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Bioorganic & Medicinal Chemistry Letters 17 (2007) 495–500
Bioorganic & Medicinal Chemistry Letters

Hemodynamic effects of potent and selective JNK inhibitors in anesthetized rats: Implication for targeting protein kinases in metabolic diseases

Gang Liu,[a,*] Hongyu Zhao,[a] Bo Liu,[a] Zhili Xin,[a] Mei Liu,[a] Michael D. Serby,[a] Nathan L. Lubbers,[b] Deborah L. Widomski,[b] James S. Polakowski,[b] David W. A. Beno,[c] James M. Trevillyan[a] and Hing L. Sham[a]

[a]*Metabolic Disease Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064-6101, USA*
[b]*Integrative Pharmacology, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064-6101, USA*
[c]*Exploratory Pharmacokinetics, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064-6101, USA*

Received 25 September 2006; revised 5 October 2006; accepted 5 October 2006
Available online 10 October 2006

Abstract—The hemodynamic effects of a series of potent and selective 4-aminopyridine carboxamide-based pan-JNK inhibitors were assessed in an anesthetized rat model. The effects of these agents on mean arterial pressure, heart rate, cardiac contractility, and peripheral vascular resistance are described, and the implication for targeting protein kinases in metabolic diseases is discussed.

The c-Jun N-terminal kinases (JNKs) are members of the mitogen-activated protein kinase (MAPK) family and regulate signal transduction in response to environmental stress, cytokines, and free fatty acids. Three distinct genes encoding JNKs have been identified (*jnk1, jnk2, and jnk3*), and at least 10 different splicing isoforms exist in mammalian cells.[1] Up-regulation of JNK expression and activity has been implicated in a number of disease states, including diabetes, obesity, inflammation, auto-immune diseases, and neuro-degenerative diseases.[2] Therefore, small molecule inhibitors of JNKs have been pursued in a number of laboratories as potential therapeutic agents for the aforementioned ailments.[3] We are particularly intrigued by the potential of inhibiting JNK1 activity for boosting the metabolic insulin-signaling cascade and ameliorating insulin resistance.[4]

Obesity and type 2 diabetes are prevalent and serious metabolic diseases. The expenses associated with treating these metabolic diseases, including diabetes and obesity, exceed 20% of the national health care budget. However, obesity and type 2 diabetes are still regarded as chronic, non-life-threatening diseases. Because the treatment regimen tends to be chronic, the safety requirement on these drugs is extremely high.

Recently, cardiovascular safety has attracted both scientific and media attention with the high-profile withdrawal of propulsid (Cisapride®)[5] and rofecoxib (Vioxx®).[6] In addition, reports of deleterious cardiovascular effects of several marketed protein tyrosine kinase inhibitors, including a monoclonal antibody,[7] have appeared in the literature. The chronic use of imatinib (Gleevec®), a Bcr-Abl tyrosine kinase inhibitor, has been linked to a striking 50% decrease in the pumping capacity of the heart, and the development of severe congestive heart failure in some patients.[8] The unanticipated cardiotoxicity of imatinib highlights the need for more vigorous preclinical and clinical evaluation of the cardiovascular liabilities of new protein kinase inhibitors.

Keywords: c-Jun N-terminal kinase inhibitor; Anesthetized rat model; Cardiotoxicity; Metabolic diseases.
* Corresponding author. Tel.: +1 847 935 1224; fax: +1 847 938 1674; e-mail: gang.liu@abbott.com

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doi:10.1016/j.bmcl.2006.10.013

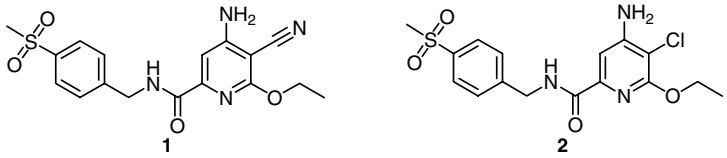


Figure 1. Selected 4-aminopyridine carboxamide JNK inhibitors reported from our laboratories.

We recently reported a series of 4-aminopyridine carboxamides, represented by **1** and **2** (Fig. 1), as potent JNK1 and JNK2 inhibitors with minimal cross-activity against other kinases.[9] These compounds also were shown to exhibit excellent selectivity (>10,000-fold) over hERG channel in a dofetilide binding assay.

Because of the stringent safety requirements for the treatment of chronic diseases such as type 2 diabetes, and the potential cardiovascular liabilities associated with protein kinase inhibitors, we initiated an assessment of the anesthetized in vivo hemodynamic effects of these JNK inhibitors early in our lead optimization process.

The pentobarbital anesthetized dog has been widely accepted as the gold standard in assessing the cardiovascular effects of new chemical entities (NCEs).[10] However, since we identified the need to screen JNK inhibitors for cardiovascular liabilities at a much earlier stage in our lead optimization program, we sought to implement a higher throughput and less compound-intensive, whole-animal model that could be applied as an early screening tool.

The acutely instrumented anesthetized rat cardiovascular model[11,12] is a predictive in vivo model of cardiovascular function that effectively interrogates lead molecules for undesirable cardiovascular effects. Studies are conducted under anesthesia (inactin) using a series of intravenous infusions and periodic collection of small (150 μL) blood samples to assess active drug levels, providing concentration-response functions for clinically relevant endpoints of mean arterial pressure (MAP), heart rate (HR), cardiac contractility, and peripheral vascular resistance (PVR). The predictive value of the model arises from its ability to identify compounds that produce effects on cardiovascular function at relevant doses or plasma concentrations, results that would preclude them from further characterization.

In this screening paradigm, a compound producing an effect greater than 15% compared to vehicle within an estimated 30-fold window is unacceptable for further characterization. Also, if MAP decreases below the minimum pressure limit (70 mmHg), the infusion is stopped and a blood sample collected. This minimum pressure level was implemented to ensure a functional cardiovascular system for determination of drug plasma levels.

One significant advantage of the anesthetized rat model is that it requires significantly less compound (<200 mg) per assay than the anesthetized dog model. In addition, easier access to animals and reduced instrumentation contribute to a greater capacity for compound throughput in the anesthetized rat assay compared to the dog assay. Such assays have been successfully implemented in the lead optimization programs for melanin concentrating hormone receptor 1 (MCH1) antagonists[13] and transient receptor potential channel vanilloid 1 (TRPV1) receptor antagonists.[14]

Therefore, from a technical standpoint, we hypothesized that the inactin-anesthetized rat model could be used as an effective screening model to establish structure–activity relationships (SAR) regarding the cardiovascular effects of our pan-JNK inhibitors. For the sake of direct comparison, 30 μg/mL (plasma concentration) was targeted as a 30× therapeutic concentration. Aminopyridine carboxamide **1** was selected as an initial compound for evaluation in the anesthetized rat model.

Compound **1** represents a potent JNK inhibitor with a cellular EC_{50} value of 318 nM against c-Jun phosphorylation in HepG2 cells. In the inactin-anesthetized rat cardiovascular screening model,[15] aminopyridine **1** decreased MAP beginning with the 10 mg/kg infusion, the maximum decrease was 41% at 90 min (Fig. 1). The MAP of two animals fell below the minimum pressure limit during the last 5 min of the final infusion. HR began to decrease slightly with the 30 mg/kg infusion while there was a slight increase in cardiac contractility (dP/dt). Maximum change for both parameters was less than 15%. PVR began to decrease during the 10 mg/kg infusion, the maximum decrease was 25% at 90 min. These preliminary data suggest that as the concentration increases, compound **1** may be acting, at least partially, as a peripheral vasodilator (decrease in vascular resistance). This is supported by the minimal effects on HR and cardiac contractility. The decrease in vascular resistance contributes to the decreases in MAP. The end of infusion (EOI) plasma concentration at 90 minutes was slightly lower than projected, but still within the relevant range. The hemodynamic profile of the other active JNK inhibitor **2** is similar to that of **1**, in terms of the changes in MAP and PVR (Table 1).

To understand whether the hemodynamic changes induced by **1** and **2** are related to the general structure of aminopyridine or related to the inhibition of JNK signaling pathway, we also profiled two inactive analogs (**3** and **4**) from the same series. As shown in Table 1, 4-hydroxypyridine **3** only reduced MAP slightly in rats at higher EOI plasma levels, while having no effect on

Table 1. Hemodynamic effects of active JNK1 inhibitors versus inactive analogs in anesthetized rat model

Class	Compound	JNK1 IC_{50} (μM)	Maximum change in MAP[a]	Maximum change in HR[a]	Maximum change in dP/dt[a]	Maximum change in PVR[a]	EOI plasma 90 min (μg/mL)[b]
Active	**1**	0.019	↓41%	↓ <15%	↑ <15%	↓25%	19.3
	2	0.024	↓20%	↓ <15%	↑ <15%	↓51%	14.7
Inactive	**3**	>10	↓ <15%	NE	NE	NE	39.9
	4	6.4	NE	NE	↑41%	↓46%	23.6

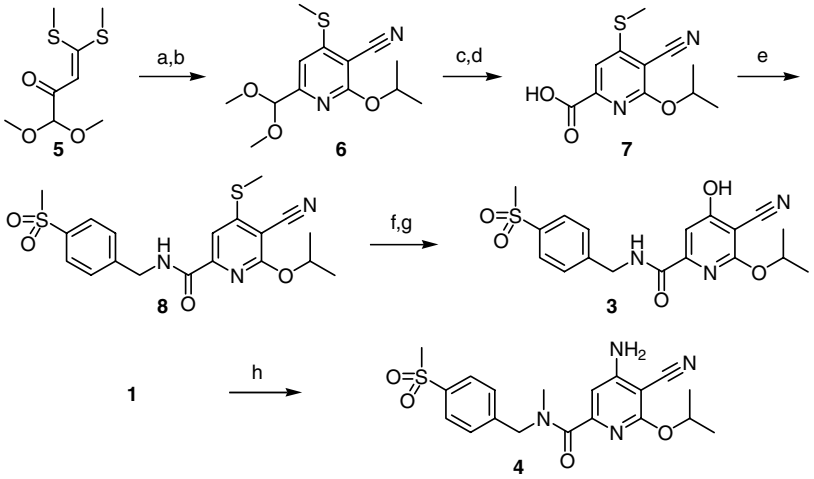
NE, no effect.
[a] Mean values based on the results from three animals.
[b] Mean values based on concentrations from three animals.

the other hemodynamic parameters measured. On the other hand, N-methylated carboxamide **4** showed a different CV profile from that exhibited by the active aminopyridine **1**, producing a large decrease in PVR (46%). The large compensatory increase in cardiac contractility (41% increase) likely contributed to maintaining MAP. These results suggest that the observed hemodynamic effects of **1** and **2** may not be linked to the general structure of aminopyridine carboxamide. However, we could not rule out the possible linkage between the hemodynamic effects and JNK activity inhibition, or potential secondary pharmacodynamic properties (off-target effects) of these structures.

The synthesis of active analogues has been reported previously.[9,16] The preparation of inactive analog **3** is described in Scheme 1. Starting from the readily available α-oxoketene dithioacetal **5**,[17] cyclization with cyanoacetamide yielded a hydroxypyridine sodium salt, which was alkylated with *i*-propyl iodide to give highly functionalized pyridine **6**. Hydrolysis of the acetal and Ag_2O oxidation of the resulting aldehyde provided the acid **7**. Condensation of **7** with *p*-methylsulfonylbenzylamine generated amide **8**. Oxidation of sulfide with *m*-CPBA to sulfone, followed by hydrolysis, afforded the 4-hydroxypyridine **3**. Straightforward methylation of **1** provided *N*-methyl carboxamide **4**.

Considerable effort was made to assign the observed cardiovascular toxicity to cross-activity at specific receptors, transporters, and ion channels via a CEREP-panel[18] affinity screening. Unfortunately, we were unable to identify a molecular target for which activity of the studied compound **1** would correlate with the observed hemodynamic effects, as cross-activity of **1** was minimal against 75 different receptors, transporters, and ion channels (<30% inhibition of control-specific binding at 10 μM). Furthermore, we could not attribute the cardiovascular toxicity to the general state of non-JNK kinase inhibition either. As shown in the Supplementary material, **1** is an extremely selective ATP competitive JNK inhibitor, exhibiting no significant inhibitory activity toward any other kinases in a panel of 77 kinases[19] at the highest concentration tested except CSF1R and ERK2, but still with a selectivity window greater than 100-fold (Fig. 2).

We then launched a broader screening campaign to identify active JNK inhibitors with an improved cardiovascular profile over that of **1**. Nine potent JNK inhibitors from the aminopyridine series were evaluated in the anesthetized rat screening to establish structural parameters responsible for cardiovascular liabilities. Since the decrease in MAP was produced by the decrease in PVR for both **1** and **2**, the model was streamlined to



Scheme 1. Reagents and conditions: (a) cyanoacetamide, Na, *t*-BuOH, reflux, 5 h; (b) *i*-PrI, DMF, 120 °C, 30 min, 80%; (c) 1 N aq HCl, THF, 65 °C, 2 h, 90%; (d) $AgNO_3$, aq KOH, EtOH, rt, over night, 75%; (e) *p*-methylsulfonylbenzyl amine, TBTU, Et_3N, DMF, rt, 30 min, 81%; (f) *m*-CPBA, CH_2Cl_2, rt, over night; (g) 3 N aq NaOH, 1,4-dioxane, 80 °C, 3 h, 65% over two steps; (h) NaH, DMF, MeI, rt, 72%.

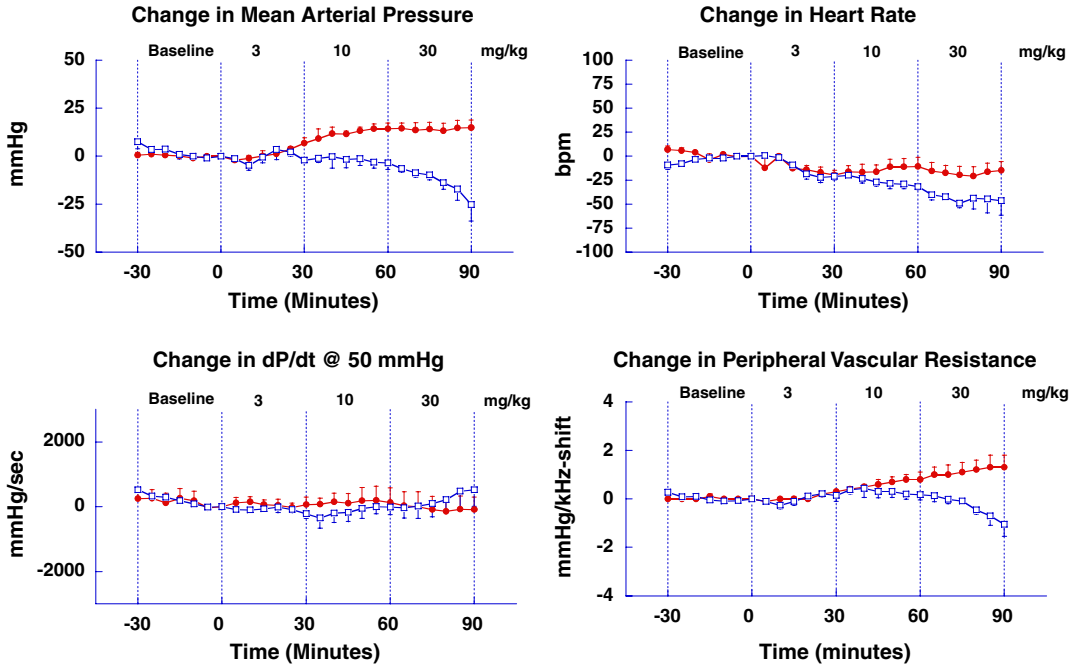
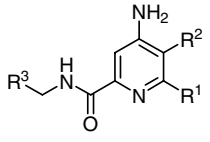


Figure 2. Hemodynamic effects induced by **1** in anesthetized rats. The red line represents those of the vehicle (10% DMSO in PEG400), and the blue line represents those of compound **1**.

maximize throughput by focusing on MAP, HR, and contractility. The results are summarized in Table 2. Once again, one common hemodynamic change was observed in these studies: a consistent, across-the-board decrease of MAP induced by these analogues (**9a**–**9i**) at comparable plasma concentrations, regardless of the structural variations at the 2-, 5-, and 6-position of the aminopyridine core. The effects on HR and cardiac contractility were generally mild to non-existent, with the exception of **9a**, which induced a large increase of heart contractility. It is worth noting that for potent JNK inhibitors **9g** and **9i**, the decrease in MAP was very close to the acceptable range (<15%), while having no effects on HR and contractility. However, the EOI plasma concentrations were found to be slightly lower than those achieved by **1** and **2**.

Table 2. Hemodynamic effect profiling of additional JNK inhibitors in anesthetized rat



9a-i

Compound	R^1	R^2	R^3	JNK1 IC_{50} (μM)	Maximum change in MAP[a]	Maximum change in HR[a]	Maximum change in dP/dt[a]	EOI plasma 90 min (μg/mL)[b]
9a	–OEt	–CN	2-Sulfonamidophenyl–	0.012	↓31%	↑22%	↑56%	25.8
9b	–OEt	–Cl	2-Sulfonamidophenyl–	0.023	↓20%	↑13%	NE	12.0
9c	–OEt	–CN	4-Chloro-3-pyridyl–	0.030	↓23%	NE	↓ >15%	
9d	–OEt	–CN	N-(5-Nitropyridin-2-yl)aminomethyl–	0.061	↓20%	NE	↑12%	
9e	–OEt	–CN	4-(Phenylsulfinyl)phenyl–	0.050	↓24%	NE	↑24%	
9f	–OEt	–CN	4-(Phenylsulfonyl)phenyl–	0.13	↓24%	↓11%	NE	
9g	Cyclopropylmethoxy	–CN	4-(Methylsulfonyl)phenyl–	0.023	↓24%	NE	NE	8.2
9h	Cyclopentane-amino	–CN	4-(Methylsulfonyl)phenyl–	0.096	↓18%	↓6%	↑13%	10.5
9i	3-Thieno	–CN	4-(Methylsulfonyl)phenyl–	0.081	↓23%	NE	NE	
10	NA			0.002	↓30%	↓22%	NE	9.0
11	NA			0.034	↓23%	↓11%	↓20%	

NA, no applicable; NE, no effect.
[a] Mean values based on the results from three animals.
[b] Mean values based on concentrations from three animals.

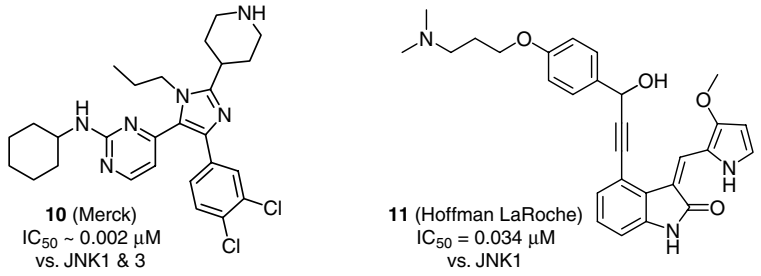


Figure 3. Selected literature JNK inhibitors evaluated in anesthetized rat model.

We also evaluated a number of JNK inhibitors reported in the literature in the anesthetized rat model. The hemodynamic effects of two of them are summarized in Table 2. The imidazole pyrimidine **10**[20] (Fig. 3) was shown to have severe cardiovascular effects. It induced a large decrease in MAP (30%) during the last infusion period. The MAP of two animals fell below the minimum pressure limit during the last 5 min of the final infusion. HR was also lowered by 22% in the final infusion period. Since **10** was a non-selective JNK and p38 inhibitor, it was difficult to interpret the origin of the hemodynamic effects of **10**. Oxindole **11**[21] (Fig. 3) decreased MAP and cardiac contractility by 23% and 20% at 90 min (end of infusion), respectively.

Thus, we discovered a hypotensive effect of a series of highly potent and selective JNK inhibitors in vivo. The decrease in MAP is likely driven in large part by the decrease in peripheral vascular resistance. Even though the hypotensive effect could be attenuated, we could not completely eliminate it through structural modification. A similar hypotensive effect was also observed with two structurally distinct classes of literature JNK inhibitors. Interestingly, our results with imatinib in anesthetized dog also indicated that it decreases arterial pressure with concomitant reduction in systemic vascular resistance (Dr. Ryan M. Fryer, unpublished internal results). The mechanism of action for the cardiovascular effect is currently not understood. The recent study with imatinib suggested that protein kinases might be involved in certain hitherto unknown cytoprotective functions in the heart.[8]

Protein kinase inhibitors have become the single largest class of drug discovery targets in the last 10 years. Most of the kinase inhibitors are developed for life-threatening oncology indications. However, there is also great interest in developing kinase inhibitors for more chronic, non-life-threatening indications, such as auto-immune diseases, diabetes, and obesity. Preclinical evaluations of the cardiovascular effects of protein kinase inhibitors have not been widely reported. This report highlighted the potential cardiovascular liability associated with serine/threonine kinase JNK inhibitors, potentially other classes of protein kinase inhibitors as well. Such potential issue should establish the importance of early screening for cardiovascular safety in kinase inhibitor programs, particularly in the area of chronic disease indications, such as diabetes and obesity.

In summary, we have described the application of established in vivo anesthetized rat cardiovascular safety screening in the JNK1 inhibitor lead selection process. This report highlights the hemodynamic effects of the aminopyridine JNK inhibitors including hypotension and a reduction in vascular resistance. Such findings underscore the value and importance of positioning in vivo cardiovascular safety screening early in the lead selection process for kinase programs. Our finding, coupled with recent observation of cardiac toxicity on the other marketed protein kinase inhibitors, calls for more vigorous screening of future kinases inhibitors, particularly for the chronic indications such as metabolic diseases.

Acknowledgments

The authors thank Ms. Sanyi Wang, Ms. Jill Clampit, and Dr. Eric Johnson for the execution of the binding, cellular, and selectivity assays.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.10.013.

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