Translational Studies of Phenotypic Probes for the Mononuclear Phagocyte System and Liposomal Pharmacology


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ABSTRACT

As nanoparticles (NPs) are cleared via phagocytes of the mononuclear phagocyte system (MPS), we hypothesized that the function of circulating monocytes and dendritic cells (MO/DC) in blood can predict NP clearance (CL). We measured MO/DC phagocytosis and reactive oxygen species (ROS) production in mice, rats, dogs, and patients with refractory solid tumors. Pharmacokinetic studies of polyethylene glycol (PEG)-encapsulated liposomal doxorubicin (PEGylated liposomal doxorubicin [PLD]), CKD-602 (S-CKD602), and cisplatin (SPI-077) were performed at the maximum tolerated dose. MO/DC function was also evaluated in patients with recurrent epithelial ovarian cancer (EOC) administered PLD. Across species, a positive association was observed between cell function and CL of PEGylated liposomes. In patients with EOC, associations were observed between PLD CL and phagocytosis ($R^2 = 0.43$, $P = 0.04$) and ROS production ($R^2 = 0.61$, $P = 0.008$) in blood MO/DC. These findings suggest that probes of MPS function may help predict PEGylated liposome CL across species and PLD CL in patients with EOC.

Introduction

Nanoparticles (NPs), which include polyethylene glycol (PEG)-encapsulated (PEGylated) liposomes, are novel drug-delivery platforms that have the potential to improve tumor drug exposure and reduce accumulation in normal tissues more so than their small-molecule counterparts (Zamboni and Tonda, 2000; Zamboni, 2005, 2008). The pharmacokinetics (PK) of NPs are dependent upon the carrier and not the drug encapsulated within the carrier until the drug gets released from the carrier (Papahadjopoulos et al., 1991; Park et al., 2004; Zamboni, 2005, 2008). The drug that remains encapsulated within NPs is an inactive prodrug, and thus the drug must be released from the carrier to be active. After the drug is released from the carrier, the PK disposition of the drug will be the same as that following administration of the noncarrier form of the drug (Zamboni and Tonda, 2000; Zamboni, 2005, 2008).

The PK disposition of PEGylated liposomal formulations of CKD602 (S-CKD602), doxorubicin (Doxil; Janssen (of J&J), Titusville, NJ), and cisplatin (SPI-077) have been evaluated in preclinical models and patients (Lee et al., 2000; Gabizon...
et al., 2003; Zamboni et al., 2007). The ability to extrapolate animal data to predict PK parameters in humans is an essential step in drug development (Gabizon et al., 2008). We have previously explored the use of allometric scaling to predict the PK of PEGylated liposomal agents across species (Caron et al., 2011). Our study indicated that while a relationship exists between species body weight and clearance (CL), there is considerable variability in PK among species, particularly when scaled by conventional and nonconventional parameters. Thus, the development of new methods of scaling and/or measures of NP interaction at the biologic level are warranted to further explore the variability observed in NP PK.

Studies suggest that the significantly high and clinically relevant interpatient variability in the PK and pharmacodynamic (PD) disposition of NP anticancer agents is related to the function of monocytes and dendritic cells (MO/DC) of the mononuclear phagocyte system (MPS) (Zamboni et al., 2011a; Zamboni et al., 2011b). PEGylated and non-PEGylated nanoparticle agents are cleared via the MPS, but PEGylated nanoparticle agents are cleared at a slower rate because of delayed and/or reduced recognition by the MPS (Dobrovolskaia et al., 2008; Caron et al., 2012). The MPS is defined as a group of cells having the ability to ingest large numbers of particles (Hume et al., 2002). These cells (comprising MO/DC circulating in the blood, fixed macrophages of various connective tissues, Kupffer cells in the liver, and macrophages in the lymph nodes, bone marrow, and spleen) serve as a potential CL pathway for NPs (Lichanska et al., 1999; Hume et al., 2002; Dobrovolskaia et al., 2008). We have previously reported a significant relationship between the PK and PD of S-CKD602 and changes in circulating monocyte numbers and absolute neutrophil count (Zamboni et al., 2011b). The results of our previous study suggest that monocytes are more sensitive to toxic effects of S-CKD602 compared with neutrophils and that the increased sensitivity appears to be related to the liposomal formulation and not the small molecule drug, CKD-602, encapsulated inside the liposome. Thus, blood monocytes may play a key role or be a surrogate marker for NP CL in patients.

Epithelial ovarian cancer (EOC) is a disease characterized by large numbers of peritoneal MO and macrophages, the primary cells of the MPS (Bookman, 2005). As a result of high relapse rates several chemotherapeutic strategies have been developed in the blood, fixed macrophages of various connective tissues, Kupffer cells in the liver, and macrophages in the lymph nodes, bone marrow, and spleen) serve as a potential CL pathway for NPs (Lichanska et al., 1999; Hume et al., 2002; Dobrovolskaia et al., 2008). We have previously reported a significant relationship between the PK and PD of S-CKD602 and changes in circulating monocyte numbers and absolute neutrophil count (Zamboni et al., 2011b). The results of our previous study suggest that monocytes are more sensitive to toxic effects of S-CKD602 compared with neutrophils and that the increased sensitivity appears to be related to the liposomal formulation and not the small molecule drug, CKD-602, encapsulated inside the liposome. Thus, blood monocytes may play a key role or be a surrogate marker for NP CL in patients.

Clinical Study Design. Baseline characteristics and treatment regimens of the 10 women enrolled are listed in Table 1. Patients were administered standard premedications, including 10 mg of dexamethasone, 25 mg of diphenhydramine, 20 mg of famotidine, and 8 mg of ondansetron, all intravenously × 1, 30 minutes prior to PLD. Patients were administered PLD at 40 mg/m2 alone or PLD at 30 mg/m2 i.v. × 1, over approximately 1 hour in combination with carboplatin infused intravenously × 1 over 30 minutes at a dose to achieve area under the curve (AUC) = 5 (Calvert equation). Serial blood PK samples were obtained at baseline prior to the administration of PLD or PLD with carboplatin; at the end of infusion; and 1, 3, 24, 48, 72, 96, 192, and 672 hours after the administration of PLD. Plasma was processed immediately, and the encapsulated and released components of PLD were separated using solid phase separation methods as described
Baseline characteristics of patients enrolled in the study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Race</th>
<th>Weight</th>
<th>BSA</th>
<th>Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52</td>
<td>AA</td>
<td>1.16</td>
<td>72</td>
<td>PLD</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>AA</td>
<td>1.94</td>
<td>73</td>
<td>PLD</td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>C</td>
<td>1.46</td>
<td>73</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>C</td>
<td>1.7</td>
<td>74</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>C</td>
<td>1.54</td>
<td>71</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>C</td>
<td>1.74</td>
<td>74</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>7</td>
<td>53</td>
<td>C</td>
<td>1.44</td>
<td>70</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>8</td>
<td>75</td>
<td>C</td>
<td>1.91</td>
<td>72</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>9</td>
<td>52</td>
<td>C</td>
<td>1.7</td>
<td>73</td>
<td>PLD + carboplatin</td>
</tr>
<tr>
<td>10</td>
<td>57</td>
<td>C</td>
<td>1.63</td>
<td>70</td>
<td>PLD + carboplatin</td>
</tr>
</tbody>
</table>

Mean ± S.D. 59 ± 10.9 C, 1 AA 78 ± 20 1.8 ± 0.2 6 PLD alone 4 PLD + carboplatin

AA, African American; BSA, body surface area; C, Caucasian.

previously (Zamboni and Tonda, 2000; Zamboni et al., 2007, 2009). Noncompartmental analysis was performed using Phoenix WinNonlin Version 6.1 to calculate PK parameters (Table 2). Blood (3 ml) was obtained at baseline, 48, 72, and 96 hours to test the function of MO/DC. At each visit, vital signs were obtained, physical examinations and blood work was performed at the discretion of the individual physician, and patients were asked about any adverse symptoms they experienced, including but not limited to nausea/vomiting, PPE, neuropathy, and stomatitis. Grade of toxicity was assessed based on light scatter properties (forward scatter versus side scatter) and subsequently plotted for histogram analysis (Supplemental Fig. 1). The proportion of positive cells (i.e., cells that exhibit fluorescence) was determined as those events, which shifted to the right out of the “negative” region on the fluorescence intensity scale. Mean fluorescent intensity (MFI) of the positive cell population served as an index of phagocytic or ROS activity.

**Phagocytosis Assay.** Twenty microliters of fluorescein isothiocyanate–labeled opsonized E. coli bacteria bioparticles (1 × 10^9 particles/ml) (Orpegen Pharma, San Diego, CA) were added to 100 μl of whole blood and incubated for 10 minutes at 37°C. Additional samples kept on ice (0°C) served as a negative control. After incubation, 100 μl of Trypan blue was added to quench extracellular fluorescence. Phagocytic activity (number of bacteria internalized per cell) was quantified as the MFI of the “positive” cells.

**ROS Production Assay.** ROS was assessed in MO/DC in response to no stimuli and to a variety of stimulants, including opsonized nonfluorescent E. coli as a phagocytic stimulus, N-formyl-methionine-leucine-phenylalanine as a physiologic peptide, phorbol 12-myristate 13-acetate (PMA) a synthetic ester, and phosphate-buffered saline as a control (no stimulus; baseline measurement). Following a 10-minute exposure to the stimulus, nonfluorescent dihydrorhodamine 123 (Orpegen Pharma, San Diego, CA) was added to the samples as a fluorogenic substrate, which, following intracellular oxidation was converted to fluorescent rhodamine 123. MFI of rhodamine 123 fluorescence served as a quantitative measure of intracellular oxidative activity.

**Statistics.** All statistical analyses were performed using SAS version 9.5 (Cary, NC) software. Simple linear regression was used to explore the linear relationship between two continuous variables, including the relationship between MO/DC or PMN cellular function and PK (CL) or PD (PFS and PPE grade). The coefficient of determination, R^2 was used to measure the linear association between PK/PD outcomes and cellular function. The relationship between CL and phagocytosis was evaluated using multiple linear regression, including a term for treatment type (PLD versus PLD + carboplatin). A Kruskal-Wallis test was used to test for differences in median MFI between patients and within patients over the course of cycle 1. A Cox proportional hazards model, using progression-free survival as the outcome variable and phagocytosis as a covariate, was used to estimate predicted progression-free survival at differing levels of phagocytosis. The value of α was set at 0.05 for all statistical tests, and P values are two-sided.

**Results**

**Relationship between Cellular Function and PEGylated Liposome PK in Preclinical Models and Patients with Refractory Solid Tumors.** The relationship between phenotypic probes of MPS function and PK of PEGylated liposomal agents was evaluated in preclinical tumor models and in patients with refractory solid tumors as part of phase I studies of PEGylated liposomal doxorubicin (Doxil; PLD), CKD-602 (S-CKD602), and cisplatin (SPI-077). There was a direct linear relationship between MPS activity and the CL of PEGylated liposomes across mice, rats, dogs, and humans. The average mean fluorescence intensity in the MO/DC population following the phagocytosis assay in the four species evaluated was correlated with CL of PLD (R^2 = 0.95), S-CKD602 (R^2 = 0.99), and SPI-077 (R^2 = 0.73) as shown in Fig. 1A. There was a similar trend observed when comparing the production of ROS across species without any stimulus (baseline) with CL of PLD (R^2 = 0.77), S-CKD602 (R^2 = 0.77), and SPI-077 (R^2 = 0.66) (Fig. 1B). The relationship was also seen between production of ROS when stimulated with PMA.
and CL of PLD ($R^2 = 0.83$), S-CKD602 ($R^2 = 0.84$), and SPI-077 ($R^2 = 0.69$).

**Phenotypic Probes Predict PLD PK in Patients with EOC.** The relationship between phenotypic probes of MPS function and PLD PK was evaluated in patients with EOC. On day 1 of the study, phagocytosis and ROS production were assessed in MO/DC prior to the start of the PLD infusion in patients with EOC ($n = 10$). A linear relationship ($R^2 = 0.43$, $P = 0.04$) was found between MFI of the phagocytic cells and PLD CL for all patients, shown in Fig. 2A. A relationship between MFI of ROS production without stimulus at baseline and PLD CL for all patients was also observed, as shown in Fig. 2B ($R^2 = 0.61$, $P = 0.008$). The relationship between the MPS function is more significant in patients treated with PLD alone ($R^2 = 0.57$ and $0.61$ for phagocytosis and production of ROS without stimulus, respectively) (Fig. 2, C and D).

There was also a relationship between the ROS probe with the addition of a stimulant, and PLD CL in all patients at baseline (Table 3). The only stimulant that did not show a strong relationship in all patients was PMA ($R^2 = 0.23$). PMA was also the only probe that had lower association in the PLD alone versus PLD + carboplatin group. However, the other oxidative burst stimulants performed similarly to the phagocytosis probe and also demonstrated stronger relationships in the cohort of patients which received PLD alone versus PLD + carboplatin.

A multiple linear regression model was also used to examine the relationship between phagocytosis and doxorubicin clearance, adjusting for treatment. The model had doxorubicin clearance as the dependent variable and phagocytosis and treatment (an indicator variable for PLD or PLD plus carboplatin) as the independent variables. This model, which results in two intercepts (intercept for PLD alone $\beta_0$ and intercept for PLD plus carboplatin $\beta_0 + \beta_{\text{treatment}}$) and a common slope ($\beta_{\text{phagocytosis}}$), suggests a positive linear association between phagocytosis and CL of PLD ($\beta_{\text{phagocytosis}} = 0.04$, $P = 0.07$) where patients with higher MPS function have a higher CL of PLD (Fig. 3). Patients on PLD plus carboplatin (dotted regression line) had somewhat lower doxorubicin clearance compared with patients on PLD only (solid regression line); however, the treatment effect was not significant ($\beta_{\text{treatment}} = 6.06$, $P = 0.38$).

The correlation between either phagocytosis or ROS production in PMNs and PLD CL failed to reach statistical significance in either the total patient population or subpopulations, suggesting that PMN are not involved in the PK of PLD. In the study, $87.4 \pm 10.9\%$ of gated MO/DC in patients tested positive to the phagocytosis or ROS probe. Therefore, differences in MFI between patients were due to cellular function variability and not the ability of the assay to detect positive events.

**Cellular Function over Time in Patients with EOC.** The cellular function of MO/DC and PMNs was also assessed over time in the first cycle of PLD with or without carboplatin. Phagocytosis measured in both MO/DC ($P = 0.85$) and PMNs ($P = 0.66$) was not significantly different in patients over the course of measurement (days 1, 3, 5, 28). The same held for ROS (no stimulus) in both MO/DC ($P = 0.37$) and PMNs ($P = 0.25$) over cycle 1. On day 1, just prior to PLD administration, the MFI of ROS (no stimulus) in all patients ranged from 7.4 to 117.0. The mean ± S.D. MFI of ROS was 39.1 ± 36.4 on day 1. The MFI for ROS in patient 3 was 117.0. Without
patient 3, the mean ± S.D. MFI for ROS of the other nine patients was 30.5 ± 25.5.

Phenotypic Probes Predict PLD PD in Patients with EOC. All patients enrolled in the study were followed until disease progression and/or PLD-related adverse events required discontinuation of PLD treatment. PLD could be stopped for grade 3/4 myelosuppression, stomatitis, PPE, or treating physician discretion. Patient 3 had rapidly progressive disease and died prior to the start of cycle 2 of PLD. Three additional patients (1, 8, and 10) had progressive disease while on PLD. For these four patients, the phenotypic probes of phagocytosis ($R^2 = 0.77$, $P = 0.02$) and ROS ($R^2 = 0.67$, $P < 0.0001$) prior to PLD administration were predictive of PFS in days (data not shown). A Cox proportional hazard model with phagocytosis as the independent variable was fit, and we determined the predicted probability of progression-free survival based on the level of MO/DC phagocytosis (Fig. 4) using the three quartiles of blood phagocytosis: Q1 = 345 (MFI), med = 486 (MFI), and Q3 = 621 (MFI).

PLD PK Predicts PD in Patients with EOC. The relationship between PLD PK and PD progression-free survival and PPE was evaluated. There was a significant association observed between encapsulated PLD exposure (AUC) and PFS (days) in the four patients receiving PLD alone who progressed while on PLD treatment ($R^2 = 0.88$, $P < 0.0001$) (data not shown). For the five patients who experienced PPE during the course of the study, there was a nonstatistically significant relationship between their exposure to PLD and the highest grade of PPE reported ($R^2 = 0.08$, $P = 0.6$) (data not shown).

Discussion

We have previously reported a relationship between physiologic parameters such as body weight, organ blood flow, and monocyte count and the PK of PEGylated liposomes in animal models and in patients with refractory solid tumors (Caron et al., 2011). In this prior study, variability in the PK, particularly CL and exposure as measured by area under the concentration versus time profile was noted across species. However, this current study looks at a plausible biologic explanation for the variability in PK of NP across species and in a clinically relevant patient population. We found that the phagocytic capacity and level of ROS production in MO/DC in blood of mice, rats, dogs, and humans is correlated with the CL of PEGylated liposomal agents across all species. This finding, in addition to our prior clinical studies of PEGylated liposomal CKD-602 (S-CKD602), prompted the development of a second clinical study that used the same phenotypic probes of MPS function to predict PLD PK and PD in patients with recurrent EOC (Zamboni et al., 2009). For the first time, we have demonstrated that a fast and inexpensive blood test of MPS function obtained prior to the administration of PLD can be used to predict PK, efficacy, and toxicity and can be used to individualize therapy. These probes may also predict PK and PD of other NP, conjugates, monoclonal antibodies, and antibody drug conjugates in animal models and in patients (Caron et al., 2012).

We observed a linear relationship between MPS activity and the CL of PEGylated liposomes across species. The phagocytic capacity and production of ROS of MO/DC was correlated with CL of the PEGylated liposomes PLD, S-CKD602, and SPI-077. The relationship was particularly noteworthy in MO/DC phagocytosis with the CL of PLD ($R^2 = 0.92$), S-CKD602 ($R^2 = 0.92$), and SPI-077 ($R^2 = 0.77$). This was the first study reporting a relationship between MPS function in blood and CL of an NP across species, including patients with cancer. The phenotypic probes developed in this study can be used to predict PK, efficacy, and toxicity and can be used to individualize therapy. These probes may also predict PK and PD of other NP, conjugates, monoclonal antibodies, and antibody drug conjugates in animal models and in patients (Caron et al., 2012).
previous findings, we performed a clinical study using circulating MO/DC in blood as a surrogate measure of the MPS function to predict PLD PK and PD (PFS and PPE toxicity). Results of the study reported here demonstrate that probes of MPS function predict PLD PK and PD. There was a linear relationship between encapsulated doxorubicin CL and both phagocytosis \((R^2 = 0.43, P = 0.04)\) and ROS activity \((R^2 = 0.61, P = 0.008)\) in blood MO/DC.

Consistent with the association between MPS probes and PLD PK, there was an association between encapsulated doxorubicin CL and both phagocytosis \((R^2 = 0.77, P = 0.02)\) and ROS activity \((R^2 = 0.67, P = 0.06)\) probes with PFS in the four patients who progressed while on PLD alone at the time of manuscript preparation. These results suggest that patients with higher MPS activity have a faster CL of PLD and a lower plasma exposure, which may be associated with less drug being available for delivery to the tumor and lower response. This relationship is further demonstrated by a Cox proportional hazard model that includes all 10 patients and assesses the relationship of the phagocytosis probe and its influence on the outcome of progression-free survival.

Our results suggest that the phenotypic probes may potentially provide valuable information toward dose individualization. Probes could be used to measure MPS function in each patient before administration of PLD, and then the dose of PLD may be adjusted based on MPS function and target plasma exposure (AUC). This is a similar process to that used to individualize carboplatin dose based on renal function and target plasma AUC (Calvert et al., 1989; Egorin et al., 1994).

### TABLE 3

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>All Patients ((R^2, r_x))</th>
<th>PLD Only ((R^2, r_x))</th>
<th>PLD + Carboplatin ((R^2, r_x))</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stimulant (baseline cell ROS production)</td>
<td>0.61, 0.64,‡</td>
<td>0.61, 0.6,*</td>
<td>0.0005, 0.4</td>
</tr>
<tr>
<td><em>E. coli</em> (particulate)</td>
<td>0.46, 0.22,*</td>
<td>0.44, 0.2</td>
<td>0.26, 0.4</td>
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<tr>
<td>fMLP (physiologic)</td>
<td>0.54, 0.57,*</td>
<td>0.48, 0.7</td>
<td>0.14, 0.4,*</td>
</tr>
<tr>
<td>PMA (synthetic)</td>
<td>0.23, 0.72</td>
<td>0.21, 0.8</td>
<td>0.59, 0.8</td>
</tr>
</tbody>
</table>

fMLP, \(N\)-formyl-methionine-leucine-phenylalanine.

‡\(P \leq 0.001; *P \leq 0.05.\)
masses abutting the liver, the largest measuring 5.2 cm. We are aware that, due to the relatively small number of subjects in this study of PLD in patients with EOC, some of our statistical comparisons are likely underpowered, which may have affected our ability to detect significant relationships. Nevertheless, we were able to observe suggestive associations between monocyte function and PLD CL, PFS, and PPE toxicity in these exploratory, rather than confirmatory analyses. Moreover, the data show that the patient with the highest probe activity had a different pathophysiology than the other nine patients enrolled. Not only did this patient have the most extensive disease burden, but the highest ROS phagocytosis in their liver. This is a paradigm shift from what is normally seen with small-molecule agents where patients with tumors in their livers have a reduced CL of drugs that are metabolized by phase I and II enzymes (Stewart et al., 1990).

One potential reason for the difference in the relationship between encapsulated doxorubicin CL and cellular functional assays between the PLD only and PLD in combination with carboplatin could be secondary to platinum effects on the cellular function of the MPS cells. The effect of platinum agents on monocytes has been explored in vitro (Nielsen, 1984). In this study, a 1 μM exposure of cisplatin for 60 minutes was shown to selectively inhibit chemotaxis, which can then also inhibit phagocytosis, in monocytes isolated from venous blood of healthy volunteers (Nielsen, 1984). Fumarulo et al. (1980) also reported an in vitro chemotaxis inhibition by cisplatin using peritoneal macrophages of the rat. In addition, an in vivo study has reported impaired blood monocyte chemotaxis in cancer patients ≥20 hours after receiving cisplatin at 20 mg/m² i.v. × 1 (Nielsen et al., 1985). On the basis of these studies and our results, the quick onset of chemotaxis and phagocytosis inhibition by cisplatin could explain a lack of functioning monocytes in the area of drug uptake and subsequently a lower NP CL.

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One MPS probe also was predictive of PPE toxicity in patients, as the ROS production at baseline was correlated with PPE grade on a scale of 0–5 ($R^2 = 0.56$); however, this will need to be validated in a larger cohort of patients. If probes may be used to determine efficacy, such as PFS, they could also indicate early in the treatment plan whether PLD is a worthwhile option for the particular patient.

When comparing the association between phagocytosis or ROS phenotypic probes and encapsulated doxorubicin CL, patient 3 consistently had the highest value in both measures. All data points were included in this study of 10 patients; however, patient 3 noticeably improves the relationship using peritoneal macrophages of the rat. In addition, an in vivo study has reported impaired blood monocyte chemotaxis in cancer patients ≥20 hours after receiving cisplatin at 20 mg/m² i.v. × 1 (Nielsen et al., 1985). On the basis of these studies and our results, the quick onset of chemotaxis and phagocytosis inhibition by cisplatin could explain a lack of functioning monocytes in the area of drug uptake and subsequently a lower NP CL.

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