CZ415, a highly selective mTOR inhibitor showing *in vivo* efficacy in a collagen induced arthritis (CIA) model

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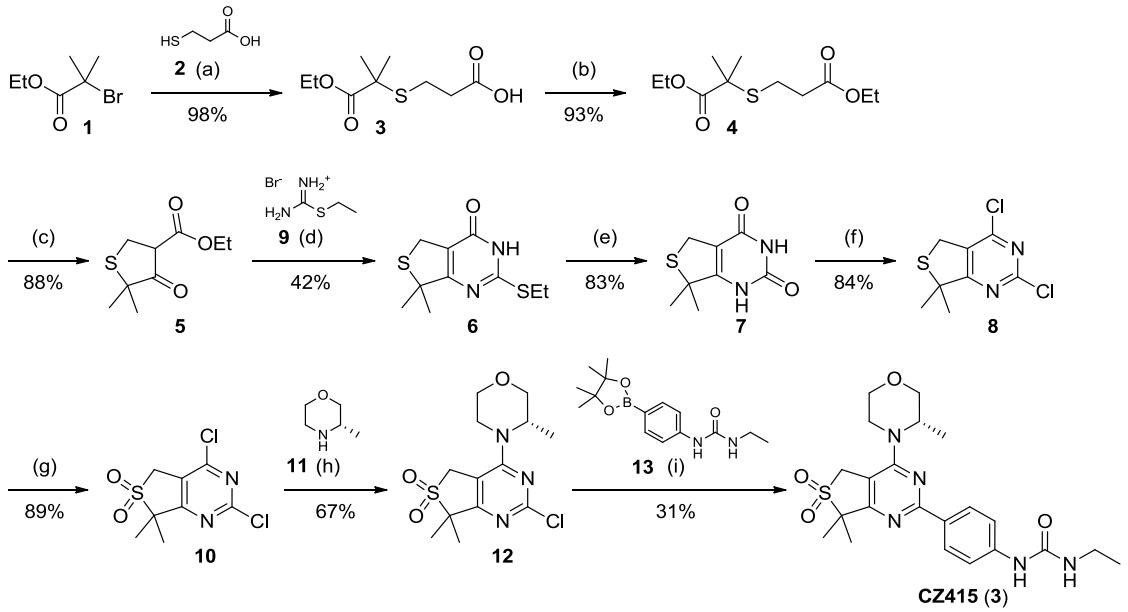
Supporting Information

General remarks:

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.
All animal studies were ethically reviewed and carried out in accordance with European legislation and guidelines for animal welfare as well as the Cellzome ethical policy on the care, welfare and treatment of animals.

Synthesis and analytical data for CZ415 (3):

The synthesis of **3** has been reported before and is outlined in **Scheme 1**.[1]
In brief, the dicarboxylate **4** was synthesized in excellent yields from bromide **1** and thiol **2** via nucleophilic substitution followed by esterification. The linear precursor **4** was then cyclized in a two step reaction into fused pyrimidine-dione **7** following a method that had been developed by Butini for a non-substituted analogue.[2] Through chlorination and subsequent oxidation, sulfone **10** was then obtained in very good yields. A regioselective nucleophilic aromatic substitution of **10** with morpholine **11** gave pyrimidine **12**, which could finally be converted into **3** utilizing Suzuki-Miyaura coupling. For structurally related pyrimidines, fused to either non-substituted or 6-membered cyclic sulfones, similar routes have been described, albeit for those oxidation of the core was carried out after introduction of the first pyrimidine substituent.[3,4]

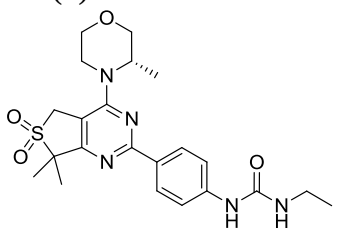


Scheme 1. Synthesis of **3**. (a) NaOH, H_2O/EtOH; (b) EtOH, reflux; (c) NaOEt, Et_2O, reflux; (d) Na_2CO_3, 40°C; (e) HCl, AcOH, H_2O, reflux; (f) $PhPOCl_2$, 135°C, then 165°C; (g) *m*CPBA, CH_2Cl_2; (h) NEt_3, DMF, 0°C to rt; (i) Na_2CO_3, $PdCl_2$(dppf), DME/H_2O, 70°C.

General remarks analytics: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz) using DMSO-d_6 as solvent. Chemical shifts are given in parts per million (ppm) (δ relative to residual solvent peak for ^{1}H and ^{13}C). LC MS analysis was performed on an Agilent Technologies 1200 series with a Venusil XBP-C18 2.1×50mm, 5µm

column. The analysis was performed at a flow rate of 0.8 mL/min with a linear gradient over 3 min (99% (water/0.04% TFA) to 90% (acetonitrile/0.02% TFA)). HPLC analyses for determination of purity were performed on the same instrument with a Phenomenex Gemini NX C18, 150×4.6 mm, 5 µm column: The analyses were performed at a flow rate of 1 mL/min with a linear gradient over 11 min (Method A: 5 to 95% acetonitrile in water, 0.1% formic acid as modifier. Method B: 5 to 95% acetonitrile in water, 0.1% ammonia as modifier).

(*S*)-1-(4-(7,7-dimethyl-4-(3-methylmorpholino)-6,6-dioxido-5,7-dihydrothieno[3,4-d]pyrimidin-2-yl)phenyl)-3-ethylurea (**3**):



^{1}H NMR (400 MHz, DMSO-d_6) δ 8.71 (s, 1H), 8.26–8.17 (m, 2H), 7.55–7.46 (m, 2H), 6.17 (t, *J* = 5.6 Hz, 1H), 4.71 (d, *J* = 15.4 Hz, 1H), 4.61 (d, *J* = 15.4 Hz, 1H), 4.47 (q, *J* = 7.1 Hz, 1H), 3.99–3.86 (m, 2H), 3.71–3.65 (m, 2H), 3.54 (td, *J* = 11.5, 2.5 Hz, 1H), 3.42 (td, *J* = 12.5, 11.9, 3.4 Hz, 1H), 3.12 (qd, *J* = 7.2, 5.5 Hz, 2H), 1.55 (d, *J* = 1.5 Hz, 6H), 1.30 (d, *J* = 6.7 Hz, 3H), 1.06 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.6 (quart.), 161.7 (quart.), 158.9 (quart.), 154.82 (quart.), 143.2 (quart.), 129.6 (quart.), 128.6 (+), 116.9 (+), 104.1 (quart.), 70.3 (–), 66.3 (–), 61.6 (quart.), 51.0 (–), 48.2 (+), 40.8 (–), 34.0 (–), 20.1 (+), 19.8 (+), 15.4 (+), 14.3 (+). LC MS *m/z* 460 $[M+H]^+$ (Rt = 2.90 min). HPLC (Method A) Rt = 9.0 min, 99% purity ($UV_{254/210\ nm}$). HPLC (Method B) Rt = 8.9 min, 99% purity ($UV_{254/210\ nm}$).

Physicochemical properties of 3:

Physicochemical properties have been discussed in the main article. A summary table is given below.

Table 1 Physicochemical properties of **3**.

Property	Value
MW	460 Da
PSA	114 Å^2
pKa	2.33
log$D_{pH7.4}$	4.31
Ligand efficiency	0.36
Solubility	149.5 µM (CLND)
	11.1 µg/mL (FaSSIF)
	60.1 µg/mL (FeSSIF)
Permeability	210 nm/s (PAMPA)
	0.47 nm/s (Papp (A-B), Caco-2)
	1.02 nm/s (Papp (B-A), Caco-2)

Metabolic stability in human hepatocytes and microsomes of CZC415:

Metabolic stability of **3** was investigated (Cyprotex) in pooled rat, dog and human liver microsomes. Microsomes (0.5 mg/mL), 0.1 M phosphate buffer pH 7.4 and **3** (1 µM; final DMSO concentration 0.25%) were pre-incubated at 37°C prior to the addition of NADPH (1mM) to initiate the reaction. The final incubation volume was 25µL. A control incubation was included for each compound tested where 0.1 M phosphate buffer pH 7.4 was added instead of NADPH (minus NADPH). All incubations were performed as replicates. Compound **3** was incubated for 0, 5, 10, 20, and 40 min. The control (minus NADPH) was incubated for 40 min only. The reactions were stopped by the addition of 50 µL methanol containing internal standard at the appropriate time points. Following protein precipitation, the sample supernatants were then analyzed by LC-MS/MS.

The intrinsic clearance per g liver was calculated from microsomal clearance measured using reported scaling factors:[5,6]

Table 2. Clearance per g liver calculated from metabolic stability.

	Rat	**Dog**	**Human**
Cl_{int} (µL/min/µg protein)	<14	<14	38 (n=2, average)
Scaling factor (mg protein/g liver)	44.8 [5]	77.9 [5]	40 [6]
Cl_{int} (mL/min/g liver)	<0.6	<1.1	1.5

Metabolic stability of **3** was investigated (Cyprotex) in pooled, cryopreserved dog and human hepatocytes. Hepatocytes (cell density of 0.5×10^6 viable cells/mL) and **3** (1 µM; final DMSO concentration 0.25%) were incubated at 37°C. Control incubations were also performed in the absence of cells to reveal any non-enzymatic degradation. Duplicate samples (50µL) were removed from the incubation mixture at 0, 10, 20, 45, 60 and 90 min (control sample at 90 min only) and added to methanol (100µL, containing internal standard) to stop the reaction. Following centrifugation, the supernatants were then analyzed by LC-MS/MS.

The intrinsic clearance per g liver was calculated from clearance in hepatocytes using reported hepatocellularity:[6,7]

Table 3. Clearance per g liver calculated from stability in hepatocytes.

	Rat	**Dog**	**Human**
Cl_{int} (µL/min/million cell)	n.d.	<3.5	≤5
Scaling factor (cells/g liver)	-	215×10^6 [7]	99×10^6 [6]
Cl_{int} (mL/min/g liver)	-	0.8	≤0.50

MS-based selectivity profiling (competition binding assays):

Experiments were performed using a quantitative strategy based on isobaric mass tags, essentially as described previously for *Kinobeads*[8] and a mixed inhibitor lipid kinase matrix.[9] LC-MS-analysis were performed as described.[10,11]

Protein fold changes are reported on the basis of tandem mass tag reporter ion areas relative to vehicle control and were calculated using a sum-based bootstrap algorithm.[8,12] Fold changes were corrected for isotope purity and adjusted for interference caused by coeluting nearly isobaric peaks as estimated by the signal-to-interference measure.[13,14]

Compound **3** was tested in 10 point dose response (10 µM start, 1:5 dilution) on *Kinobeads* using a mixed HEK293/K-562/Placenta lysate and in 8 point dose response (10 µM start, 1:4 dilution) on the lipid kinase matrix using a mixed HeLa/Jurkat/K-562 lysate.
For determination of apparent dissociation constants (K_d^{app}), measured IC_{50} values were corrected for the influence of the immobilized ligand on the binding equilibrium using the Cheng-Prusoff equation.[15]
A summary data table including all proteins identified in both experiments is provided as a separate file.

Cellular assay monitoring phosphorylation of mTOR downstream targets:
Assessment of pS6RP and pAkt levels after treatment with **3** in HEK294T17 cells. MesoScaleDiscovery (MSD) assays K150DGD-3 (Phospho-S6RP (Ser240/244)) and K151CAD-3 (Phospho-Akt (Ser473)) were used as readout, PI-103 (Calbiochem, #528100) served as as positive control:

- Cells were seeded in 90 µL DMEM containing 2% FCS at $4x10^4$ cells/well (for pS6RP S240/244 assay) or $8x10^4$ cells/well (for pAKT S473 assay) in a 96well U-bottom plate. The plate was then incubated for 1h in a humidified incubator (37ºC, 5% CO_2) to allow cells to adhere
- Cells were treated in dose response with **3** or controls in 0.1% DMSO:
    - Compound **3**: 3 µM start, 8 points 1:3 dilution steps, n=2. Positive control: 1 µM PI-103 (n=8). Negative control: DMSO (n=8)
    - 10 µL of 10x compound concentration in 1% DMSO / 99% (DMEM 2% FCS) were added to the cells followed by 2 h incubation in a humidified incubator (37ºC, 5% CO_2)
- Cells were lysed by addition of 10 µL 5x Complete Lysis Buffer and gentle shaking at 4°C for 15 min

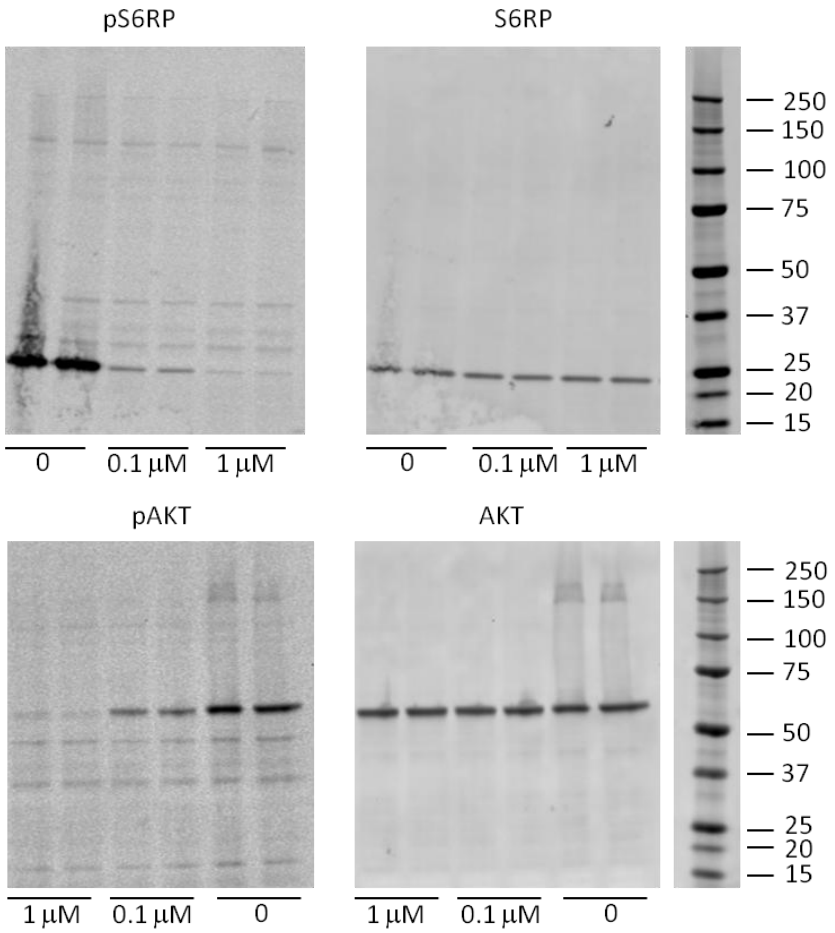
The readout was performed according to the instructions provided by the supplier, in details:

- MSD plate preparation: The MSD plate was incubated with Blocking Solution (150 µL per well) for 60 min at room temperature and then washed 4 times with 150 µL/well Tris Wash Buffer
- 25 µL/well cell lysate as prepared above were dispensed into the blocked MSD plate and incubated for 1 h at room temperature
- The plate was washed 4 times with with 150 µL/well Tris Wash Buffer
- 25 µL/well Detection antibody was added to the plate and incubated for 1 h at room temperature
- The plate was washed 4 times with with 150 µL/well Tris Wash Buffer
- 150 µL/well diluted Read Buffer T, with surfactant, were added and the plate was analyzed on a MSD Sector Imager 2400 plate reader

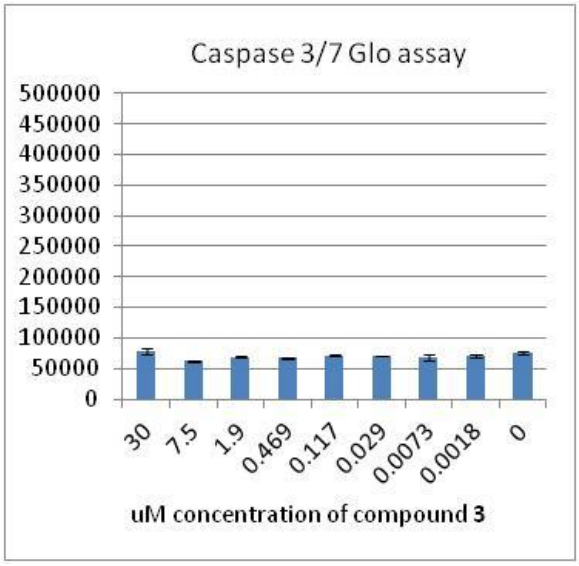
IC_{50} values were determined as average of 2 independent experiments.

Western Blot analysis of compound 3 effects on phosphorylation of S6RP and Akt:
HEK293 cells were incubated for 3 hours with 0.1 % DMSO vehicle and 0.1 and 1 µM concentrations of compound **3** (n=2). Following lysis, samples were separated on denaturing NuPAGE 4-12% SDS gels and then wet transferred onto Polyvinylidene fluoride (PVDF) membrane (Western blot). Membranes were incubated overnight with either rabbit-anti-phospho-S6 ribosomal protein (Cell signaling technology, CST, #2211L, Ser235/236) and mouse-anti-S6 ribosmal protein (CST #2317, monoclonal-within full length protein) or rabbit-

anti-phospho-AKT (CST #9271L, Ser473) and mouse-anti-AKT (BD Biosciences #610861, monoclonal). Following incubation with the respective secondary antibodies, IR dye 800CW donkey anti-rabbit (Licor #926-32213) and IR dye 680 RD donkey anti-mouse (Licor #926-68072), the membranes were scanned using an Odyssey Infrared imager.
Following treatment with **3**, a concentration dependent decrease of S6RP and AKT phosphorylation and corresponding stable levels of S6RP and AKT, was detected.



Compound 3 does not induce apoptosis:



HEK293 cells were incubated for 5 hours with different concentrations of compound **3**. Following lysis, the activity of Caspase 3/7 was measured with the ApoTox Glo assay (n=2) according to the manufacturer instructions (Promega) using Caspase-Glo®3/7 substrate and Caspase-Glo®3/7 buffer. The luminescence counts were measured with a Perkin Elmer Envision 2103 multilabel reader.
The luminescence detected in cells treated with compound **3** was comparable to the control sample of untreated cells indicating lack of Caspase 3/7 activity and therefore lack of apoptosis induced by **3** treatments.

Whole blood assay monitoring interferon gamma (IFNγ) release:

Assessment of IFNγ release after treatment with **3** upon αCD3/αCD28 and IL-2 stimulation in whole blood, using MSD Human IFN-γ Ultra-Sensitive kit K151AEC-2 as readout:

- Human whole blood was dilute 1.4x with RPMI medium and distributed into a 96well U-bottom plate (175 µl/well)
- The samples were treated in dose response with **3** or controls in 0.2% DMSO:
  - Compound **3**: 10 µM start, 10 points 1:3 dilution steps, n=2. Stimulated and unstimulated controls: DMSO (n=8)
  - 25 µL of 10x compound concentration in 2% DMSO / 98% RPMI were added followed by 1 h incubation in a humidified incubator (37ºC, 5% CO_2)
- The blood samples were then stimulated with αCD3/αCD28 and IL-2:
  - 50 µL 5x αCD3/αCD28 in RPMI (each at1 µg/mL final concentration) and 50 µL IL-2 in RPMI (10 ng/mL final concentration) were added, followed by 18 h incubation in a humidified incubator (37ºC, 5% CO_2)
  - 50 µL RPMI was added were added to unstimulated controls
- The plate was centrifuged for 5 min at 250×g and then 40 µL of plasma was collected from each well
- MSD plate preparation: The MSD plate was incubated with Human Serum Cytokine (HSC) assay diluents (25 µL/ well) for 30 min at room temperature
- 25 µl of the plasma sample was added to a MSD plate containing the blocking solution and incubated 2 h at room temperature
- The plate was washed 3 times with with 150 µL/well PBS-T
- 25 µL/well Detection antibody was added to the plate and incubated for 2 h at room temperature
- The plate was washed 3 times with with 150µL/well PBS-T
- 150 µL/well 2x Read Buffer T were added and the plate was analyzed on a MSD Sector Imager 2400 plate reader

IC_{50} values were determined as average of 4 independent experiments.

***In vivo* pharmacokinetic study:**

Pharmacokinetics and oral bioavailability of **3** were investigated (WuXi AppTec) in male SD rats following administration of a single intravenous (1 mg per kg body weight) or oral dose (3 mg per kg body weight). The dosing vehicle used was 5% DMSO / 95% (10% Kleptose in water) for oral gavage. The intravenous dosing vehicle was 5% DMSO / 95% (10% Kleptose in saline). EDTA-K_2 blood for pharmacokinetic analysis was withdrawn and blood/plasma samples were analyzed by LC-MC/MS for levels of **3**.

Table 4. Experimental groups rat PK study.

Group	N	Route	Dose Level (mg/kg)	Dose (mg/mL)	Dose (mL/kg)	Blood sampling time post dose
1	3	IV	1	0.5	2	5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h
2	3	PO	3	0.6	5	15 min, 30min, 1 h, 2 h, 4 h, 6 h, 8 h

Anti-CD3 acute mouse model:

The study was performed at Heidelberg Pharma GmbH.

Male C57BL/6J mice were predosed with **3** or vehicle 60min before stimulation with anti-CD3 antibody (10 µg/200µL/mouse, intraperitoneal injection). 15 min after stimulus, a blood sampling was performed, mice were terminated and spleens were dissected.

Table 5. Experimental groups anti-CD3 acute mouse model.

Group	N	Compound	Route	Dose Level (mg/kg)	Dose (mg/mL)	Dose (mL/kg)	Stimulus antibody
1	4	Vehicle	PO	0	0	0	- (PBS)
2	6	Vehicle	PO	0	0	10	aCD3
3	6	**3**	PO	1	1	1	aCD3
4	6	**3**	PO	3	1	3	aCD3
5	6	**3**	PO	10	1	10	aCD3

Vehicle: 5% DMSO / 95% (10% Kleptose in water)

Snap frozen spleens were lyzed in buffer containing containing 0.5% Igepal and 10% glycerol (detailed composition see table below). After homogenisation (sonication 60sec, 60% power (~75W), 60% pulse), lysates were centrifuged for 30 min at 30,000 g, 4°C and supernatants were snap frozen. Lysates were kept at -80°C until further analysis.

Table 6. Buffer for preparation of mouse spleen lysates (anti-CD3 acute mouse model)

Component
50 mM Tris-HCl, pH8
280 mM NaCl
0.2 mM EDTA
2 mM EGTA
0.5% Igepal
10% glycerol
500 µL/25 mL Phosphatase Inhibitor Cocktail 2 (Sigma Aldrich)
500 µL/25 mL Phosphatase Inhibitor Cocktail 3 (Sigma Aldrich)
20 mM NaF
1 tablet/25 mL cOmplete™ EDTA-free Protease Inhibitor Cocktail Tablets (Roche)
2 mM PMSF
water

1.5 mL buffer for lysis of 1 spleen

The mouse spleen lysates were then analyzed in two different assays for quantitative determination of phospho-S6RP and phospho-Akt. To do so, Meso Scale Discovery assay kits K150DGD-3 (Phospho-S6RP (Ser240/244)) and K151CAD-3 (Phospho-Akt (Ser473)) were utilized. The assays were performed according to the instructions provided by the supplier using the following incubation times and amounts of lysate per data point:

- for pS6RP Ser240/244: plate incubated 2 h at room temperature with 25 µL lysate diluted 1:5, 2 h incubation with detection antibody (room temperature)
- for pAKT Ser473: plate incubated overnight at 4°C with 100 µL neat lysate, 2 h incubation with detection antibody (room temperature)

Assay results were corrected for protein amounts in lysate, that were determined using the Thermo Scientific Pierce BCA Protein Assay Kit according to the suppliers instructions.

Terminal drug levels were determined from blood sampling for calculation of EC_{50}s.

Collagen induced arthritis (CIA) mouse model:

The study was performed at Bolder BioPATH Inc.

Male DBA/1OlaHsd mice with semi-established type II collagen arthritis were challenged with an injection of 150 µL of Freund's Complete Adjuvant containing 2 mg/mL bovine type II collagen on day 0 and again on day 21 (intradermal injection at base of tail). Treatment was initiated after enrollment on day 22 and continued twice daily at 12 hour intervalls through study day 33. On study days 25–34, onset of arthritis occured. Mice were terminated on study day 34 via cervical dislocation.

Table 7. Experimental groups CIA mouse model.

Group	N	Collagen challenge	Compound	Route	Dose Level (mg/kg)	Dose (mg/mL)	Dose (mL/kg)
1	4	-	-	-	0	0	0
2	10	+	Vehicle	PO	0	0	10
3	10	**+**	**3**	PO	10	1	10

Vehicle: 5% DMSO / 95% (10% Kleptose in water)

The development of arthritis was determined by evaluation of body weights and clinical arthritis scores. Mice were weighed on study days 22, 24, 26, 28, 30, 32, 34 (prior to necropsy). Daily clinical scores were given for each of the paws on arthritis days 22-34 using the following criteria:

- 0 = normal
- 1 = 1 hind or fore paw joint affected or minimal diffuse erythema and swelling
- 2 = 2 hind or fore paw joints affected or mildl diffuse erythema and swelling
- 3 = 3 hind or fore paw joints affected or moderate diffuse erythema and swelling
- 4 = 4 hind or fore paw joints affected or marked diffuse erythema and swelling
- 5 = Entire paw affected, severe diffuse erythema and severe swelling, unable to flex digits

For determination of drug levels, mice were time dosed on study day 31 a blood sampling was performed.

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