Online Supplement to:

Penetration of Topical Diclofenac into Synovial Tissue and Fluid of Osteoarthritic Knees: a Multicenter, Randomized, Placebo-Controlled, Pharmacokinetic Study

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Bioanalytical Methodology

Blood samples were centrifuged at 1500 G at 4°C for 10 minutes, and the isolated plasma was frozen and shipped to the bioanalytical laboratory. Synovial tissue and fluid samples were retrieved during surgery, frozen immediately, and shipped to the bioanalytical laboratory. Bioanalysis was performed by FARMOV S (Pty) Ltd (Bloemfontein, South Africa) for analysis of diclofenac concentrations in synovial tissue, synovial fluid, and plasma, and by Synexa Life Sciences, Ltd (Cape Town, South Africa) for prostaglandin E₂ (PGE₂), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα) levels in synovial tissue and fluid.

For analysis of diclofenac concentrations, synovial tissue samples were pulverized and ground to a fine powder in liquid nitrogen. The powdered samples were transferred to microfuge tubes with methanol and metal balls, vortexed and
homogenized twice using a Bead Blaster™ 24 (Benchmark Scientific, Sayreville, NJ), and centrifuged. The supernatants were stored at −70°C until sample preparation.

Diclofenac concentrations in synovial tissue, synovial fluid, and plasma were assayed by high-performance liquid chromatography tandem mass spectrometry, using diclofenac-d4 as the internal standard. Liquid-liquid extraction was performed with a mixture of hexane and dichloromethane as the extraction solvent for synovial tissue and fluid samples, and hexane and ethyl acetate for plasma samples. The extracts were dried under nitrogen and reconstituted in a solution mixture of acetonitrile and ammonium acetate. The sample extracts were injected into a chromatography system equipped with an autosampler and an Agilent Zorbax Eclipse XDB-C18 (150 x 4.6 mm) 5 µm analytical column. The autosampler was fitted with a cooling device to keep the samples at ~5°C. Mobile phase (acetonitrile and ammonium acetate) was delivered isocratically. Multiple reaction monitoring was done with a Sciex API5500 mass spectrometer (Sciex, Framingham, MA), with electrospray ionization in negative mode. Mass-to-charge ratios (m/z) in unit resolution was set at 293.9 ± 0.1 for diclofenac deprotonated precursor ion and 249.8 ± 0.1 for product ion were used; m/z for the internal standard diclofenac-d4 was 299.5 ± 0.6 and 255.5 ± 0.6 for deprotonated precursor and product ions, respectively.

Data collection and analysis were performed using Analyst® version 1.6.2 (Sciex) and Watson LIMS™ version 7.4.2 software (ThermoFisher Scientific, Waltham, MA). The lower limit of quantitation was 0.23 ng/g in synovial tissue, 0.10 ng/mL in synovial fluid, and 0.098 ng/mL in plasma. Over 3 consecutive validation runs, between-run accuracy had to be within 15% over the range (and within 20% at the lower limit of quantitation),
and between-run precision had to be ≤15% (20% at the lower limit of quantitation). Across the range of quantitation, the percent coefficient of variation (%CV) ranged from 0.1% to 2.9% in synovial tissue, 0.9% to 5.5% in synovial fluid, and 0.9% to 2.9% in plasma; the percent bias was −0.9% to 1%, −1.7% to 3.0%, and −9.3% to 4.8% in synovial tissue, synovial fluid, and plasma, respectively.

PGE₂ concentrations in synovial tissue and fluid were measured using an enzyme-linked immunosorbent assay (ELISA). Following dilution, samples were analyzed using a PGE₂ multiformat enzyme immunoassay kit (DetectX®; Arbor Assays, Ann Arbor, MI) and BioTek® Gen5 version 1.11.5 software (BioTek, Winooski, VT). The lower limit of quantification was 2.62 ng/mL in synovial tissue and 0.013 ng/mL in synovial fluid. Percent relative error (%RE) for PGE₂ detection was −3.2% to 3.2%; %CV was 2.9% to 11.2%.

IL-6 and TNFα were measured with an electrochemiluminescence assay with a Meso Scale Discovery (MSD) Sector Imager 6000 electrochemiluminescence reader (Meso Scale Diagnostics, Rockville, MD). Following dilution, samples were analyzed using a V-PLEX human cytokine multiplex proinflammatory panel (excluding proinflammatory markers other than IL-6 and TNFα). Microplate wells were coated with antibodies against IL-6 and TNFα before the samples were added; bound analytes (IL-6 and TNFα) were then detected with SULFO-Tag conjugated antibodies. MSD Read buffer was added, and electricity was applied to plate electrodes via the MSD Sector Imager leading to light emission. Analysis was done using MSD’s Discovery Workbench version V4.0.12.1 software. The range of quantification was 3.16–976.00 pg/mL for IL-6 and 1.38 to 496.00 pg/mL for TNFα in both synovial tissue and fluid. Intra-assay
accuracy (%bias) and precision (%CV) for IL-6 and TNFα were both 20% (25% upper and lower limit of quantification).
### Supplemental Table S1. Complete Inclusion and Exclusion Criteria

| Inclusion Criteria                                                                 | Exclusion Criteria                                                                 |
|-----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| • Written informed consent provided                                               | • Study site personnel involved in the study conduct and their family members,     |
| • Men and women ≥50 years of age at screening                                      | study site staff otherwise supervised by the investigator, or employees of the     |
| • Diagnosis of knee OA with radiographic evidence within the last 6 months         | Sponsor who were directly involved with the study                                   |
| • Kellgren-Lawrence Grade ≥2 and scheduled unilateral knee arthroplasty            | • Participation in a study involving an investigational drug within 1 month prior to|
| • Good general physical health and deemed fit for surgery by the investigator,     | entry or during study participation                                                |
| • no clinically relevant abnormalities identified by medical history, physical     | • Acute or chronic medical or psychiatric condition or laboratory abnormality that |
|   exam including vital signs, 12-lead ECG, and laboratory testing                   | could increase the risk associated with the study or the investigational product   |
| • Body mass index 17.5 to <40 kg/m² and total body weight >50 kg                    | or interfere with interpretation of the study results                              |
| • Willingness to comply with scheduled visits, treatment plan, laboratory testing  | • Pregnancy or breastfeeding                                                       |
| • Women of childbearing potential and at risk for pregnancy had to agree to use    | • Known or suspected intolerance or hypersensitivity to the study materials or     |
|   highly effective contraception throughout the study for ≥21 days after the last   | their ingredients                                                                   |
|   dose of study treatment                                                          | • History of asthma, angioedema, urticaria, or acute rhinitis precipitated by      |
| • Positive urine drug screen during screening (day -7)                              | acetylsalicylic acid or other NSAIDs                                                |
| • Any condition possibly affecting drug absorption (eg, gastrectomy)               | • Broken or diseased skin, skin wound, or other open injury around the knee        |
| • History of regular alcohol consumption >14 drinks/week within 6 months of        | • Inability or unwillingness to comply with lifestyle guidelines or investigator    |
|   screening                                                                        | instructions                                                                       |
| • Previous enrollment in this study                                                | • Use of one of the prohibited treatments                                          |
| Prohibited Treatments |
|-----------------------|
| • Prescription or nonprescription drugs (unless deemed necessary by investigator), NSAIDs, COX-2 inhibitors, or dietary supplements within 7 days or 5 half-lives (whichever was longer) prior to first dose of study treatment and during the study.  
  o Participants had to be willing to avoid use of topical or systemic analgesics or anti-inflammatory treatments other than study medication and rescue medication(s) during the washout period and treatment period |
| • Any intra-articular or peri-articular procedures or injections in either knee within the previous 3 months |
| • Any systemic treatment with corticosteroids within the previous 6 weeks (topical corticosteroids applied to sites other than the knees were permitted up to screening visit only) |
| • Any chondroprotectant or disease-modifying OA drugs (eg, glucosamine or chondroitin sulfate) unless dose was stable over the month prior to screening and would be maintained throughout the study |
| • Any systemic anti-inflammatory or analgesic drugs at screening if 5 times their elimination half-life exceeded 7 days (ie, half-life was >33.6 hours) |
| • Anticoagulants (eg, warfarin, heparin) in the week prior to screening or anti-aggregants within the month prior to screening with the exception of anticoagulant therapy for surgery and aspirin at stable low doses started at least 1 month before randomization and maintained at a stable dose throughout the study |
| • Any other investigational drugs within the month prior to screening or 5 half-lives before the first dose of study medication (whichever was longer) |
Supplemental Table S2. Significant Protocol Deviations, All Randomized Participants

| Deviation                                                                 | Diclofenac Diethylamine 2.32% w/w Gel (N=30), n (%) | Placebo Gel (N=17), n (%) |
|---------------------------------------------------------------------------|------------------------------------------------------|---------------------------|
| Any significant protocol deviations                                        | 30 (100)                                             | 17 (100)                  |
| Inadequate/inappropriate collection, handling, processing, or storing of study samples | 26 (86.7)                                             | 13 (76.5)                 |
| Incorrect storage of study treatment                                       | 12 (40.0)                                             | 6 (35.3)                  |
| Inadequate execution, completion, or documentation of informed consenta   | 11 (36.7)                                             | 6 (35.3)                  |
| Study procedure performed by staff member not appropriately delegated      | 9 (30.0)                                              | 5 (29.4)                  |
| NSAIDs and corticosteroids administered between screening and collection of synovial samples | 5 (16.7)                                              | 3 (17.6)                  |
| Failure to maintain subject’s confidentiality information                   | 3 (10.0)                                              | 0                         |
| Exclusion criteria met, but participant was still enrolled and provided samples | 3 (10.0)                                              | 0                         |
| One or more investigational product doses was missed, including incorrect administration | 1 (3.3)                                               | 2 (11.8)                  |
| Rescue medical misuse                                                     | 1 (3.3)                                               | 0                         |
| Participant was incorrectly randomized                                     | 1 (3.3)                                               | 0                         |

aA majority of the consent-related protocol deviations (9/11 in the diclofenac group and 5/6 in the placebo group) were the result of informed consent being executed by a site staff member who was not adequately qualified, based on the site delegation of duties and signature log.
### Supplemental Table S3. PGE$_2$, IL-6, and TNF$_\alpha$ Levels in Synovial Tissue and Fluid, Analysis Population

|                        | Synovial Tissue Concentration | Synovial Fluid Concentration |
|------------------------|-------------------------------|-----------------------------|
|                        | Diclofenac Diethylamine 2.32% w/w Gel (N=29) | Placebo Gel (N=16) | Diclofenac Diethylamine 2.32% w/w Gel (N=29) | Placebo Gel (N=16) |
| **PGE$_2$, ng/mL**     |                               |                             |                               |                             |
| Number (%) with quantifiable level$^a$ | 28 (96.6%) | 16 (100%) | 29 (100%) | 16 (100%) |
| Median                 | 36.74                        | 46.25                       | 0.08                           | 0.08                         |
| Range                  | 1.3–576.4                    | 2.3–273.7                   | 0.02–3.20                      | 0.04–1.24                    |
| Geometric mean$^b$     | 32.35                        | 28.26                       | 0.09                           | 0.13                         |
| 95% CI                 | 16.37, 63.92                 | 11.30, 70.69                | 0.062, 0.135                   | 0.076, 0.219                 |
| Ratio of LS Means (95% CI) $P$ value$^c$ | 1.14 (0.37–3.59) $P=0.8123$ | 0.71 (0.37–1.37) $P=0.2945$ |
| **IL-6, pg/mL**        |                               |                             |                               |                             |
| Number (%) with quantifiable level$^a$ | 1 (3.4%)                    | 0                           | 24 (82.8%)                     | 16 (100%)                    |
| Median                 | NC                           | NC                          | 11.23                          | 15.43                        |
| Range                  | NC                           | NC                          | 1.58–884.82                    | 5.48–472.62                  |
| Geometric mean$^b$     | NC                           | NC                          | 13.99                          | 24.28                        |
| 95% CI                 | NC                           | NC                          | 7.95, 24.62                    | 11.35, 51.97                 |
| Ratio of LS Means (95% CI) $P$ value$^c$ | NC                           | 0.58 (0.22–1.49) $P=0.2473$ |
| **TNF$_\alpha$, pg/mL** |                               |                             |                               |                             |
| Number (%) with quantifiable level$^a$ | 0                           | 0                           | 10 (34.5%)                     | 4 (25.0%)                    |
| Median                 | NC                           | NC                          | 0.69                           | 0.69                         |
| Range                  | NC                           | NC                          | 0.69–5.38                      | 0.69–1.91                    |
| Geometric mean$^b$     | NC                           | NC                          | 1.00                           | 0.87                         |
| 95% CI                 | NC                           | NC                          | 0.82, 1.21                     | 0.67, 1.13                   |
| Ratio of LS Means (95% CI) $P$ value$^c$ | NC                           | 1.15 (0.83–1.58) $P=0.3986$ |

$^a$Levels below the limit of quantification (LOQ) were replaced by LOQ/2. PGE$_2$ LOQ = 2.62 ng/mL in synovial tissue and 0.013 ng/mL in synovial fluid. IL-6 LOQ = 3.16 pg/mL in both synovial tissue and fluid. TNF$_\alpha$ LOQ = 1.38 pg/mL in synovial tissue and fluid. However, for 5 participants (diclofenac n=3; placebo n=2) with synovial tissue levels of PGE$_2$ <LOQ, the laboratory was able to quantify PGE$_2$ using altered dilution factors, so these quantified values were used rather than LOQ/2. In addition, 1 participant in the
diclofenac group had PGE2 >LOQ in synovial fluid but the value was not quantifiable due to insufficient volume for dilution; this patient's PGE2 concentration was set to the upper LOQ (3.20 ng/mL).

bBased on \( t \)-test using log transformed data; results are back transformed into original scale for the geometric mean and its 95% CI.

cDifference between diclofenac and placebo concentrations based on \( t \)-test; \( P \) values are two sided using 0.05 level of significance.

CI, confidence interval; IL-6, interleukin-6; LS, least squares; NC, not calculable; PGE\(_2\), prostaglandin E\(_2\); TNF\(\alpha\), tumor necrosis factor alpha.