Published in final edited form as:

Neurotoxicology. 2015 March ; 47: 88–98. doi:10.1016/j.neuro.2015.02.005.

New Tools for the Quantitative Assessment of Prodrug Delivery and Neurotoxicity

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Abstract

Systemic off-target toxicities, including neurotoxicity, are prevalent side effects in cancer patients treated with a number of otherwise highly efficacious anticancer drugs. In the current study, we have: 1) developed a new analytical metric for the *in vivo* preclinical assessment of systemic toxicities/neurotoxicity of new drugs and delivery systems; and 2) evaluated, in mice, the *in vivo* efficacy and toxicity of a versatile and modular NanoDendron (ND) drug delivery and imaging platform that we recently developed. Our paclitaxel-carrying ND prodrug, ND^{PXL}, is activated following proteolytic cleavage by MMP9, resulting in localized cytotoxic chemotherapy. Using

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Supporting Information: Detailed procedures for behavior assays, corroborating toxicity and behavior data. This material is available via the internet at: <http://www.journals.elsevier.com/neurotoxicology/>.

Author Contributions: The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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click chemistry, we combined ND^{PXL} with a traceable beacon, ND^{PB}, yielding ND^{PXL}-ND^{PB} that functions as a theranostic compound. *In vivo* fluorescence FRET imaging of this theranostic platform was used to confirm localized delivery to tumors and to assess the efficiency of drug delivery to tumors, achieving 25-30% activation in the tumors of an immunocompetent mouse model of breast cancer. In this model, ND-drug exhibited anti-tumor efficacy comparable to nab-paclitaxel, a clinical formulation. In addition, we combined neurobehavioral metrics of nociception and sensorimotor performance of individual mice to develop a novel composite toxicity score that reveals and quantifies peripheral neurotoxicity, a debilitating long-term systemic toxicity of paclitaxel therapy. Importantly, mice treated with nab-paclitaxel developed changes in behavioral metrics with significantly higher toxicity scores indicative of peripheral neuropathy, while mice treated with ND^{PXL} showed no significant changes in behavioral responses or toxicity score. Our ND formulation was designed to be readily adaptable to incorporate different drugs, imaging modalities and/or targeting motifs. This formulation has significant potential for preclinical and clinical tools across multiple disease states. The studies presented here report a novel toxicity score for assessing peripheral neuropathy and demonstrate that our targeted, theranostic NDs are safe and effective, providing localized tumor delivery of a chemotherapeutic and with reduced common neurotoxic side-effects.

Keywords

Protease-mediated Drug Delivery; Paclitaxel; Protease-mediated Imaging; Peripheral Neurotoxicity; Neuropathy; Theranostic Agents; In vivo assessment; toxicity assessment

1. Introduction

The treatment of cancer patients with a variety of highly effective anti-neoplastic agents is generally accompanied by off-target systemic toxicities that limit the dosage, with neurotoxicity being one of the most severe and often dose-limiting non-hematological morbidities of chemotherapy (Cavaletti et al., 2011; Argyriou et al., 2012; Grisold et al., 2012). The neurotoxicity of anticancer drugs, including the widely used and efficacious platinum drugs and antitubulins such as paclitaxel (PXL), is most often manifested as peripheral neurotoxicity, commonly called peripheral neuropathy (Cavaletti et al., 2011; Grisold et al., 2012; Gradishar et al., 2005). Notably, patients treated with taxane-containing regimens including PXL or nab-PXL, an often prescribed nanoparticulate albumin-bound clinical formulation of PXL, frequently develop such peripheral neurotoxicity (Gradishar et al., 2005) that may present as the dose-limiting toxicity for these and other therapies. Neurological impairment, including sensory neuropathy, that results from treatment with a number of widely used anticancer drugs, is often such a severe and persistent side-effect that the dosage must be reduced or the drug replaced with alternate and potentially less-effective therapy (Grisold et al., 2012); thus, the potential benefit to patients of otherwise effective drugs can be abrogated by debilitating neurotoxicity.

There are methods to assess some drug-induced toxicities, for example, chemotherapy-induced liver toxicity can be revealed, both in preclinical studies and clinical practice, by measurements of plasma alanine transaminase (ALT) or aspartate transaminase (AST). By

contrast, neurotoxicities are more difficult to detect and/or evaluate particularly during preclinical drug development. Unfortunately, despite the prevalence of drug-induced neurotoxicity that may result, at least in part, from off-target effects of anticancer drugs on peripheral nerves and the dorsal root ganglia of primary sensory neurons (Argyriou et al., 2012), the mechanisms that lead to this pathology remain poorly understood (Kudlowitz and Muggia, 2013; Gornstein and Schwarz, 2014). Thus, in practice, the off-target induction of peripheral neurotoxicity in either animals or patients on chemotherapy is not only challenging to predict, prevent and treat but also can be late-onset (Argyriou et al., 2008; Frigeni et al., 2011; Pachman et al., 2011; Grisold et al., 2012). This is a particular problem during preclinical testing of new drugs in small animals, since the methods to assess such chemotherapy-induced peripheral neurotoxicity in such models are limited. Consequently, the debilitating and persistent neurotoxic side-effects of new therapeutics and/or drug formulations often do not become evident until after initiation of clinical trials and sometimes not until after extensive clinical use (Grisold et al., 2012; Gradishar et al., 2005). Thus, not only is there is a critical need for drug formulations that reduce or eliminate peripheral neuropathy, but the problem is exacerbated by the lack of reliable preclinical tools to assess such chemotherapy-induced peripheral neurotoxicity or other off-target effects of new therapies. The work presented here addresses both of these issues.

Some of the systemic toxicities associated with cytotoxic chemotherapeutics can be ameliorated by formulation of drugs as nanomaterials (Davis et al., 2008; Ahmed et al., 2012; Kanapathipillai et al., 2012; Ranganathan et al., 2012) and/or as non-toxic and essentially inactive prodrugs that require *in vivo* activation to yield the cytotoxic agent, e.g., via cleavage by proteinases, such as the matrix metalloproteinases (MMPs) (Zawilska et al., 2013). Using a design based on our proteolytic beacons (McIntyre et al., 2004; McIntyre and Matrisian, 2009; McIntyre et al., 2010), we recently demonstrated a PXL prodrug that is not toxic to tumor cells *in vitro* until activated by MMP9 (Samuelson et al., 2013). In these studies, we report the synthesis of a similar MMP9-activated PXL prodrug, ND^{PXL}, constructed on a clickable NanoDendron (ND) scaffold and an analogous proteolytic beacon, ND^{PB}. By linking ND^{PXL} to either ND^{PB} or another ND to enhance solubility, we have produced a set of prototypical MMP9-activated ND reagents (Fig. 1) that were tested for *in vivo* efficacy and toxicity in mouse models of breast cancer. Combined as ND^{PXL}-ND^{PB}, the MMP9-activated prodrug and imaging beacon NDs provide *in vivo* quantification of not only delivery of the ND to the tumor, but also an estimate of the activation of the prodrug at the tumor target from the measured cleavage of the intrinsic ND beacon. The self-reporting ND^{PXL}-ND^{PB}, a bifunctional theranostic (Kelkar and Reineke, 2011) agent, was used *in vivo* to measure the efficiency of tumor-associated activation of the prodrug in each of two preclinical models of breast cancer, in each of which the anti-tumor efficacy of ND^{PXL} was assessed.

Given the confounding morbidity of PXL and nab-PXL therapies (Gradishar et al., 2005), a major focus of the studies described here was an extensive assessment of the systemic toxicity of therapeutic doses of our novel prodrug formulation of PXL as compared with nab-PXL, a currently used clinical formulation of PXL. Following extensive behavioral studies of drug-treated versus control animals, we derived an overall toxicity score (TS) for

each mouse from a combination of primarily somatosensory nociception and motor coordination behavioral metrics. This novel TS metric provides a quantitative *in vivo* assessment of the development of drug-induced peripheral neurotoxicity within the various study cohorts and revealed subtle, but significant peripheral neurotoxicity in mice treated with nab-PXL but not those treated with ND^{PXL}. The results presented here show that the ND^{PXL} prodrug delivery system retains anti-tumor efficacy in the absence of debilitating peripheral neurotoxicity that attends the administration of nab-PXL, an extensively prescribed clinical formulation of PXL.

2. Methods

2.1. Materials

Generation 3 (G3)-polyester dendrons were purchased from Polymer Factory (Stockholm, Sweden). AF750 and AF700, with either maleimido- or N-hydroxysuccinimidyl- (NHS-) activating groups, were purchased from Molecular Probes Inc. (a division of Thermo Fisher Scientific USA). Mice (6-8 week old FVB or *nu/nu*) were obtained from Jackson laboratories. Animal studies were conducted with the approval of, and according to guidelines provided by, the Vanderbilt University IACUC. All animals were housed in an AAALAC-approved facility. Nab-PXL was obtained through the Vanderbilt Pharmacy as Abraxane® from Abraxis Bioscience, LLC (now Celgene Corp.). All other reagents were purchased from Fisher Scientific or Sigma-Aldrich chemical companies unless otherwise stated.

2.2. ND construction

The nanodendrons were constructed from G3-polyester dendrons with either an alkyne or azide terminus (Malkoch, 2002). Briefly, the periphery of the hydroxyl-dendron was modified to contain aldehyde groups. Then a linking peptide, including an AVRWLLTA sequence for MMP9 selectivity (Kridel et al., 2001; Chen et al., 2003; Samuelson et al., 2013), was attached via Dpr(AOA) coupling chemistry. Paclitaxel was attached to the N-terminus of the peptide using published procedures to give ND^{PXL} (Majoros et al., 2006). Our MMP-beacons, originally on a polyamidoamine (PAMAM) scaffold, include a two-color FRET probe with sensor and reference dyes for quantifying MMP activation (McIntyre et al., 2004; McIntyre and Matrisian, 2009; McIntyre et al., 2010). For these studies, novel beacons were made from AVRWLLTAC peptide attached to the ND as will be described in detail elsewhere (Scherer et al., 2015). NIR fluorescent dyes AF750-maleimide (reference) and AF700-NHS (sensor) were added sequentially to give ND^{PB}.

2.3. Phantom Preparation

Phantoms were prepared with either the sensor or reference dye bound to PAMAM, (McIntyre et al., 2004; McIntyre et al., 2010), or ND^{PXL}-ND^{PB} (not protease treated) or MMP9-treated ND^{PXL}-ND^{PB}, or ND^{PB} without ND^{PXL}, each in matrigel and injected subcutaneously into *nu/nu* mice, to create a localized phantom (Fig. 2A). Each mouse was injected up to six times with different matrigel samples (different dye and/or beacons concentrations) and imaged (Pearl® Impulse, LI-COR Biosciences, licor.com) using the 700 and 800 nm channels.

2.4. Imaging and Pharmacokinetics

Mouse images were acquired using the Pearl® Impulse, 700 and 800 channels, or the IVIS200 imaging system (PerkinElmer Inc., perkinelmer.com) with green fluorescent protein (GFP), Cy5.5 or ICG filters. Luciferase imaging was as described previously (Thiolloy et al., 2009) except that bioluminescence was recorded 10 minutes post-i.p. administration of luciferin (120 mg/kg). For imaging beacons in tumor-bearing mice, pre-injection images were obtained using both the IVIS200 and Pearl® Impulse systems, followed by retro-orbital injection of either ND^{PB} (1.0 nmol in 100µl of sterile 0.9% saline) or ND^{PXL}-ND^{PB} (0.5 nmol in 80 µl sterile 0.9% saline); multiple images were acquired in the first 30 minutes post injection and additional image sets usually at 60 and 90 min and up to every hour till 6 hours and overnight (20-24 hours). At each time point, both the reference and sensor channels were imaged, using either 800/ICG or 700/Cy5.5 channels, respectively, with either the Pearl Impulse or IVIS imaging systems, so as to calculate the sensor-to-reference (S/R) ratio of the beacon fluorescence after background subtraction (McIntyre et al, 2010). In most images after i.v. injection of ND^{PB} or ND^{PXL}-ND^{PB}, the lower part of the abdomen was either rotated or covered to shield against the fluorescence of the excreted beacon that was usually retained in the bladder. Pharmacokinetics of ND^{PB} was calculated by measuring the rate of clearance of the reference (AF750) and sensor (AF700) fluorescence from the plasma following retro-orbital i.v. injection (1.0 nmol in 100µl of sterile 0.9% saline); plasma was recovered from blood samples (up to three time points per mouse) that were collected into heparinized tubes at different times (up to 120 min); data from a number of mice (as indicated) were combined for analysis. The rate of clearance appeared to be consistent with a first order process, as indicated by approximately linear semi-log plots of sensor or reference fluorescence versus time (Figures S3 and S4), and clearance half-times were calculated by linear fit of these plots. In mice without tumors, the first data point for the sensor fluorescence in plasma (at ~10 min post injection) appeared to deviate from first-order and this point was excluded from calculations of clearance half-time; there was a consistently slightly faster clearance of the sensor as compared with the reference fluorescence.

2.5. Tumor models

Orthotopic tumors were induced in either FVB or *nu/nu* mice by injecting 1×10^6 cells into a single mammary fat pad, usually number 8 on the right thorax. The immunocompetent FVB tumor model used R221A-luc cells, originally derived from a spontaneous mammary tumor in an MMTV-polyoma virus middle T antigen (PyVT) transgenic mouse (Martin et al., 2008). The *nu/nu* xenograft tumor model used MDA-MB-231-GFP cells (Johnson et al., 2011). Palpable tumors were observed after 5-7 days in immunocompetent and 13-15 days in *nu/nu* tumor models.

2.6. Treatment Cycle

Mice were given i.p. injections of the equivalent of 12.5 mg/kg in paclitaxel (either as ND^{PXL}-ND or nab-PXL) or vehicle every day (12 doses) in the *nu/nu* model, and every second day (6 doses) in the FVB models (with or without tumors). In a pilot study, FVB tumor-bearing mice, 3-4 mice per cohort, were treated with either 4.1 or 12.5 mg/kg

paclitaxel (either as ND^{PXL}-ND or nab-PXL) every second day (6 doses) and another cohort of were also administered 25 mg/kg nab-PXL every second day (6 doses). Nab-PXL was administered in sterile saline (vehicle 1) and ND^{PXL}-ND was administered in 12-30% DMSO with sterile saline (vehicle 2). For the *nu/nu* model, 12 % DMSO was used and, for the FVB studies, 15-30% DMSO was used, depending on the batch of ND^{PXL}-ND. Mice receiving different therapies were housed together.

2.7. Efficacy studies

Treatment began when tumors were palpable; tumors averaged $30 \pm 5.6 \text{ mm}^3$ in the *nu/nu* tumor model and $9.8 \pm 2.5 \text{ mm}^3$ in the immunocompetent tumor model. Tumor volumes, monitored by imaging (luciferin or GFP) were measured by caliper as recorded by a researcher who was blinded to the treatments. Drug efficacy is defined as the inhibition of tumor growth rate as compared with tumors in vehicle-treated mice.

2.8. Toxicity and mouse behavior

For AST and ALT, blood was collected into heparinized tubes at sacrifice and the plasma was recovered prior to being shipped to the Yale School of Medicine's mouse metabolic core (<http://mouse.yale.edu/testing/options/analytical.aspx>) for analysis. A number of behavioral tests (see Supplemental Information, Methods and "The Vanderbilt Neurobehavioral Laboratory", <http://www.vandymouse.org/>) were conducted to monitor nociception, sensory and motor coordination in mice treated with ND^{PXL}-ND, nab-PXL or vehicle. The mice were exposed to the behavioral tests prior to being scored (training period). The schedule for drug treatments and the various behavioral tests are illustrated in Supplemental Figs. S1 and S2. For the non-tumor study, FVB mice (6-8 weeks old) were allowed to acclimate for 1 week. In week 2, the mice were introduced to the examiner and behavioral tests on at least three separate days, but no data were recorded. In week 3, behavior scores were recorded to obtain a pre-therapy baseline for each mouse. Behavior was assessed for signs of drug-induced peripheral neuropathy before, during and after the course of treatment. Behavior tests were performed every 2-3 days throughout the treatment course (days 1-12). Behavior was monitored for up to 68 days after the first treatment and was divided into the drug (days 1-12), short (days 13-20) and long (days 21-68) periods (Fig. S2). Data was analyzed for individual periods, combinations of periods and over the whole study and is presented as indicated. All the behavioral responses were recorded by an individual blinded to treatment and further randomized by housing mice with different treatments in the same cages. The behavior testing in tumor-bearing mice was less extensive due to logistics of coordinating imaging and efficacy measurements in the same mice as behavior testing. Tumor-bearing FVB mice were scored only on day 12 of treatment. Mice were acclimated to equipment prior to testing; however some tests were only conducted before/during or after therapy (as noted). The resulting data from each test, scored as described in the Supplementary Information, were statistically analyzed (see section 2.9, below) to compare the treatment groups and over different time periods. In addition, a toxicity score (TS) was calculated using the tests that had differences between the 3 cohorts. To obtain a toxicity score for each mouse, each behavioral test was converted to a uniform scale (0-100). For the *nu/nu* tumor-bearing mice, behavior scores from day 10-13 were used to calculate the toxicity score; for the FVB non-tumor model, behavior scores from eight

weeks after therapy were used. Final toxicity scores were obtained by averaging the scaled scores from the individual tests and the toxicity scores of mice in different cohorts were evaluated using analysis of variance (ANOVA) and, after confirming that the ANOVA showed an overall significance, we used t-tests to conduct all pairwise comparisons, with p-values adjusted for multiplicity. For FVB non-tumor mice, the TS for each mouse was calculated from toe pinch, Von Frey, pinna, pole climb, hot plate and total reflexes tests; for *nu/nu* mice, TS was calculated from toe pinch, Von Frey, hot plate, hanging wire and % liver mass scaled scores.

2.9. Statistics

The mixed effect model with log transformation was used to detect tumor growth rate differences between mice groups. Survival analysis was applied to calculate the marginal hazard ratio with corresponding 95% confidence interval (CI) for the hanging wire and hot plate, which are the behaviors recorded over time. The generalized estimating equations (GEE) model was applied to odds ratio (OR) of success/failure between different mice groups for the pinch, reflexes, and pole climb, which are the behaviors recorded as a score. The observation proportion with exact 95% confidence interval was used to summarize the sensation response of mice in the Von Frey test. All the statistics analysis were performed with R version 2.15.0 (Team, 2011) alone with the “survival” (Therneau and Grambsch, 2000), “geepack” (Yan and Fine, 2004; Højsgaard et al., 2006), and “binom” (Dorai-Raj, 2009) packages. All the p-values are two-sided and considered significant at values <0.05.

3. Results and Discussion

3.1. ND prodrug design and preparation

NDs targeted to the tumor microenvironment were designed with a number of adaptable parameters as needed for preclinical and clinical applications including a modular ND scaffold to facilitate adjusting the size, activation target and attached therapeutic (or imaging modality) plus an option to click two individual NDs together to provide dual-functionality (Scherer et al., 2015). To highlight this platform, we synthesized a ND for drug delivery (ND^{PXL}) and a ND for imaging (ND^{PB}). For imaging drug delivery, ND^{PB} was clicked to ND^{PXL} (ND^{PB}-ND^{PXL}); for testing *in vivo* toxicity and efficacy, ND^{PXL} was clicked to an un-modified ND (ND^{PXL}-ND) (see Fig. 1). For preclinical (and ultimately clinical) studies, production of adequate quantities in high yield and purity is required (Hu et al., 2010). ND^{PB}-ND^{PXL} was synthesized in 1-3 mg batches, enough for imaging and quantifying drug delivery for ~150 injections in mice. The therapeutic ND^{PXL}-ND was routinely synthesized in batches of ~200 mg of HPLC-purified product (80-90% overall yield), providing material for six doses at 12.5 mg/kg in 40 mice. Scale-up from 5 to 200 mg batches of ND^{PXL}-ND did not present any problems, suggesting that even larger batches are possible with little or no changes to the synthetic procedure.

3.2. ND beacons as tools to estimate drug delivery

The ND molecular beacon, ND^{PB}, was designed to quantify MMP9 activity *in vivo* using a cleavable peptide linker as a fluorescence switch with an internal reference analogous to our previous beacons (Scherer et al., 2008; McIntyre et al., 2010). The ND beacon consists of a

fluorescent sensor (S) that is activated by MMP9 and an internal reference (R) that is independent of protease activity. The reference allows for pharmacokinetic tracking *in vivo* (McIntyre and Matrisian, 2003; McIntyre et al., 2004; Scherer et al., 2008; Scherer et al., 2008; McIntyre et al., 2010). We have now quantified the pharmacokinetics of beacon clearance by measuring both reference and sensor fluorescence in plasma samples collected over time (Supplementary data, Figs S3 and S4). The clearance of the reference, that tracks the ND delivery scaffold, is consistent with first-order kinetics yielding a plasma half-life 174 and 77 minutes for FVB and tumor-bearing FVB mice, respectively. The somewhat faster clearance from the blood of tumor-bearing animals may, in part, reflect reduced intrinsic stability of ND^{PB} in these mice versus mice without tumors. In this regard, in both groups of animals, the sensor appeared to be cleared from the blood >2-times faster than clearance of the reference, with ~60 and 22 minute half-lives for the sensor in FVB mice without and with a tumor, respectively. The sensor clearance kinetics may deviate somewhat from first-order (Figures S3 and S4), perhaps reflecting some cleavage of the probe in the plasma and/or accessible extravascular volume as well as its distribution and elimination; this appears to be somewhat enhanced in tumor-bearing animals. In comparison, the plasma half-life of nab-PXL (Abraxane®), 6.2 hours in Balb/C mice (Ernsting et al., 2012), is at least 2-fold longer than for the NDs. A long circulation time is an advantage for reducing the frequency of dosing, but has the disadvantage of potentially increasing systemic toxicities due to more time for accumulation in healthy tissues.

The reference signal of the NDs also makes possible the quantification of beacon activation by measuring the sensor-to-reference (S/R) ratio. Since the peptide linker in ND^{PB} used the same sequence as the linker in ND^{PXL}, the cleavage of ND^{PB}-ND^{PXL}, assessed from the beacon S/R fluorescence ratio over the tumor, provides a surrogate measure of PXL delivery to the tumor microenvironment. The cleavage and quantification of the ND beacon was demonstrated *in vivo* with subcutaneous matrigel phantoms (Fig. 2A). The sensor and reference signals were discriminated in mice with a ~3.5-fold increase in S/R ratio between the untreated beacon and the protease-cleaved phantoms (Supplemental Fig. S5). Similar phantoms were used to generate a calibration plot for calculating the percent cleavage of ND^{PB} or ND^{PB}-ND^{PXL} in the tumor.

We confirmed that ND^{PB} was activated in MMP9-expressing mammary tumors using two orthotopic mouse models, one established with MDA-MB-231-GFP cells in athymic *nu/nu* mice (Johnson et al., 2011) and the other with R221A-luc cells in syngeneic, immunocompetent FVB mice (Martin et al., 2008). Representative images show the reference fluorescence distributed throughout the mouse, while the sensor was primarily activated in the tumor (Figs. 2B & 2C). Minimal activation of the beacon observed outside the tumor appears to be in the vicinity of the salivary glands that secrete MMP9-rich saliva (Fingleton et al., 2004). The overall signal in the tumor decreased over time, but the S/R ratio in the tumor region remained constant within error (Fig. 2D) and averaged 5.6 ± 0.4 (n = 36 mice). By comparison with the beacon calibration phantoms (see Supplementary Fig. S5), we calculated peptide cleavage of ND^{PXL}-ND^{PB} to be 10 (±3) % in the immunodeficient (*nu/nu*) and 25-30% in the immunocompetent mouse models. In the immunocompetent mice, infiltrating MMP9-laden stromal cells in the tumor

microenvironment may provide an additional source of MMP9 (Martin et al., 2008) that contributes to the enhanced intratumoral activation of the beacon in these tumors.

3.3. Efficacy of ND^{PXL}-ND vs. Nab-PXL

Anti-tumor efficacy of ND^{PXL}-ND was assessed as inhibition of the rate of tumor growth in immunocompetent and immunodeficient mammary tumor models (see Methods). The immunocompetent tumor model tested efficacy in mice with an intact immune system recognized to play an integral role in cancer development and progression (Martin and Matrisian, 2007). In the immunocompetent tumor model, the tumor size, visualized by molecular imaging (Fig. 3A), was reduced following treatment with either ND^{PXL}-ND or nab-PXL. In a pilot study (n = 3 or 4 mice/cohort), the measurement of tumor volumes over time (Fig. 3B) and at day 12 (Fig. 3C), indicates a dose-dependent reduction in tumor size with either nab-PXL or ND^{PXL}-ND. In a much larger study (n = 20 or 22 per cohort), treatment with the same total dose of paclitaxel as either nab-PXL or ND^{PXL}-ND resulted in reduced growth rates that were nearly equivalent, 21 and 20 mm³/day, respectively, reflecting a 46% and 47% inhibition of tumor growth rate relative to tumors in vehicle-treated mice (Fig. 3D, & Supplemental Tables S6 and S7). A higher dose of nab-PXL, 25 mg/kg, inhibited nearly all tumor growth in this mouse model, yielding a growth rate of 1.1 mm³/day (Supplemental Tables S6 and S7). Although ND^{PXL}-ND was found to be insufficiently soluble for delivery at higher concentrations, the NDs are designed for facile modification that could increase solubility to achieve higher dosing in future studies.

The efficacy of ND^{PXL} versus human tumors was tested in a xenograft model of breast cancer established in immunocompromised mice. Such mice bearing MDA-MB-231 tumors, treated daily with nab-PXL had smaller tumors visualized by imaging GFP (Fig. 3E) and an 84% decrease in tumor growth rate as compared to those in vehicle-treated mice (Fig. 3F and Supplementary Tables S4 & S5); however, the difference in growth rate did not reach significance (p=0.076) due primarily to variability in the tumor growth rate in the vehicle-treated controls (Fig. 3F and Table S5). In contrast, ND^{PXL}-ND, also dosed daily in this model, was less effective than nab-PXL, inhibiting tumor growth by only 40%. Thus, ND^{PXL}-ND was significantly less effective in this model as compared with the immunocompetent model where 46% inhibition was observed in mice dosed only every second day, i.e., at half the cumulative dose of PXL. As noted above, imaging with ND^{PB} indicated that less than half the activation of the beacon occurred in the tumor microenvironment in the *nu/nu* tumor model compared to the immunocompetent tumor model. Although *in vitro* studies suggest that MDA-MB-231 and R221A cells are equally sensitive to PXL and MMP9-cleaved ND^{PXL} (Samuelson et al., 2013), reduced efficacy of ND^{PXL} in the *nu/nu* model is consistent with reduced activation of ND^{PXL}-ND in the immunodeficient tumor microenvironment, as measured by reduced activation of the beacon in this setting. The NDs are activated by MMP9 (Samuelson et al., 2013). While MMP9 is expressed by tumor cells, it is also highly concentrated in infiltrating inflammatory cells (Hojilla et al., 2008; Martin et al., 2008), that, in the immunocompetent mice, may have provided an additional source of MMP9 that enhanced the intratumoral release of PXL from ND^{PXL}-ND^{PB} and efficacy. In the tumor setting, as shown in the results reported here (Fig. 3), the reduced activation and efficacy of the ND^{PB}-ND^{PXL} in the MDA-MB-231 model of

breast cancer as compared with the immunocompetent FVB model, highlights the utility of our theranostic ND for assessing prodrug activation in proximity of the tumor, i.e., higher activation of the beacon in the tumors of FVB versus *nu/nu* mice, as measured by imaging, results in enhanced efficacy as measured by the inhibition of tumor growth and *vice versa*. In concert, the two tumor models demonstrate enhanced efficacy of ND^{PXL}-ND in the tumors that exhibit the higher cleavage of the prodrug scaffold; this result is consistent with lack of cytotoxicity of PXL attached to the ND scaffold, as was observed *in vitro* (Samuelson et al., 2013) and imperative in any prodrug formulation.

3.4. Chemotherapy-induced peripheral neurotoxicity revealed by mouse behavioral tests

Assessment of general systemic toxicities of ND^{PXL}-ND and nab-PXL by measures of body mass, liver mass and plasma ALT and AST revealed no significant differences in any metrics (Supplementary Figs. S27-S30) with the exception of a 30% reduction in liver mass at necropsy of the tumor-bearing *nu/nu* mice treated with nab-PXL (Fig. S27), indicating some hepatotoxicity from nab-PXL treatment, not found in mice treated with ND^{PXL}-ND. However, such whole-body metrics provide no measure of neurotoxicity although peripheral neurotoxicity is well-documented in PXL-treated patients and is not improved in patients treated with nab-PXL (Grisold et al., 2012; Gradishar et al., 2005). Neurotoxicity, particularly peripheral neuropathy, is difficult to detect in preclinical studies with PXL or other drugs and/or formulations. To address this issue, we have now developed a behavioral toxicity score designed to assess chemotherapy-induced peripheral neurotoxicity of drugs, including nanomaterials and prodrugs in preclinical development. Recently, after our studies were in progress, it was reported (Renn et al., 2011) that behavior response could reveal both attenuated mechanical allodynia and cold hyperalgesia in oxaliplatin-induced neuropathy in BALB/c mice, validating our approach. To fully characterize the ND^{PXL} compounds as compared with a clinical formulation, nab-PXL, we have adapted mouse behavior metrics to provide quantifiable assessment of neurological differences that result from drug treatment. Metrics for sensory and motor coordination were evaluated in the two mouse tumor models (see Methods) and in FVB mice. While some tests showed no change upon treatment with either nab-PXL or ND^{PXL}-ND (see Supplementary Figs., S13-S16 and S21-S23, a number of behavioral metrics were informative. Metrics for toe pinch, total reflexes, Von Frey paw withdrawal response, hot plate, hanging wire and pole climb tests revealed differences between cohorts, notably for mice treated with nab-PXL as compared with the vehicle-treated controls. In all three mouse models, impairments indicative of peripheral neuropathy were observed in mice treated with nab-PXL, but not in ND^{PXL}-ND or vehicle-treated mice (Figs. 4 and 5, Table 1, Supplemental Figs. S6-S26 and Supplemental Tables S1-S3 and S8-S10). In tumor-bearing immunodeficient mice, the cohort treated with nab-PXL performed poorly on the hanging wire test of sensorimotor function (Figs. 4A and S10); during treatment, the nab-PXL-treated mice had a tendency to fall off before the 60 second limit (Fig. 4A) and, when scored over the whole study, had significantly ($p < 0.02$) poorer performance as compared with vehicle-treated or ND^{PXL}-ND-treated mice (Fig. S10). Furthermore, in tumor-bearing *nu/nu* mice, the somatosensory toe pinch (Figs. 4B & S6 and Table 1) and Von Frey (Supplementary Figs. S7, S8, and S32) tests demonstrated changes indicative of peripheral neuropathy from nab-PXL treatment, but not in ND^{PXL}-ND-treated versus vehicle-treated animals. Analyses of toe-pinch response

data to assess peripheral nociception in the three cohorts of tumor-bearing *nu/nu* mice during the treatment cycle (Fig. 4B, left panel) and throughout the whole study (Fig. 4B, right panel) showed significantly reduced responsiveness only in the nab-PXL-treated mice compared with vehicle-treated controls. Statistical analyses of the toe-pinch data (Table 1) showed a higher odds ratio (OR) for a low score, reflecting poorer performance ($p < 0.05$) for nab-PXL-treated versus vehicle-treated animals at each timeframe of the study with $p = 0.015$ during the drug treatment timeframe and $p = 0.004$ for the whole study period. Von Frey tests of paw withdrawal response, analyzed either as individual scores or as the median score per mouse for each of the study periods (before, during or after), and for either the right or left foot responses (Figures S7 and S8, respectively), showed no significant effect of the treatments although, with Abx, there appears to be a trend towards a higher median score for the individual mice, despite averaging all scores during treatment. Focusing on only the Von Frey data towards the end of treatment (days 10-13, Figure S32) reveals a significantly poorer response (higher toxicity score) in the Abx-treated versus vehicle-treated mice, but not in animals treated with ND^{PXL}-ND. Therefore, the Von Frey filament tests for paw withdrawal response in the *nu/nu* tumor-bearing mice suggest that, towards the end of treatment, nab-PXL-treated mice may have slightly reduced sensitivity, i.e., tactile allodynia in the hindlimbs compared to ND^{PXL}-ND-treated mice. The tumors in these animals precluded extensive after-treatment studies in sufficient animals to accurately assess long-term changes in behavioral responses. However, the toe-pinch data (Fig. 4B and Table 1), corroborated by analogous but less significant changes in Von Frey responses, demonstrate that nab-PXL-treated tumor-bearing FVB mice have reduced nociception, particularly in the middle digit of their hindlimbs, which was not evident in the cohorts of ND^{PXL}-ND- or vehicle-treated mice that were tested simultaneously within these blinded studies (Fig. 4B & Table 1).

Ideally, both acute and persistent neurotoxic effects of ND^{PXL}-ND and nab-PXL would be examined in tumor-bearing mice; however, the physical constraints of large mammary tumors that often required early necropsy, precluded long-term assessment in tumor-bearing animals. Long-term follow-up for peripheral neuropathy was assessed in mice without tumors from before therapy until 8 weeks after treatment with nab-PXL, ND^{PXL}-ND or vehicle. Mice treated with nab-PXL exhibited signs of acute and persistent peripheral neuropathy with reduced somatosensory nociception and motor coordination as revealed by scoring total reflexes, toe pinch and pole climb tests (Fig. 5) as well as other behavior assessments (Supplemental Figs. S16-S24 and Tables S1-S3 and S8-S10). Short-term behavior assessment in tumor-bearing FVB mice mirror the observations made in the study of FVB mice without tumors. As noted above, in tumor-bearing immunodeficient mice, nab-PXL treatment reduced toe pinch nociception, an observation not found with ND^{PXL}-ND or vehicle treatment (Table 1) and corroborated by other tests for neurotoxicity (see Supplementary Figs S6-S15 and S31-S35). While only two behavioral tests were conducted on the tumor-bearing immunocompetent mice (Supplemental Figs S25 & S26), results were analogous to those observed in the other two mouse models. The results tend to mimic the clinical progression of PXL toxicity that exhibits as distal neuropathy with less prominent motor involvement, and also in the relative time to onset, i.e., first the loss of sensory perception followed by loss of motor skills, and the persistent nature of PXL-induced

peripheral neuropathy (Argyriou et al., 2008; Pachman et al., 2011). Since all behavioral studies were conducted on treated mice that had received the same total dose of PXL, either as nab-PXL or as ND^{PXL}, the reduced toxicity of ND^{PXL}, that becomes only partially activated *in vivo* and predominantly at the tumor site, shows that the uncleaved prodrug formulation of PXL has significantly reduced systemic toxicity as compared with nab-PXL, consistent with our previously reported *in vitro* studies (Samuelson et al., 2013).

3.5. Toxicity Score

Behavior assessments that exhibited significant differences between cohorts were scaled for each mouse and then combined to obtain an overall toxicity score, with 100 being the most toxic (see Methods and Supplementary Methods). Due to differences in temperament of the two strains of mice, the results from each mouse strain were scored independently. For FVB mice, the mean of the toxicity scores were 14.5, 18 and 57 for vehicle, ND^{PXL}-ND and nab-PXL cohorts respectively, (Fig. 6, left panel, and Supplemental Figs. S36-S41). For *nu/nu* mice, the mean of the toxicity scores were 18.7, 16.3 and 51.8 for vehicle, ND^{PXL}-ND and nab-PXL cohorts, respectively (Fig. 6, right panel, and Supplemental Figs S31-S35). Overall, the toxicity scores show a significantly higher systemic toxicity including neurotoxicity of nab-PXL compared to the ND formulation indicating that ND^{PXL}-ND is less toxic to healthy tissues than is the albumin-bound nab-PXL, reported to exhibit systemic toxicities similar to other formulations of PXL (Gradishar, 2005; Gradishar et al., 2005). The reduced *in vivo* toxicity of PXL attached to the ND scaffold is consistent with the lack of *in vitro* cytotoxicity of the uncleaved prodrug (Samuelson et al., 2013). The ability to “turn off” toxicity of PXL, as in the uncleaved ND^{PXL}, is an essential feature of any prodrug system, and likely accounts for the elimination of detectable systemic side effects in mice treated with this prodrug formulation.

3.6. Relating Toxicity and Efficacy

Efficacy, the ability to eliminate diseased cells/tissues, is always the top priority in any therapy. Toxicity can limit efficacy by producing such debilitating damage to healthy cells/tissues that administration of effective drugs needs to be discontinued or reduced. Evaluating new therapies for efficacy in relation to toxicity is a challenge. To evaluate the systemic effects of nab-PXL as compared to ND^{PXL}-ND, tumor inhibition and toxicity measured in individual mice were compared graphically for the *nu/nu* model (Fig. 7). As illustrated in Fig. 7, nab-PXL was efficacious as measured by the inhibition of tumor growth, but at the cost of higher toxicity. ND^{PXL}-ND treatment resulted in consistently reduced toxicity, while maintaining appreciable efficacy, i.e. >60% tumor growth inhibition in 5 of 8 animals. The results show that when accounting for toxicity, the NDs outperform nab-PXL, a current clinical formulation of PXL. This reduction in systemic toxicity suggests that higher doses of drug could be administered as the ND formulation for longer periods of time, so as to further improve efficacy with this formulation, i. e., therapies that are working to stop or slow cancer progression but with reduced systemic toxicity could be continued with less concern for damage to healthy tissues. Thus, a major benefit of delivering chemotherapeutics with this ND formulation of PXL is a significant reduction of systemic toxicities associated with nab-PXL and an expectation that this kind of prodrug targeted

chemotherapy would lead to improvement in the overall quality of life for patients receiving PXL therapy.

3.7. Summary

We report a proteinase-activated prodrug formulation of PXL that is designed to target cancer by exploiting the upregulation of tumor-associated MMP9 to achieve localized delivery of the cytotoxic payload. The MMPs, including MMP9, are active in the extracellular microenvironment and therefore present ideal targets for activation of such prodrugs since MMPs are generally expressed at low levels in non-pathological tissue (Coussens et al., 2002; Martin and Matrisian, 2007) but are often highly expressed during tumor progression (Liotta et al., 1980; Matrisian, 1999; Coussens et al., 2000; Fidler, 2002; Hiratsuka et al., 2002; Wang et al., 2002; Martin and Matrisian, 2007). MMP-activated compounds have been previously developed by us and others both as contrast agents for imaging tumors (Bremer et al., 2002; McIntyre et al., 2004; McIntyre and Matrisian, 2009; McIntyre et al., 2010; Achilefu, 2012; Akers et al., 2012) and as tumor-targeted prodrugs (Zawilska et al., 2013). The novel bifunctional, ND^{PXL}-ND^{PB}, described here is a prodrug formulation of PXL coupled with a tumor-imaging beacon, to combine both imaging and prodrug modalities in a single “theranostic” compound to provide metrics for both the efficiency of prodrug delivery and *in vivo* activation in the tumor microenvironment. It is notable that there is enhanced activation of beacon and prodrug in the immunocompetent versus immunodeficient tumor models, a difference that may be due to infiltrating inflammatory cells in the former. This result suggests that an MMP9-activable prodrug formulation might also be useful for targeted treatment of inflammation and/or infection, either of which may complicate direct clinical application of ND^{PXL} to the treatment of cancer. The modular ND system has potential for further development to optimize diverse properties, depending on the target: modifying the “blank” ND can tune solubility, pharmacokinetic parameters, circulation time and more; the peptide sequence can be selected to serve as a substrate for other MMPs proteinases; other drugs could be delivered in place of PXL; and, for more clinically relevant imaging, gadolinium-based (MRI) or radiolabeled (PET/SPECT) proteolytic beacons could be incorporated (Lepage et al., 2007; Lebel et al., 2008; Jastrzebska et al., 2009) in place of the optical ND^{PB} that is particularly useful for the preclinical studies reported here. Thus, NDs represent a versatile platform that minimize peripheral neurotoxicity and offer potential as a universal delivery system to minimize the systemic toxicity of a variety of cytotoxic drugs while maintaining efficacy. We anticipate that the toxicity score described here will serve, in preclinical assessment of novel drugs and/or formulations, to enhance the assessment of neurotoxicity, including the debilitating peripheral neuropathy that often accompanies treatment with otherwise efficacious chemotherapeutics.

4. Conclusions

In this manuscript, we report new tools for targeting drug delivery to tumors and for assessing systemic toxicities of drugs via a novel toxicity score for quantifying, in mice, the development of peripheral neurotoxicity, a common confounding morbidity of chemotherapy. The toxicity score, based primarily on a combination of established mouse

behavioral metrics, reveals chemotherapy-induced peripheral neurotoxicity (neuropathy) in mice treated with nab-PXL, a routinely used clinical formulation of PXL. We also describe a prodrug theranostic platform that not only targets the delivery of a cytotoxic drug to tumors with reduced systemic toxicity but also provides imaging metrics for prodrug activation in the tumor microenvironment. In an immunocompetent preclinical model of breast cancer, the novel ND prodrug formulation of PXL, ND^{PXL}-ND, had efficacy comparable to nab-PXL, but with significantly reduced systemic toxicity and peripheral neuropathy as revealed by lower toxicity scores, also found in two additional mouse models. Thus, these NDs not only inhibit tumor growth, but are less toxic than nab-PXL. The behavioral metrics and derived toxicity score described here have significant potential for widespread application during the preclinical stage of drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported in part by Susan G. Komen for the Cure® Grant KG090434 (J.O.M.), NCI P30 CA68485 (small animal imaging center, VUIIS), NSF IGERT, 0333392 (R.L.S.), DOD BCRP, BC073450 (R.L.S.) and NCI R25CA092043 (L.E.S.). Behavioral work was performed at the Vanderbilt Mouse Neurobehavioral Core, which is supported in part by P30HD15052. The sponsors had no involvement in: study design; collection, analysis or interpretation of data; the writing of the manuscript; or the decision to submit the article for publication. MA was supported in part by NIH grants R01 ES07331 and R01 ES10563. We thank: Michelle Martin and Barbara Fingleton for providing R221A-luc cells and advice on the orthotopic immunocompetent FVB tumor model; Conor Lynch for providing training in tissue culture and animal procedures; John Allison for providing training and expertise on mouse behavior assessment; Paul E. Matrisian for assistance with quantifying the beacon; and John Wikswo and Todd Peterson for advice on the manuscript and data presentation.

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Highlights

- We developed a nanodendron prodrug (NDdrug) to deliver paclitaxel (PXL) to tumors
- We used optical imaging to quantify the localized release of prodrug in tumors
- NDs are safe and NDdrug has anti-tumor efficacy in immunocompetent mice
- A novel toxicity score (TS) of mouse behavior revealed nab-PXL neurotoxicity
- NDdrug-treated mice had a lower TS and reduced peripheral neurotoxicity/neuropathy

Figure 1. Schematic representations of the NanoDendrons (NDs) structures and cleavage (left side) and mechanism of action (right side), illustrating NDs within the vasculature and extravasated into tumor tissue, an MMP9-rich microenvironment. The proteolytic beacon, ND^{PB}, includes both reference (R) and sensor (S) fluorophores; the prodrug, ND^{PXL}, carries PXL (D) attached to the MMP9-cleavable peptide (shown in heavy black).

Figure 2. Imaging of ND activation *in vivo* for calibration (A) and as a drug delivery surrogate (B-D). In each image (A-C), green, red and yellow represent reference, sensor and co-localization respectively. (A) *In vivo* calibration of ND^{PXL} via subcutaneous matrigel phantoms. In this mouse, the phantoms include: PAMAM-AF750 (23 nM, **1**), PAMAM-AF700 (10nM, **2**), ND^{PB} (40 nM, **3**), MMP9-treated ND^{PB} (40 nM, **4**) and PAMAM-AF750 (7 nM, **5**). (B&C) Representative images for measuring ND^{PB} activation in FVB mice with R221A, (B) and *nu/nu* mice with MDA-MB-231, (C) orthotopic mammary tumors. (D) Quantification of ND^{PB} activation used to estimate PXL delivery to the tumor.

Figure 3.

Efficacy of ND^{PXL}-ND and nab-PXL (Abx) versus vehicle in two pre-clinical models of orthotopic mammary cancer; an immunocompetent model of FVB mice with R221A tumors (panels A to D) and a human mouse model of *nu/nu* mice with MDA-MB-231 tumors (panels E & F). Panels A & E: White light images of typical mice overlayed with either bioluminescence from luciferin in FVB mice (panel A) or GFP fluorescence from tumors in *nu/nu* mice (panel E), each before treatment and after a 12 day cycle with either ND^{PXL}-ND, nab-PXL (Abx), each 12.5 mg/kg, or vehicle. Panels B & C: Tumor volume (median with max and min denoted by the vertical bar, panel B) measured with calipers for mice (n = 3 or 4 per cohort) treated with either ND^{PXL}-ND or Abx (either 12.5 or 4.1 mg/kg as indicated) or vehicle; individual tumor volumes at day 12 of treatment (Panel C). Panels D & F: Tumor volume (mean ± SEM) measured with calipers for mice treated with either ND^{PXL}-ND (Red), Abx (Blue), each 12.5 mg/kg, or vehicle (black); panel D, R221A tumor model (n = 20 or 22 mice per cohort, Tables S6 & S7); panel F, MDA-MB-231 tumor growth (8 to 15 mice/cohort, Tables S4 & S5) was normalized to the individual mouse tumor volume on the first day of treatment.

Figure 4.

Behavioral tests of mice hosting the MDA-MB-231-GFP (*nu/nu*) model of human breast cancer revealed altered behavior in mice treated with nab-PXL (Abx) but not those treated with ND^{PXL}-ND or vehicle, indicating neurotoxic effects of Abx but not ND^{PXL}-ND. Panel A: Hanging wire test results during drug-treatment indicate a trend towards poorer performance in Abx-treated mice. The dotted line and gray bar are the average and SEM, resp., for vehicle-treated mice. Panel B: The toepinch test scored for each test in all mice shows a larger number of low scores in Abx-treated versus vehicle-treated mice, i.e., a poor response not observed in the ND^{PXL}-treated mice, indicating reduced sensitivity in Abx-treated mice during the treatment cycle (left side) and throughout the whole study (right side). The gray bar identifies the range of scores defined as normal behavior for statistical analysis.

Figure 5.

Representative behavior tools to assess and compare the PXL-induced peripheral neuropathy of ND^{PXL}-ND and nab-PXL (Abx) in FVB mice. Sensory reflexes were scored for a combined total reflex score (panel A), and sensitivity to a toe pinch (panel B); motor coordination was assessed by the pole climb test (panel C). Each graph represents the mean $\pm$ SEM of either all measurements acquired in a given study period (panels A & B) or of individual mouse performance for each day (panel C).

Figure 6.

Calculated toxicity scores for non-tumor FVB (left) and *nu/nu* tumorbearing (right) mice treated with either ND^{PXL}-ND or nab-PXL (Abx) versus vehicle. The toxicity score for each mouse was calculated from several tests (scaled 0-100) in which higher scores reflect poorer performance indicative of toxicity; the mean (+/- SEM) of all mice in each cohort are denoted by horizontal bars. **, p<0.05; ***, p<0.005.

Figure 7. Efficacy and toxicity comparison in *nu/nu* tumor-bearing mice treated with either ND^{PXL}-ND (open circles) or nab-PXL (solid squares). Each dot represents a mouse from day 12 of the study. The rectangle denotes the region with acceptable efficacy (> 60%) but low toxicity score (< 25).

Table 1

Statistical analyses of toe-pinch assessment of *nu/nu* mice with MDA-MB-231 mammary tumors.

	Drug			Drug & Short			Whole Study		
	OR	95% CI	p-value (m1, m2)*	OR	95% CI	p-value (m1, m2)	OR (n1, n2)**	95% CI	p-value (m1, m2)
Vehicle 1 vs. Vehicle 2	1.75	0.19-16.0	0.62 (30, 26)	3.44	0.29-40.3	0.325 (46, 36)	1.86 (8, 7)	0.30-11.6	0.51 (79, 66)
ND^{PXL}-ND vs. Vehicle	1.46	0.30-7.05	0.638 (34, 56)	0.69	0.11-4.51	0.698 (51, 82)	1.02 (9, 15)	0.25-4.13	0.973 (105, 145)
Abx vs. Vehicle	10.7	1.57-72.5	*0.015 (34, 56)	10.3	1.36-78.3	*0.024 (53, 82)	5.55 (9, 15)	1.72-17.9	*0.004 (99, 145)

All individual toe-pinch scores, see Figure 4, panel B, in each segment of the study, as indicated, were analyzed with a generalized estimating equations (GEE) model to calculate the odds ratio (OR) of performance different from normal between various mice groups (as indicated), the associated exact 95% confidence interval (CI), and two-sided p-values (see Methods).

* m1 and m2 are the number of measurements analyzed in that study period.

** n1 and n2 are the number of mice per cohort.