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Identification of an Adamantyl Azaquinolone JNK Selective Inhibitor

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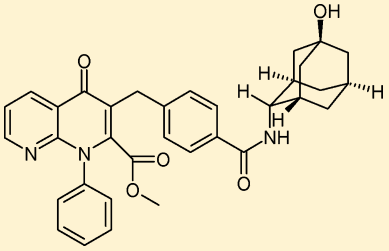
Supporting Information

ABSTRACT: 3-[4-((1*S*,2*S*,3*R*,5*S*,7*S*)-5-Hydroxyadamantan-2-ylcarbamoyl)benzyl]-4-oxo-1-phenyl-1,4-dihydro-[1,8]naphthyridine-2-carboxylic acid methyl ester (**4**) was identified as a novel, druglike and selective quinolone pan JNK inhibitor. In this communication, some of the structure−activity relationship of the azaquinolone analogues leading to **4** is discussed. The focus is on how changes at the amide functionality affected the biochemical potency, cellular potency, metabolic properties, and solubility of this class of JNK inhibitors. Optimization of these properties led to the identification of the adamantyl analogue, **4**. **4** achieved proof of mechanism in both rat and mouse TNF-α challenge models.



KEYWORDS: *JNK selective inhibitor, kidney disease, adamantyl azaquinolone*

The c-jun NH_2-terminal kinases (JNK-1, JNK-2, and JNK-3) are members of the mitogen activated protein (MAP) kinase family. Whereas JNK-1 and JNK-2 are ubiquitously expressed throughout the body, JNK-3 is found primarily in the brain, heart, and testes. The JNKs, also known as "stress activated protein kinases", are activated in response to a number of natural stress stimuli, including reactive oxygen species, UV light,[1] and hypoxia,[2,3] as well as some chemical entities, such as the cancer drug Cisplatin.[4] Activation of JNK by internal or external stimuli can be detected in an experimental setting by measuring phosphorylation of the eponymous substrate c-jun.[4]

The JNK kinase family has attracted attention as a potential target for multiple therapeutic indications such as rheumatoid arthritis (RA), asthma, diabetes, and neurological disorders.[5] JNK has also been shown to have increased activity in various chronic kidney diseases and acute kidney injury (AKI).[6] The focus of our program was the development of novel JNK-selective inhibitors that may be clinically effective in treating AKI. AKI can have many different origins, but it is most frequently encountered clinically as a complication of cardiac surgery, as a consequence of sepsis, or as the nephrotoxic side-effects of pharmaceutical agents including some chemo-therapeutics, antibiotics, and nonsteroidal anti-inflammatory drugs. AKI is a relatively common complication that can rapidly lead to renal failure, which is associated with a high mortality rate. Currently, no pharmaceutical agent has been approved for the treatment of AKI. However, nonselective JNK inhibitors (i.e., CC-401,[7] SP600125[8]) have been shown to decrease damage markers in rodent renal ischemia/reperfusion (I/R) models and in Cisplatin nephrotoxicity studies.

Critical issues have impeded progress in the discovery of agents to treat AKI. For example, there is increased debate over the validity of available animal models and how these models translate in terms of predicting efficacy in human disease, as well as the identification of rapidly detectable and sensitive biomarkers and the determination of appropriate physiological and clinical end-points.[9,10] Identification of selective compounds will be important to help answer and guide the biological investigation of this disease.

An initial investigation of the quinolone series for the indication of RA and asthma resulted in the identification of a compound of interest, **1** (Figure 1).[11] In our investigation of inhibitors of JNK for the treatment of AKI, **1** was profiled, but its potency in human kidney (HK-2) cells and microsomal clearance values did not appear compelling to warrant further profiling. The deficiencies of **1** were confirmed when it did not achieve the necessary levels of JNK inhibition when challenged with TNF-α *in vivo* (data not shown; the criteria used to determine adequate inhibition of JNK will be described later in this paper). Improved solubility was also a key challenge in advancing the quinolones toward clinical candidate selection for the indication of AKI. Since most AKI patients develop the condition as a complication of surgery and are unable to take medications orally, the preferred delivery route is intravenous administration. Thus, it seemed there were few compounds from the original exploration of the quinolone series which presented adequate *in vitro* potency coupled with an acceptable biopharmaceutical profile.[11,12]

However, closer examination of the structure−activity relationship (SAR) from the RA/asthma program revealed an underexplored subset of compounds, the 8-azaquinolones.[12] A number of compounds in the 8-aza series had been prepared containing a 7-methyl group as in **2** (Figure 1). While inclusion of a methyl vs a hydrogen at the 7-position imparted improved

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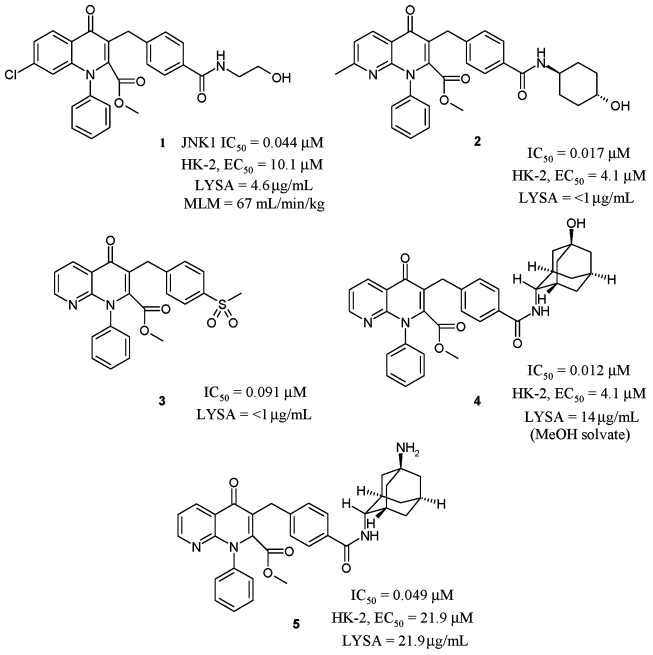


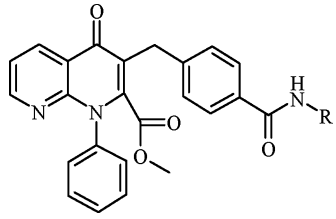
Figure 1. Structures and properties of **1**−**5**, showing mean values from at least two experiments. HK-2 EC_{50}:[15] Cellular potency was determined by the ability of compounds to prevent TNFα-stimulated accumulation of phospho-c-jun in human proximal tubule (HK-2) cells; LYSA solubility measurement (acetonitrile/water, 70:30, at 25 °C).

potency, it also resulted in decreased metabolic stability.[12] Only a single analog, **3**, was synthesized with no substitution at the 7-position in the original investigation of the azaquinolone series. **3** served as our starting point for SAR exploration in the AKI indication with the goal of improving the solubility while maintaining the *in vitro* and cellular potency.[12] In this communication, the results from the synthetic exercises to probe the SAR of the azaquinolone series which led to **4** are introduced. The main emphasis of this investigation involved profiling various amides at the *para* position of the benzyl ring (Table 1). This work culminated in the identification of **4**, a *trans*-hydroxyadamantyl azaquinolone isolated as the methanol solvate.[13,14] **4** achieved a compromise between JNK biochemical potency, cellular potency, solubility, and acceptable rodent PK. Furthermore, no other kinases in a screen of >450 kinases were inhibited more than 50% at the testing concentration of 10 μM (Supporting Information). This result allowed us to conclude that the *in vitro* and *in vivo* activities observed were most likely based solely on JNK activity and not due to inhibition of other kinases (Cerep panel).

In order to help guide chemistry efforts and to try to understand the exquisite selectivity of the quinolone/azaquinolone based compounds, we determined the crystal structures of JNK1β1-JIP with many of our compounds. Figure 2 shows the binding of a *trans*-aminoadamantyl azaquinolone, **5** to JNK1.

The backbone NH from methionine 111 in the kinase hinge donates a hydrogen bond to the carbonyl of the azaquinolone, and this is the only compound−hinge hydrogen bond interaction. The conformation of the main chain of methionine 111 is quite unique among the kinases that we have observed. Generally, the residue at this position is oriented such that the backbone carbonyl points directly into the ATP binding site

Table 1. (A) *In Vitro* Properties of 1, 4, and 6−11[a]; (B) Oral PK Parameters Obtained for 4, 6, and 8−11; (bottom) IV PK Parameters Obtained for 4 and 9[b]



A.

Compound	R	Activated JNK1 (2 μM ATP) IC_{50} (μM)	HK-2 EC_{50} (μM)	MLM (mL/min/kg)	mHep (mL/min/kg)	LYSA (pH=6.5) (μg/mL)
1		0.044	10.1	67 (h)	31.7 (m)	4.6
4	*trans*-adamantyl-OH (methanol solvate)	0.012	4.1	59 (m)	10.9 (l)	14
6		0.012	3.3	83 (h)	-	17
7	*cis*-adamantyl-OH	0.047	8.5	81 (h)	-	25
8		0.044	9.9	44 (m)	24.7 (l)	41
9		0.054	14	41 (m)	8.7 (l)	78
10		0.045	9.0	58 (m)	52.9 (m)	103
11		0.045	21	45 (m)	33.2 (m)	138

B.

Oral PK

compound	AUC (h*ng/mL)	C_{max} (ng/mL)	MRT (h)	t_{max} (h)	Plasma (8 h exposure - μM)	Kidney	Liver
4[a]	22355	13600	2.38	0.25	0.56	0.86	2.96
6[a]	1280	1140	1.42	0.25	0.02	0.03	0.23
8[b]	16213	7370	3.14	0.25	0.74	1.42	4.0
9[a]	33500	14500	3.45	0.25	0.81	1.89	5.57
10[b]	7012	3840	2.03	0.25	0.38	0.62	2.49
11[b]	7482	4370	1.73	0.25	0.38	0.86	4.49

IV PK

compound	AUC_{0-24h} (h*ng/mL)	CL (mL/min/kg)	Vdss (L/kg)
4[a]	9890	8.4	0.30
9[b]	9620	8.5	0.73

[a]MLM (mouse microsomes; mL/(min·kg)); *l* = low clearance (<27.4); *m* = medium clearance (27.4-63.9); *h* = high clearance (>63.9); mHep (mouse hepatocytes): *l* (<27.6), *m* (27.6−63.9), *h* (>63.9). Mean values from at least two experiments. [b](B) Experiments performed in C57 mice dosed po at 30 mg/kg; IV at 5 mg/kg; a: 20% DMSO/80% PEG 400; b: 5% DMSO/30% PEG monooleate/65% PEG 400. All numbers are the mean average ($n \geq 2$).

and accepts a hydrogen bond from the ligand; it would clash with a carbonyl from a small molecule. In the JNKs, this backbone carbonyl is pointed more perpendicularly from the cleft, allowing compounds such as the quinolones/azaquinolones to bind without clashing. We believe that this is the primary basis for the excellent selectivity seen for this class.

The aryl chain then leads away from the ATP pocket. The phenyl sits in a ∼8 Å wide hydrophobic channel flanked by the side chain of isoleucine 32 and the peptide plane of aspartate 112/alanine 113 from the end of the hinge. The amide linked

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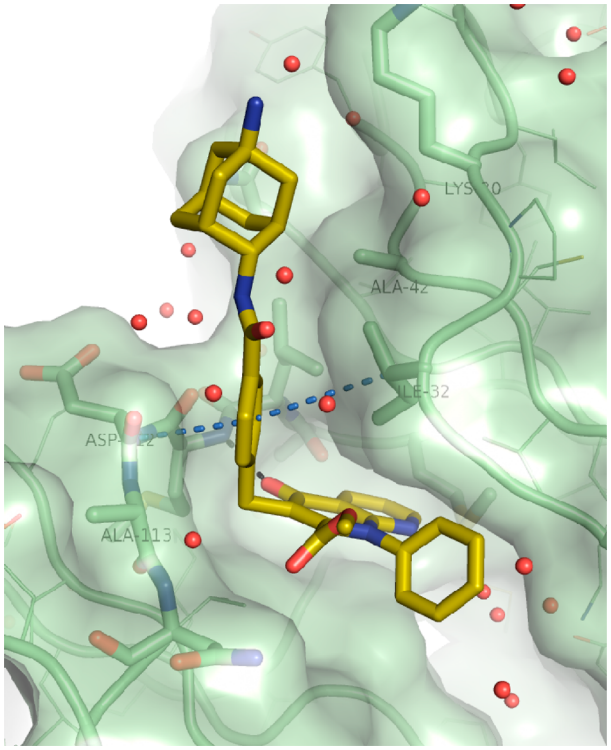


Figure 2. X-ray crystal structure of **5** with JNK1.

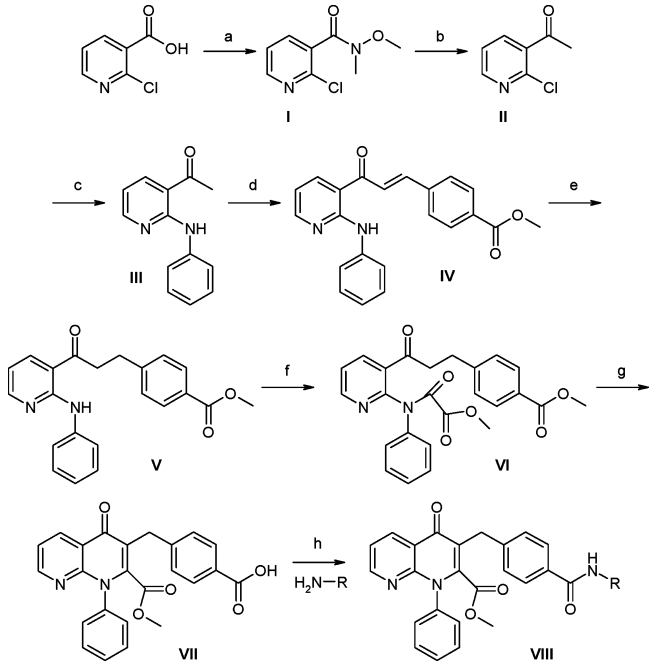
trans-adamantyl moiety then reaches the outside of the *N*-terminal lobe of the kinase, generally making only hydrophobic interactions with the protein. This shallow pocket where the adamantyl sits may also represent another element that is helpful for determining JNK selectivity: alanine 42, which is at the base of this cleft, in other kinases is often a lysine, whose side chain would fill and therefore eliminate the shallow pocket.

A general synthetic approach for the 8-aza analogs is shown in Scheme 1.[14] The synthesis of the azaquinolone scaffold was initiated by synthesizing the Weinreb amide, **I**, of 2-chloronicotinic acid followed by installation of the methyl ketone by treatment with methyl Grignard. Displacement of the chloride of **II** with aniline under acidic conditions provided **III**. A subsequent aldol condensation resulted in the formation of the α,β-unsaturated ketone, **IV**, which was ultimately reduced under standard hydrogenation protocols. Installation of the glyoxylate ester set up the system for base initiated cyclization and dehydration with concomitant ester hydrolysis to the azaquinolone core, **VII**. Amide formation under standard conditions using various amines allowed exploration of the SAR at this position via compounds such as **VIII**.

A set of amines identified from our lab shown to increase the potency of the quinolone class in the biochemical assay were coupled onto the *p*-benzoic acid position (Table 1A).[12] The values reported in Table 1 are the average of at least two separate experiments, and it is typical for duplicate values to be within 2-fold of each other.

This exercise resulted in many analogs which exhibited good *in vitro* potency, high solubility (LYSA: lyophilized solubility assay),[16] and moderate microsomal stability. It appeared that the changes in the amide functionality did not dramatically alter the biochemical activity. However, these changes resulted in significant changes in solubility, and as the solubility increased, the cellular potency decreased noticeably.[17] This trend is most clearly observed by examining the data for **7**−**11**. All have

Scheme 1. General Scheme for the Synthesis of Amides in the 8-Azaquinolone Series[a]



[a]Reagents and conditions: (a) *N*,*O*-hydroxylamine hydrochloride, benzotriazole-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate, rt, 93%; (b) 3.0 M MeMgCl in THF, 0 °C, 72%; (c) aniline, *dl*-10-camphorsulfonic acid, 1,4-dioxane, 80 °C, 36%; (d) methyl-4-formylbenzoate, 25 wt % NaOMe in MeOH, rt, 95%; (e) 10% Pd/C, H_2, EtOAc, CH_2Cl_2, rt, 81%; (f) methyl 2-chloro-2-oxoacetate, toluene, 130 °C, used without further purification; (g) K_2CO_3, MeOH, 85 °C; (h) EDC-HCl, HOBT, DIPEA, CH_2Cl_2, rt, 12−82%.

comparable *in vitro* potencies, but the cellular potency suffered as the solubility increased. An exception to this trend was **4**. **4** exhibited moderate solubility and clearance, good *in vitro* potency, and acceptable cellular potency. The properties of **4** can be contrasted to the original lead of the quinolone series, **1**, which exhibited lower *in vitro* and cellular potency, lower solubility, and higher microsomal clearance (Table 1A).

To further delineate the differences between the analogs, PK experiments were performed and tissue exposures were measured (Table 1B). Analogs **4**, **6**, and **8**−**11**, which exhibited a range of cellular potencies, were dosed orally in mice at 30 mg/kg. Of these compounds, only **4**, **8**, and **9** exhibited adequate plasma and kidney exposure (Table 1B). Based on the PK results, although **4**, **6**, and **8**−**11** exhibited moderate to high clearance in microsomes, it was shown that the hepatocyte data correlated better with *in vivo* clearance (Table 1A).

An IVPK was performed on **4** and **9** to compare clearance and AUC parameters (Table 1B). Since the PK parameters of **4** and **9** were quite similar, the decision to further investigate **4** was based on its overall profile. Only **4** combined good exposure numbers with an acceptable cellular potency in HK-2 cells (4.1 μM). It appeared that **4**, isolated as the methanol solvate, provided a molecule that attained a balance between solubility and cellular activity while moderating microsomal clearance.[13,17] Based on these results, further profiling of **4** was pursued.

Due to limited solubility of the quinolone series in general, a DMSO-containing formulation was used to profile the PK

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Table 2. Oral and IV PK Parameters Obtained for 4[21] [a]

Oral PK						
species	formulation	dose (mg/kg)	C_{max} (ng/mL)	AUC (h·ng/mL)	t_{max} (h)	$t_{1/2}$ (h)
rat	DMSO	30	8030	48300	4.0	2.4
C57 mouse	DMSO	30	13600	22400	0.25	
rat	MBP	75	30100	252000	4.0	2.0
C57 mouse	MBP	75	18100	49200	1.0	1.62

IV PK							
species	formulation	dose (mg/kg)	CL (mL/min/kg)	Vd (L/kg)	AUC extrap. (h·ng/mL)	MRT (h)	$t_{1/2}$ (h)
rat	DMSO	5	6.4	0.61	16310	1.8	2.4
mouse	DMSO	5	8.4	0.30	9890	0.6	0.6

[a]Compound was dosed orally to noncannulated rodents. DMSO: 20% DMSO, 80% PEG 400 solution. MBP: 20% drug loading, 2% Klucel in water pH 4.0. IV formulation strength: 1 mg/mL. DMSO: 5% DMSO, 20% PEG 400, HPBCD in water. All numbers are the mean average ($n \geq 2$).

properties of compounds initially. This formulation allowed a larger set of analogs to be studied. However, DMSO itself can have protective effects on the kidney in rodent models of AKI, and thus, its use could confound the interpretation of results obtained in disease models.[18,19] The DMSO based formulation was replaced with an amorphous formulation, MBP (micro-precipitated bulk powder).[20] The PK experiments performed with **4** in rats and mice using the DMSO-based and MBP formulations are shown in Table 2. Several important points emerged from these PK experiments. First, in either formulation, clearance was comparable in the mouse and in the rat. Second, the MBP formulation resulted in comparable, or superior, PK to that seen using the DMSO-containing formulation. Third, when the rat was dosed at 75 mg/kg in MBP with **4**, the exposure was maintained for over 8 h at a level 2-fold higher than the predicted EC_{50}, a level predicted to effect a significant inhibition of kidney JNK (Supporting Information).

To confirm this prediction, we performed "proof of mechanism" (POM)[22] experiments using **4** in the rat, based on a TNF-α challenge model. In this paradigm, rats were dosed with **4** (or vehicle) followed some time later by intravenous administration of TNF-α (2.5 μg/kg), which causes JNK activation in multiple tissues, including the kidney. A control group received only saline IV. Thus, by quantifying the accumulation of p-c-jun in the kidney 1 h after TNF-α/saline (by Western blot), it was possible to estimate the degree of JNK inhibition achieved. Figure 3 shows that when TNF-α was dosed 7 h after **4**, p-c-jun accumulation in the kidney was only 52% of the level in rats that did not receive **4**. This significant reduction of signal confirmed the prediction based on the PK. Since the degree of JNK inhibition required for efficacy in disease models is unknown, it was important to establish a dosing regimen that would significantly inhibit kidney JNK for 24 h. Thus, we repeated the TNF-α challenge in rat, dosing **4** at 0 and 12 h (bid dosing) or 0, 8, and 16 h (tid dosing), with the TNF-α introduced at 23 h. At 24 h, the kidneys were harvested, and the results for p-c-jun content are shown in Figure 3. The data demonstrated that, with multiple dosing, significant continuous inhibition of kidney JNK was achieved.

In summary, a pan JNK selective azaquinolone analogue, **4**, showing promising *in vitro* and biopharmaceutical properties was identified. From PK studies, **4** exhibited higher kidney and plasma exposure in rats than in mice. MBP formulation of **4** with dosing at 75 mg/kg in rat provided sufficient drug exposure at the 8 h time point to significantly blunt TNF-α induced c-jun phosphorylation in kidney. The results shown for

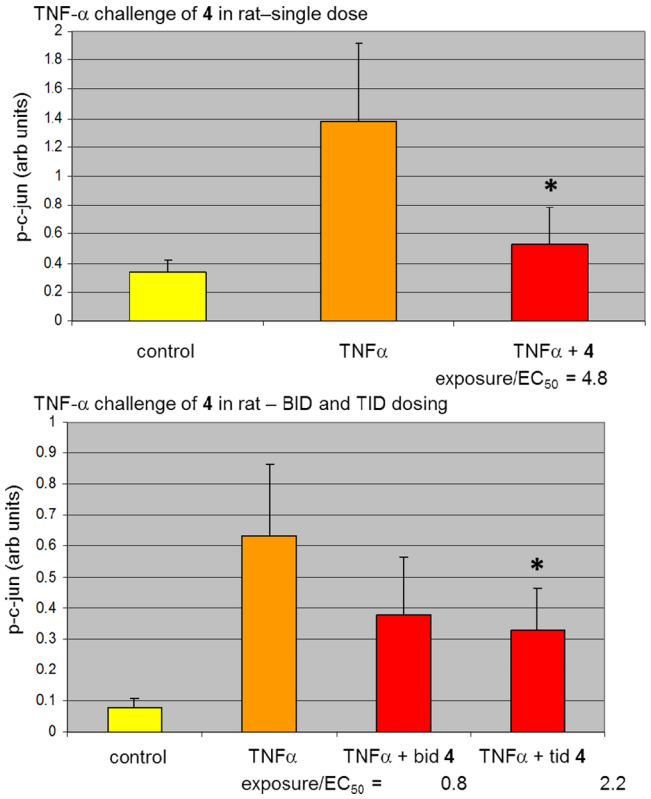

Figure 3. **4** lowered kidney p-c-jun at 8 h in the TNF-α challenge model in rat. 75 mg/kg po in MBP formulation. *$p < 0.05$ relative to TNF-α alone.

4 serve to clarify the differences in potency and properties between **4** and the RA/asthma lead, **1**. Although **1** and **4** were members of the same quinolone series, **4** exhibited enhanced biopharmaceutical properties, improved potency for the AKI indication, and achieved POM for JNK inhibition in the TNF-α challenge model. Incorporation of the *trans*-hydroxyadamantyl moiety into the azaquinolone series and isolation of its methanol solvate provided the key to obtaining a balance between the biochemical, *in vitro*, and *in vivo* properties.[13]

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, crystallographic methods, kinase selectivity data, and assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

JNK, c-jun NH_2-terminal kinase; RA, rheumatoid arthitis; AKI, acute kidney injury; I/R, ischemia/reperfusion; SAR, structure activity relationship; HK-2 cells, human kidney cells; TNF-α, tumor necrosis factor-α; LYSA, lyophilized solubility assay; JIP, JNK interacting protein; ATP, adenosine triphosphate; THF, tetrahydrofuran; EDC-HCl, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; DIPEA, *N*,*N*-diisopropylethyl amine; HOBT, *N*-hydroxybenzotriazole; EtOAc, ethyl acetate; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; IV, intravenous; PK, pharmacokinetics; MBP, microprecipitated bulk powder; MRT, mean residence time; MLM, mouse liver microsomes; AUC, area under the curve; Vdss, volume of distribution; mHEP, mouse hepatocyte; HPBCD, hydroxypropyl-beta-cyclodextrin

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(17) HK-2 cellular assay: stimulates or stresses the cell by applying TNF-α (an established cytokine/stressor known to stimulate JNK); dose compound; measure how well the drug/compound blunts the excursion of TNF-α by measuring the amount of c-jun phosphorylation present relative to rats exposed to vehicle.

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(21) Aliquots (0.025 mL) of rodent plasma were protein precipitated with acetonitrile. The extracts were diluted with water and analyzed by LC/MS/MS.

(22) POM experiment: *in vivo* measurement of how well the drug/compound blunts the excursion of TNF-α by measuring the amount of c-jun phosphorylation present relative to rats exposed to vehicle; proof of mechanism.