Examining the Pharmacodynamics and Pharmacokinetics of a Diclofenac Poly(lactic-co-glycolic) Acid Nanoparticle Formulation in the Rat


Abstract. - OBJECTIVE: Nonsteroidal anti-inflammatory drugs (NSAIDs) are assembled into two categories; cyclooxygenase (COX-1) sparing inhibitors of COX-2 and non-selective NSAIDs. Diclofenac (DICLO) is a non-selective NSAID that has been linked to serious side effects including gastric ulcers and renal injury. In this study, we examine the effect of poly(lactic-co-glycolic) acid nanoformulation on DICLO-associated adverse events and pharmacokinetics using a nanoparticle (NP) formulation previously developed in our laboratory.

MATERIALS AND METHODS: Rats were administered a single dose of methylcellulose (VEH), blank NP, DICLO (10 mg/kg), or a DICLO-NP suspension equivalent to the DICLO dose. Urinary and blood parameters were measured at baseline and following treatment. Duodenal and gastric prostaglandin E₂ (PGE₂) and duodenal myeloperoxidase (MPO) were collected to assess inflammation at 24 hrs post-treatment.

RESULTS: The mean percent change from baseline in sodium excretion rate (µmol/min/100 g body weight) differed significantly from VEH in the NP (p < 0.0001), DICLO (p < 0.0001), and DICLO-NP (p = 0.0001) groups. The differences among groups did not reach significance for plasma sodium or potassium concentrations, potassium excretion rate, gastric PGE₂, or intestinal biomarker concentrations. Regarding renal histopathology, DICLO produced considerably more necrosis compared to VEH; while DICLO-NP did not elicit notable differences from VEH.

CONCLUSIONS: Our results suggest that over the duration and dosage examined, DICLO-NP may reduce renal necrosis without influencing other side effects or drug characteristics.
Promising results, in terms of renal and gastrointestinal side effect reduction, have been seen with the use of drug nanoformulation. Nanoparticles (NPs) can be formulated to exhibit favorable qualities, such as large surface area or tissue-specific targeting, which contribute to the utility of this drug delivery system. Several types of NPs have been devised using various materials including the polymer, poly(lactic-co-glycolic) acid (PLGA). In an effort to alter the side effect profile of DICLO, a NP formulation (DICLO-NP) was recently developed in our laboratory. This study sought to evaluate the gastrointestinal and renal side effects as well as the pharmacokinetics of the new DICLO dosage form.

Materials and Methods

Chemicals
The drug of interest, DICLO, and methylcellulose were acquired from MP Biomedical (Solon, OH, USA) and Science Stuff Inc. (Austin, TX, USA), respectively. Flufenamic acid, PLGA (MW 30,000 Da, 50:50 copolymer composition), and didodecyldimethylammonium bromide (DMAB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fischer Scientific Laboratory (Fair Lawn, NJ, USA) was the vendor for high-performance liquid chromatography (HPLC)-grade water, acetonitrile, acetic acid, and ethyl acetate.

Preparation and Characterization of PLGA-NP Formulation
This NP formulation was prepared using a method previously optimized by our laboratory. Briefly, fifty milligrams of PLGA was dissolved, stirred for 30 min at 750 rpm, in 3 mL of ethyl acetate along with 45 milligrams of DICLO. DMAB (0.25% w/v) was stirred and heated in 6 mL of water until dissolved. The organic phase was added under moderate stirring to the aqueous phase in a drop wise manner. The emulsions were sonicated for 5 min (20 kHz) after which 25 mL of water was added. The organic phase was allowed to evaporate over 4 hrs under constant stirring. Following centrifugation at 18,665 g, the finished product (supernatant) was collected. NP parameters, including size (diameter) and zeta potential, were determined on a NICOMP particle sizer (Particle Sizing Systems, Port Richy, FL, USA). Drug entrapment efficiency was calculated based on the drug entrapped in NP, as evaluated by UV spectroscopy, relative to total drug utilized.

Animals and Drug Administration
Male Sprague-Dawley rats (265-300 g) from Charles River Laboratories (Raleigh, NC, USA) were used for all experiments. Each animal was housed in a 12 hrs light-dark cycle with ambient humidity and temperature along with unrestricted access to food (2020X, Harlan Teklad) and water; however, upon dosing, food access was removed. Each animal was fitted with jugular vein cannula by the vendor. The study protocol was reviewed and approved by the Animal Care Committee of East Tennessee State University.

Study Design
All four treatment groups (n = 6), 0.5% methylcellulose solution (VEH), blank NP, DICLO (10 mg/kg) in methylcellulose, and a PLGA-NP equivalent dose (DICLO-NP), were administered respective treatments using a stainless steel oral gavage tube. The VEH group rats were selected from a group of animals which would serve as controls for two studies. Administration of NP allowed for an examination of PLGA pharmacodynamics. The DICLO dose chosen for this study was therapeutically equivalent to a rofecoxib dose which had previously elicited sodium excretion rate changes in rats. As such this dose would be sufficient to serve as a positive control for an adverse effect which may be alleviated through nanoformulation. Urine samples (12 hrs) were collected through housing the animals in metabolic cages subsequent to dosing.

Isoflurane was used to anesthetize the rats twenty-four hours post-treatment during which animals were exsanguinated using cardiac puncture. Tissues (stomach, proximal 8 cm of duodenum, and kidneys) were collected for analysis.

Renal Function Parameters
Change in Urine Flow Rate
The total urine volume in milliliters was divided by collection duration (12 hrs) to assay urine flow rate. Each value was normalized using 100 g body weight (B.W.) before the mean percent change from baseline was calculated for each group.

Change in Urinary and Plasma Electrolytes
In addition to baseline and post-treatment urine collection, one blood sample was taken prior to dosing and another prior to euthanasia to examine
electrolyte concentrations. Millimolar electrolyte (sodium and potassium) concentrations were evaluated using an EasyLyte analyzer (Medica Corporation, Bedford, MA, USA). Electrolyte excretion rates were determined using concentration (mM) detected in urine, urine volume (mL), and collection duration (hr). Rates were normalized by 100 g B.W. and presented as mean percent change from baseline.

**Kidney Histopathological Assessment**
A board certified pathologist (blinded to treatment groups) evaluated paraffin embedded, hematoxylin and eosin stained kidney sections. Each section was given a score ranging from 0 to 3 in terms of tubular dilatation (normal, mild, moderate, or severe) and necrosis (0, 10, 25, > 25%).

**Gastrointestinal Inflammatory Factors**

**Gastric and Intestinal PGE$_2$**
An enzyme-linked immunosorbsent assay (ELISA) kit for detecting picograms per milliliter concentrations of PGE, was purchased from Antibodies-Online Inc. (Atlanta, GA, USA). The assay was carried out in accord with manufacturer’s instructions using homogenized gastric and duodenal tissue then assayed using MyAssays software (MyAssays Ltd, Sussex, UK).

**Intestinal MPO**
Levels of myeloperoxidase (MPO) were measured with an ELISA kit from Kamiya Biomedical Company (Seattle, WA, USA). Homogenized samples were assayed following manufacturer’s instructions with ng per mL reported. The standard curve used was determined using MyAssays software.

**Chromatographic Conditions**

**Analysis Equipment and Solution Preparation**
HPLC was orchestrated on a Shimadzu system (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) fitted with a LC20AB solvent delivery system coupled with a DGU-20A Prominance degasser. A SIL-20A HT auto sampler was used to load samples which then flowed through a CTO-20A column oven (C18 column). Drug signal was observed using a SPD-M20A diode array detector (280 nm) and the system was controlled using a CBM-20A communication bus module. DICLO concentrations were determined by a method described previously 10. Using a mobile phase consisting of acetonitrile, water, and acetic acid (50:50:0.25) and a flow rate of 0.75 mL/min, chromatographic separation of DICLO was accomplished. One hundred microliters of DICLO in methanol (ranging from 50 to 50,000 ng/mL) and 50 microliters of flufenamic acid (10,000 ng/mL) in acetonitrile, the internal standard, were added to the blank plasma. Following the addition of 2 mL of acetonitrile, samples were vortexed (30 sec) then centrifuged for 15 minutes (2,500 g). A Labconco CentriVap Concentrator (Kansas City, MO, USA) was used to evaporate the collected organic phase. Samples were reconstituted with 200 µL mobile phase; then 115 µL was transferred to injection vials in order to inject 100 µL. Signal height ratios were used to quantify drug concentration. This assay had a lower limit of detection of 50 ng/mL and a lower limit of quantitation of 100 ng/mL with a coefficient of variation of 2.67%.

**Pharmacokinetic Analysis**
Nine-time points (0, 0.5, 1, 2, 4, 6, 8, 12, and 24 hrs) were examined in the pharmacokinetic blood sampling scheme; however, DICLO was undetectable at time points 12 and 24 in both formulations. Various pharmacokinetic parameters, half-life ($t_{1/2}$), maximum plasma concentration ($C_{max}$), area under the plasma concentration-time curve from time zero to infinity (AUC$_{0-\infty}$), apparent oral clearance (CL$_{oral}$), and apparent volume of distribution (Vd/F), were calculated using the non-compartment component of Phoenix WinNonlin 6.3 (Certara USA, Inc., Princeton, NJ, USA). In the absence of a calculable elimination phase rate constant, rats were removed from the analysis.

**Data Treatment and Statistical Analysis**
The mean percent change from baseline, ($\text{post-treatment} - \text{baseline})/\text{baseline} \times 100$, and standard error of the mean (SEM) were calculated for the rate of urine flow and electrolyte excretion along with plasma electrolyte concentrations. All data, unless otherwise stated, are presented as mean ± SEM. One-way ANOVA through PROC GLM in SAS (SAS Institute Inc., Cary, NC, USA) was used for gastrointestinal and urinary parameter analysis. Pharmacokinetic parameters were analyzed using Student’s $t$-test. IBM SPSS Statistics software version 21 (Armonk, NY, USA) was used to identify outliers in each data set apart.
from histological examination. Significance in this study was set at \( p < 0.05 \).

Kruskal-Wallis one-way analysis utilizing pairwise comparisons, post hoc testing which detected minimal significant difference between groups, was conducted on histological values\(^\text{11}\). The tabulated familywise error rate (significance at 0.05 and adjusted for sample size) was used for comparisons of the mean-of-ranks differences. Using a “z” of 2.576, five comparisons were made. Three outliers, two in dilatation and one in necrosis, were removed prior to statistical analysis.

Results

Characteristics of Diclofenac-loaded PLGA-NPs

The DICLO-NP formulation (\( n = 3 \)) for this study presented with a mean diameter of 221.03 ± 1.71 nm and a drug entrapment efficiency of 76.38 ± 0.33%. The zeta-sizer also indicated a mean zeta potential of 20.86 ± 0.47 mV.

Renal Function Assessments

Mean percent changes (compared to baseline) in urine flow rate did not attain to significance (Figure 1; \( p = 0.2790 \)). Urinary sodium excretion rate mean percent changes (Figure 2) presented with significant differences among groups (\( p = 0.00002 \)). The NP (-52.18 ± 6.14%; \( p < 0.0001 \)), DICLO (-53.55 ± 6.39%; \( p < 0.0001 \)), and DICLO-NP (-49.40 ± 11.98%; \( p = 0.0001 \)) groups were each significantly decreased compared to VEH (17.82 ± 3.14%). While, as seen in Figure 3, the mean percent changes in urinary potassium excretion rate showed no significant difference (\( p = 0.1185 \)) among groups. No significant difference was found in either sodium (\( p = 0.2638 \)) or potassium (\( p = 0.0929 \)) plasma concentration mean percent change values among treatment groups, Figures 4 and 5, respectively.

Histopathological Assessments

Sections from the VEH group (Figure 6-A) showed mild tubular dilation in every kidney sampled; however, no necrosis was observed in any kidney from the VEH group. The NP group (Figure 6-B) showed tubular dilatation ranging from mild to moderate with necrosis ranging from none to moderate. Represented in Figure 6-C, sections from the DICLO group showed tubular dilatation and necrosis ranging from mild to severe. Finally, the DICLO-NP group, as shown in Figure 6-D, presented moderate tubular dilatation and mild necrosis in every kidney. Overall, the DICLO group had significantly more necrosis compared to VEH; while DICLO-NP group did not differ significantly from the VEH.

Specific histological mean-rank scores are enumerated in Table I. Statistical analysis of

![Figure 1. Mean Percent Change in Urine Flow Rate. Mean percent change from baseline of urine flow rate in groups treated with vehicle (VEH; \( n = 6 \)), nanoparticles (NP; \( n = 6 \)), diclofenac (DICLO; \( n = 6 \)), or diclofenac-loaded nanoparticles (DICLO-NP; \( n = 6 \)). The values are expressed as percent change ± standard error of the mean. The values were not significantly different, \( p \geq 0.05 \).](image-url)
the histology analysis demonstrated a significant difference, tie-adjusted H score 9.60 (k = 4, tabulated Chi Square = 7.82), among the treatment groups for renal dilation. Post hoc two group comparisons of the VEH group (mean-rank 6.0) to the NP group (mean-rank 11.3), DICLO (mean-rank 13.9), or DICLO-NP group (mean-rank 16.5) demonstrated no significant differences. Additionally, no differences were observed in the comparison of the DICLO group to the

Figure 2. Mean Percent Change in Sodium Excretion Rate. Mean percent change from baseline of sodium excretion rates in groups treated with vehicle (VEH; n = 3), nanoparticles (NP; n = 4), diclofenac (DICLO; n = 6), or diclofenac-loaded nanoparticles (DICLO-NP; n = 4). The values are expressed as percent change ± standard error of the mean. *p < 0.05, significantly different from VEH.

Figure 3. Mean Percent Change in Potassium Excretion Rate. Mean percent change from baseline of potassium excretion rates in groups treated with vehicle (VEH; n = 2), nanoparticles (NP; n = 5), diclofenac (DICLO; n = 5), or diclofenac-loaded nanoparticles (DICLO-NP; n = 6). The values are expressed as percent change ± standard error of the mean. The values were not significantly different, p ≥ 0.05.
DICLO-NP group (mean ranks 13.9 vs. 16.5), or NP group to the DICLO-NP group (mean ranks 11.3 vs. 16.5).

For the renal necrosis histology analysis, again there was a tie-adjusted H score of 12.05 that was significant (k = 4, tabulated = 7.82). Post hoc group comparisons of the VEH group (mean-rank 5.0) to the NP (mean-rank 11.9) and DICLO-NP (mean-rank 14.5) groups demonstrated no significant differences. However, there was a significant difference between the VEH group (mean-rank 5.0) and the DICLO (mean-rank 17.0) in the two group comparison.

**Gastrointestinal Inflammatory Factors**

The differences in gastric PGE$_2$ (Figure 7) among groups did not reach significance ($p = 0.5345$). As shown in Figure 8, no significant
changes were noted in the groups when comparing intestinal PGE$_2$ concentrations ($p = 0.9963$). There was also no notable difference (Figure 9) when comparisons were made within the treatment groups in regard to intestinal MPO ($p = 0.2623$).

**Pharmacokinetics of Diclofenac**

The plasma concentration time curves for both formulations are given in Figure 10, while pharmacokinetic parameters are presented in Table II. The $t_{1/2}$ ($p = 0.2395$), $C_{max}$ ($p = 0.9134$), AUC$_{0-\infty}$ ($p = 0.7258$), CL$_{oral}$ ($p = 0.6650$), and Vd/F ($p = 0.5331$) were not significantly changed between formulations.

**Discussion**

The use of NSAIDs is limited by two major side effects: gastrointestinal erosion and diminished renal function. Nanoparticles are a group of colloidal drug delivery systems which may be used to alter drug side effects. By performing analysis of renal and gastrointestinal parameters, and pharmacokinetics, we compared DICLO and DICLO-NP formulations.

Urine flow rate did not differ significantly among groups (Figure 1). This result is supported by a previous study in which urine flow rate was unchanged in rats 24 hrs following a 30 mg/kg dose. In that study, no significant change from baseline occurred until the fourth day of dosing. Sodium excretion rate exhibited notable differences among NP, DICLO, and DICLO-NP when compared to VEH (Figure 2). The 30 mg/kg DICLO dose from a previous study also produced a decrease in sodium excretion at 24 hrs post-dose. As the effect of DICLO and DICLO-NP did not vary from each other, this parameter may not be altered by nanoformulation. As displayed in Figure 3, potassium excretion rate demonstrated no significant changes. Similarly, no change was seen in the 30 mg/kg dose study at 24 hrs either. For this study, electrolytes were analyzed after only one day of exposure while the study

Table I. Histopathological assessment of tubular dilatation and necrosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>n</th>
<th>Mean-Rank</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>n</th>
<th>Mean-Rank</th>
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<tbody>
<tr>
<td>VEH</td>
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<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
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<td>0</td>
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<td>11.3</td>
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<td>1</td>
<td>2</td>
<td>0</td>
<td>6</td>
<td>11.9</td>
</tr>
<tr>
<td>DICLO</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>13.9</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>17.0*</td>
</tr>
<tr>
<td>DICLO-NP</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>16.5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>14.5</td>
</tr>
</tbody>
</table>

Tubular dilatation and necrosis scores in the groups treated with methylcellulose (VEH), empty nanoparticles (NP), diclofenac (DICLO), diclofenac-loaded nanoparticles (DICLO-NP). *p < 0.05, significantly different from VEH.
mentioned collected electrolyte rates following up to four days of exposure at which point significant change was detected. Therefore, these results coincide with research that, like other NSAIDs, DICLO-LO-associated side effects are dose-dependent and affected by exposure time\textsuperscript{15}. Short exposure and single dosing may have limited the observable effect of DICLO or DICLO-NP on electrolyte excretion rates in this study.

Neither sodium nor potassium plasma concentrations displayed any significant difference among groups as shown in Figure 4 and Figure 5. These results are supported by Stokes et al\textsuperscript{16} who found unaltered plasma electrolyte concentrations when utilizing DICLO (50 mg; given 3 times-a-day for 14 days) in patients with hypertension controlled by a diuretic and/or a beta blocker. The lack of change in plasma electrolytes concentrations could also be indicative of the body’s capacity of maintaining homeostasis of electrolytes and the work of hormone mediators.

Histological assessment demonstrated a significant difference between the treatment groups for renal necrosis as seen in Table I. Overall, the
DICLO group had significantly more necrosis compared to VEH; while the DICLO-NP group did not differ significantly from the VEH as shown in Figure 6. Renal papillary necrosis has been associated with the use of nonselective NSAIDs, such as DICLO\textsuperscript{17}. These results imply that DICLO administration amplified necrosis levels compared to VEH; while DICLO-NP did not at this dosage and interval of exposure.

There were no significant changes among groups in regard to gastric PGE\textsubscript{2}, intestinal PGE\textsubscript{2}, or intestinal MPO as seen in Figures 7, 8, and 9.

**Table II.** The pharmacokinetic parameters of diclofenac following a single oral dose of diclofenac (10 mg/kg) or a PLGA nanoparticle equivalent.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>n</th>
<th>t\textsubscript{1/2} (hr)</th>
<th>C\textsubscript{max} (µg/mL)</th>
<th>AUC\textsubscript{0-∞} (µg.h/mL)</th>
<th>Cl\textsubscript{app} (L/h/kg)</th>
<th>Vd/F (L/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DICLO</td>
<td>5</td>
<td>4.44 ± 0.58</td>
<td>1.25 ± 0.28</td>
<td>6.18 ± 0.78</td>
<td>1.75 ± 0.26</td>
<td>11.52 ± 2.54</td>
</tr>
<tr>
<td>DICLO-NP</td>
<td>5</td>
<td>6.36 ± 1.40</td>
<td>1.22 ± 0.16</td>
<td>6.55 ± 0.65</td>
<td>1.60 ± 0.19</td>
<td>13.78 ± 2.37</td>
</tr>
</tbody>
</table>

DICLO-diclofenac; DICLO-NP-diclofenac-loaded nanoparticles. Values expressed as mean ± standard error of the mean. Values were not significantly different, \( p \geq 0.05 \).
respectively. In another study, DICLO (50 mg/kg with six hours of exposure) treatment reduced PGE2 gastric production and exhibited the maximum rating of gastric lesions in rats\(^9\). In the same study, statistically significant increases in gastric MPO were detected after DICLO was administered. As found by Fornai et al\(^9\), fourteen days of DICLO (8 mg/kg), given daily reduced intestinal PGE2 levels in rats. Additionally, DICLO increased MPO levels in both the jejunum and ileum. Our results do not correspond with these outcomes possibly due to differences in treatment duration and dosing level. This may explain why the PGE2 and MPO level changes were nonsignificant among groups in our study.

The pharmacokinetics of DICLO-NP showed no significant changes in any of the measures recorded (Figure 10). In a study conducted by Manvelian et al\(^9\), a proprietary nanoformulation of DICLO (35 mg) yielded an AUC\(_{0-\infty}\) similar to a 50 mg dose in humans under fasted and fed conditions. The change in bioavailability is difficult to determine because different doses were compared. Other studies\(^11,22\), examining DICLO ophthalmic nanoformulations (NP or nano-composite) in rabbits, have found increased bioavailability. This suggests that increased bioavailability is possible; however, in our study, this particular PLGA-NP formulation of DICLO did not change the systemic exposure of the drug.

**Conclusions**

In this work, nanoformulation of DICLO did not significantly alter expected DICLO-associated effects or systemic exposure; however, there was a reduction in renal necrosis. Altogether, the DICLO-NP dosage form appears to be an improvement upon the typical formulation, DICLO.

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**Conflicts of interest**

The authors declare no conflicts of interest.

**References**

Effect of PLGA diclofenac nanoparticles in rats


