against both cisplatin- and oxaliplatin-induced neurotoxicity (Kelley et al., 2014). Therefore, we wanted to determine whether APX2009 has a similar protective effect after oxaliplatin treatment, which would also support our hypothesis that repair of oxidative DNA damage participates in the regulation of the platinum crosslink removal as shown with E3330 (Kelley et al., 2014; Kim et al., 2015). As shown in Fig. 5A, a 72-hour treatment with 10 or 20 μM APX2009 protected the sensory neuronal cultures from cell killing caused by a 24-hour exposure to oxaliplatin. In a similar manner, pretreating neuronal cultures with APX2009 for 48 hours prior to and throughout exposure to oxaliplatin for 24 hours prevented the oxaliplatin-induced decrease in CGRP release from sensory neurons (Fig. 5B).

APX2009 also significantly reduced the phosphorylation of H2AX after 24- and 48-hour treatments of oxaliplatin (Fig. 5C), indicating that its neuroprotective effects may be attributable to reduced DNA damage.

**Treatment of a Human PDAC Three-Dimensional Tumor Model with APX2009.** Although 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 coculture 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 et al., 2016). We assessed the effects of APX2009-induced cytotoxicity on the area and intensity of tumor cells both alone and in coculture with CAFs. Spheroids composed of patient-derived PDAC cells

![Graph](image-url)
(Pa03C; labeled red) and CAF19 cells (labeled green) were treated with APX2009 (Fig. 6), and we evaluated the area and intensity of red and green fluorescence separately as markers for each cell type (Fig. 6). Interestingly, CAFs were not significantly affected by APX2009 treatment, again suggesting that nontumorigenic cells can tolerate the effects of APE1 inhibition more than tumor cells. These data are similar to what is observed with E3330 (Logsdon et al., 2015) but show effectiveness at lower dose levels, validating APX2009 as a potential PDAC therapeutic agent while also showing CIPN-protective indications.

In addition, we determined the ED50 values for tumor cell killing in two neuroblastoma cell lines: IMR32 (p53wt, MYCN amplified) and SK-N-SH (p53wt, MYCN nonamplified) (Supplemental Figs. 1, C and D, and 3). All three compounds had reduced ED50 values compared with E3330, which were 7- to 10-fold greater in IMR32 cells and 4- to 6-fold greater in SK-N-SH cells (Supplemental Figs. 1, C and D, and 3). For APX2009, tumor cell killing was increased 4.3- to 8.7-fold compared with E3330 for either the neuroblastoma or three-dimensional pancreatic tumor cell models (Supplemental Fig. 2). These tumor cell killing data are consistent with the increased effect on APE1 function demonstrated by the EMSA and transactivation data (Supplemental Figs. 1 and 2).

**Discussion**

The development of CIPN is one of the major concerns for patients during and after cancer treatment and during survivorship, which can limit patients from receiving truly therapeutic levels of these drugs and can have a significant effect on patient quality of life. The platinum agents are still one of the major families of chemotherapy used as standard of care 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 chemotherapy side effects (e.g., nausea, hair loss, bone marrow failure; Zafar et al., 2010), there are no standard,

![Fig. 6.](image-url)
effective treatments to prevent or reverse CIPN. The American Society of Clinical Oncology 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 development of new drug treatments based on the 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, BER and APE1 (Vasko et al., 2005, 2011; Jiang et al., 2008; 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 APE1 expression increased the neurotoxicity produced by anticancer treatment, whereas augmenting APE1 activity lessened the neurotoxicity (Vasko et al., 2005, 2011; Jiang et al., 2008, 2009). In addition, we demonstrated that APE1’s DNA repair function, not its redox signaling function, is crucial for sensory neuron survival and function (Vasko et al., 2011). We also identified a small molecule (E3330) that has Investigational New Drug approval, is slated for clinical trials, and has shown protective effects in preclinical models for CIPN. Although E3330 is a targeted inhibitor of APE1’s redox function, it seems that E3330 can also enhance the DNA repair (APE) activity of APE1 in the setting of sensory neurons. Although this seems counterintuitive, on closer inspection it is not so unexpected. E3330, and the new second-generation compounds described in this article, 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 Fig. 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 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 IC50 values (Supplemental Figs. 2 and 3).

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 neuroprotection in isolated sensory neurons and in vivo (Fig. 2) and previously with E3330 (Kelley et al., 2014). We demonstrated that reducing the activity of the DNA BER pathway by reducing APE1 expression increased the neurotoxicity produced by anticancer treatment, whereas augmenting APE1 activity lessened the neurotoxicity (Vasko et al., 2005, 2011; Jiang et al., 2008, 2009). In addition, we demonstrated that APE1’s DNA repair function, not its redox signaling function, is crucial for sensory neuron survival and function (Vasko et al., 2011). We also identified a small molecule (E3330) that has Investigational New Drug approval, is slated for clinical trials, and has shown protective effects in preclinical models for CIPN. Although E3330 is a targeted inhibitor of APE1’s redox function, it seems that E3330 can also enhance the DNA repair (APE) activity of APE1 in the setting of sensory neurons. Although this seems counterintuitive, on closer inspection it is not so unexpected. E3330, and the new second-generation compounds described in this article, 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 Fig. 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 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 IC50 values (Supplemental Figs. 2 and 3).

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., 2012) 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 United States. Preclinical and clinical PK and toxicity data of E3330 in a noncancer paradigm demonstrate reasonable and unremarkable toxicity (Investigational New Drug application number 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 effort based on modifying the various chemical aspects of E3330 as shown in Fig. 1 and Supplemental Fig. 1. Through these efforts, we identified three compounds (APX2007, APX2009, and APX2032) that demonstrated increased potency for APE1 targeting in our biochemical screening assays (Fig. 1) as well as tumor killing (Fig. 6; Supplemental Figs. 1 and 3). Of these three compounds, APX2009 emerged as a leading candidate because it did not show any negative effects on dorsal root ganglia neuronal cultures alone or in combination with cisplatin or oxaliplatin (Figs. 2–5) and had tumor killing similar to the other two compounds at significantly lower levels than E3330 (Supplemental Figs. 1–3). APX2007 and APX2032 did not protect nearly as well against 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 (Figs. 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 then E3330. APX2009 also demonstrates an increased antitumor activity (approximately four to nine times) at lower doses than E3330 in our antitumor studies using two cancer tumor types and a three-dimensional tumor model (Fig. 6; Supplemental Figs. 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 Fig. 2), suggesting that its pharmacokinetic profile might be beneficial in treating patients. In summary, APX2009 increases tumor cell killing, PK, and P450 metabolism half-life compared with E3330 and has more effective APE1 stimulation than E3330. This supports its potential to increase CIPN protection and tumor cell killing (Supplemental Fig. 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.

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Authorship Contributions

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References

