Bioorganic & Medicinal Chemistry Letters 22 (2012) 1068–1072

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Facile synthesis of tetracyclic azepine and oxazocine derivatives and their potential as MAPKAP-K2 (MK2) inhibitors

Ashwin U. Rao [a,*], Dong Xiao [a], Xianhai Huang [a], Wei Zhou [a], James Fossetta [b], Dan Lundell [b], Fang Tian [b], Prashant Trivedi [b], Robert Aslanian [a], Anandan Palani [a]

[a] Department of Medicinal Chemistry, Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA
[b] Department of Immunology, Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

ARTICLE INFO

Article history:
Received 21 October 2011
Revised 26 November 2011
Accepted 28 November 2011
Available online 6 December 2011

Keywords:
Azepine
Oxazocine
Tetracyclic
MK2

ABSTRACT

Facile synthesis of two new series of tetracyclic azepine and oxazocine analogs is described. These analogs were evaluated for their potential as MAPKAP-K2 (MK2) inhibitors and several were found to be potent at inhibiting MK2 with a non-ATP competitive binding mode.

Published by Elsevier Ltd.

Mitogen-activated protein kinases (MAPKs) belong to the Ser/Thr kinase family, to control cytoskeletal remodeling and regulate the cell cycle. Because of their biological roles, MAPK have been targeted for various chronic inflammatory diseases such as rheumatoid arthritis (RA), Alzheimer's disease, atherosclerosis and cancer.[1] MAPKAP-K2 (MK2), a direct downstream substrate of p38, plays a crucial role in signaling and synthesis of proinflammatory cytokines, such as TNFα, IL-6 and IFNγ.[2] MK2 knockout mice show a strong reduction in disease incidence and disease severity in arthritis models.[3] As MK2 is downstream of p38, its inhibition is expected to produce the same beneficial effect as p38 MAPK inhibition but with reduced side effects. In spite of the strong rationale for MK2 inhibitors for treating human diseases, direct proof of concept in clinical settings has yet to be demonstrated. Several ATP-competitive inhibitors have been published recently, and efforts are continuing to identify novel and more selective MK2 inhibitors.[4] In contrast, non-ATP competitive inhibitors may provide distinct advantages with respect to these issues. In this study we wish to disclose our discovery efforts in targeting this kinase with a non-ATP competitive binding mode and our strategy towards further modification of our lead **1** obtained from the high-throughput Automated Ligand Identification System (ALIS)[5] screening hit **I** leading to the tetracyclic series (Fig 1).

A medicinal chemistry effort was carried out to optimize this encouraging hit into a lead by substitution of the secondary amide in **I** with various alkyl and heteroaryl groups.[6] We hypothesized based on empirical reports that rigidity in the molecule would render proper orientation in the binding pocket since the amide bearing substituents are conformationally flexible and rotation about the amide bond is extremely facile. With this effort we have identified structurally restricted compounds of type **2** and **3** with improved activity (Fig 2).[7] We hypothesized that further restricting the conformation by combining the structural features of **2** and **3** resulting in tetracyclic derivatives may improve MK2 activity and selectivity. We focused our attention on the development of a practical synthetic route to tetracyclic azepine derivatives **4** and oxazocine derivatives **5**.

The synthesis started with preparing the appropriate intermediate **8** as outlined in Scheme 1. Conversion of 5-(4-chlorophenyl)-furan-2-carboxylic acid ethyl ester **9** to benzhydryl alcohol **12** was achieved by reacting **9** with Knochel base and trapping the Mg intermediate with aldehyde **8** (Scheme 2).[8] Reduction of the nitro group was effected with Zn in acetic acid to afford **15** in 90% yield. Triethylsilane reduction of **15** in TFA afforded the benzyl derivative **18** in 59% yield. Upon treatment with LiHMDS, **18** smoothly underwent cyclization to give **21** in 78% yield. Likewise, azepinones **22** and **23** were synthesized in a similar manner described for **21** in 95% yield. The 4-fluorophenyl derivative **24** was obtained from bromide **23** by Suzuki cross-coupling.[9] Compound **23** provides an expedient way to incorporate other aryl groups through changes on the left-hand side of the azepine core. Deprotection of the *N*-Cbz group with TMSI afforded the tricyclic analogs **25** and **26**.

To construct the fourth ring in the molecule, we decided to activate the lactam with $POCl_3$ to generate the reactive chloroiminium

* Corresponding author.
E-mail address: ashwin.rao@merck.com (A.U. Rao).

0960-894X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2011.11.113

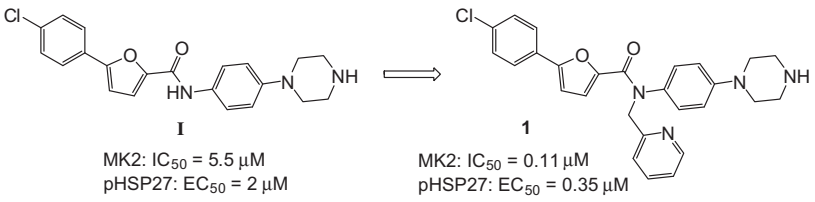


Figure 1. ALIS hit-to-lead modification.

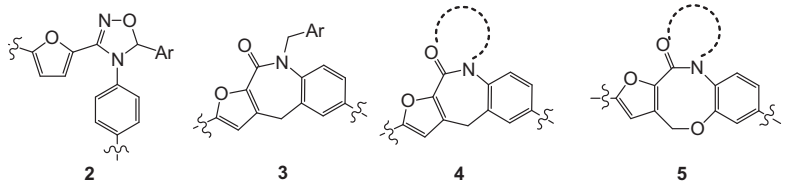
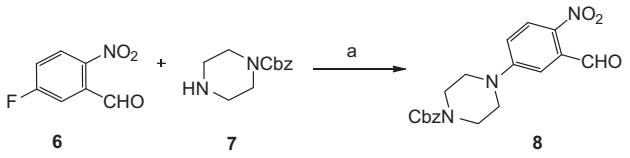


Figure 2. Conformational restraint on hit-to-lead compound **1**.

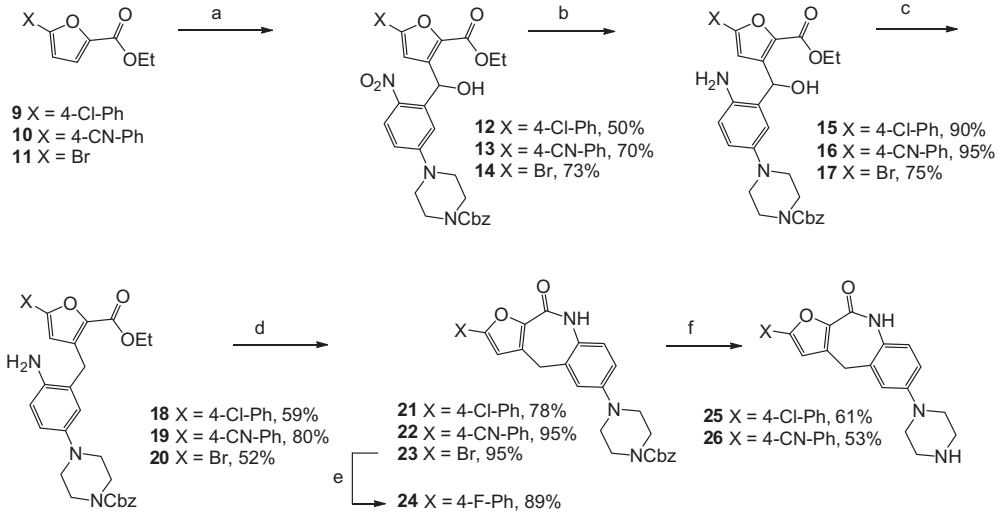


Scheme 1. Synthetic route for the preparation of intermediate **8**. Reagents and conditions: (a) DIEA, DMF, 80 °C, 12 h, 76%.

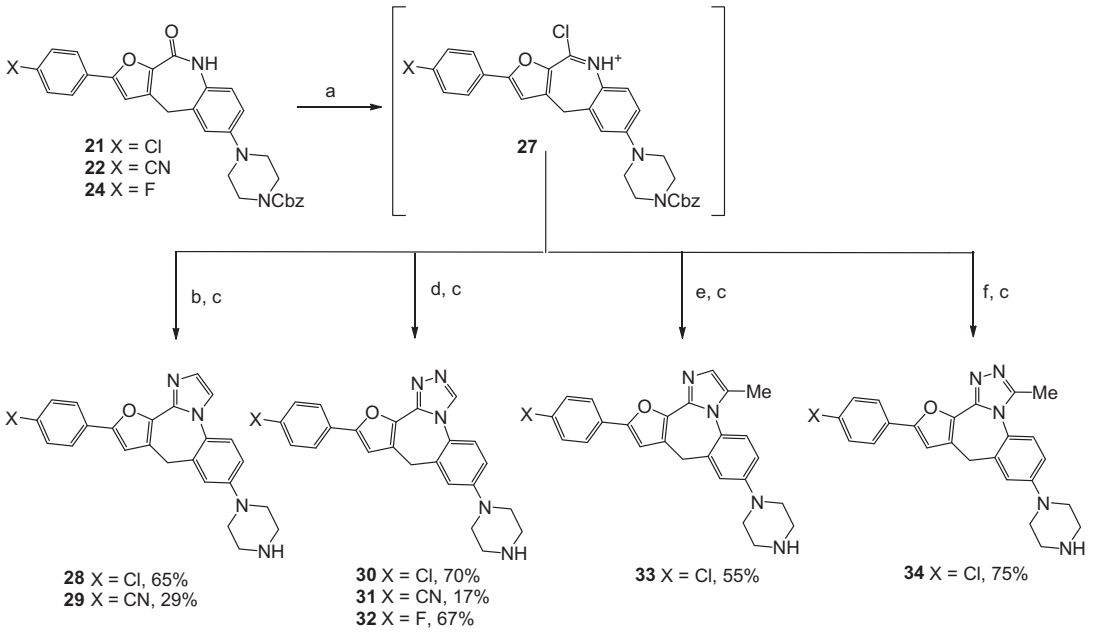
ion intermediate **27** (Scheme 3). Thus $POCl_3$ treatment of **21** generated the active intermediate **27** which upon reaction with aminoacetaldehyde dimethyl acetal followed by a reflux under acidic conditions conveniently led to the preparation of tetracyclic derivative **28**. In a similar fashion, the chloroiminium ion intermediate **27** generated from **21**, **22** and **24** were coupled with formic hydrazide to afford triazoloazepines (**30**–**32**). The analogous methyl substituted imidazo- and triazolo-azepine (**33** and **34**) were obtained by heating **27** with propargylamine and acetic hydrazide, respectively.[10] Considering the ring constraint with the tetracyclic systems, these imidazole and triazole forming conditions are useful techniques.

The synthesis of oxazocine derivative started with the conversion of the commercially available 5-(4-chlorophenyl)-2-formylfuran-3-carboxylic acid ethyl ester **35** to dihydroimidazole by reacting with 1,2-ethylenediamine followed by NBS treatment (Scheme 4).[11] Oxidation with iodophenyl acetate[12] afforded imidazole derivative **36**, followed by the reduction of ester **36** using DIBAL-H to afford **37**. N-Arylation of imidazole **37** with 3-fluoro-4-iodonitro benzene in the presence of Cs_2CO_3 gave a 76% yield of **38** which upon treatment with *t*-BuOK gave a low yielding (25%) nitro derivative **39**. Reduction of nitro group using Zn in acetic acid gave the aniline intermediate in moderate yields, which was subsequently dialkylated with bis(2-bromoethyl)amine hydrogen bromide and basic alumina under neat conditions to yield **40**.

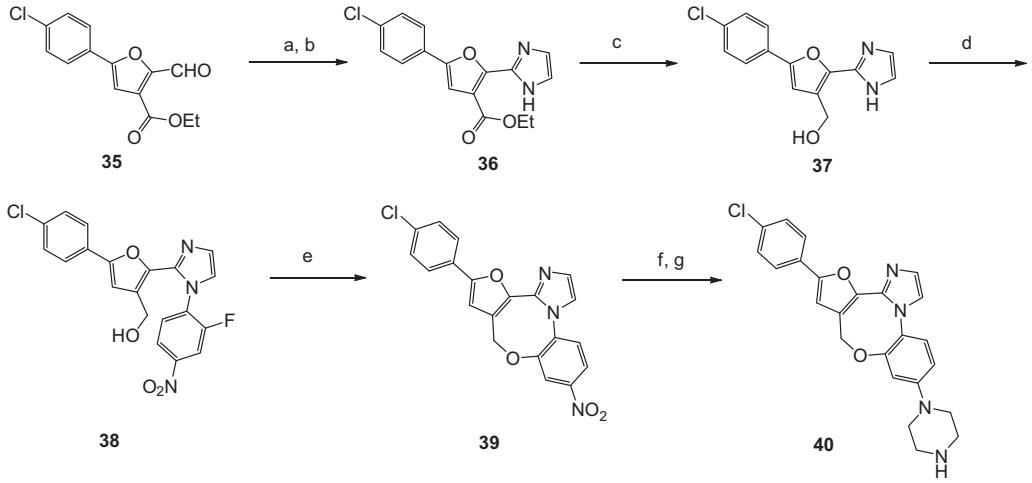
An alternate approach for the preparation of oxazocinone derivatives using **35** required the preparation of **45** and this synthesis was carried out with relative ease as shown in Scheme 5. Treatment of **35** with TMPMgCl·LiCl and quenching with DMF provided aldehyde **39**. Reduction of aldehyde **41** to alcohol was achieved using $NaBH_4$ followed by the formation of the nitro derivative **43**, which was prepared using standard Mitsunobu conditions from **42**, obtained by a similar route as in Scheme 1.[13] Reduction of **43** to amine **44** followed by ring closure using LiHMDS generated



Scheme 2. Synthetic route for the preparation of azepinone derivatives **21**–**26**. Reagents and conditions: (a) TMPMgCl·LiCl, **8**, THF, −30 °C, 2 h; (b) Zn, AcOH, 1 h; (c) Et_3SiH, TFA, CH_2Cl_2, 1 h; (d) LiHMDS, THF, 0 °C, 3 h; (e) $Pd(dppf)_2Cl_2{\cdot}CH_2Cl_2$, K_3PO_4, dioxane, 100 °C, 6 h, 80%; (f) TMSI, CH_2Cl_2, 1 h.



Scheme 3. Synthetic route for the preparation of **27**–**34**. Reagents and conditions: (a) $POCl_3$, 100 °C, 2 h; (b) (i) aminoacetaldehyde dimethyl acetal, THF, reflux, 2 h; (ii) 4 N HCl, reflux, 2 h; (c) TMSI, CH_2Cl_2, 1 h; (d) formic hydrazide, THF, reflux, 2 h; (e) (i) propargylamine, THF, reflux, 2 h; (ii) 4 N HCl, reflux, 2 h; (f) acetic hydrazide, THF, reflux, 2 h.

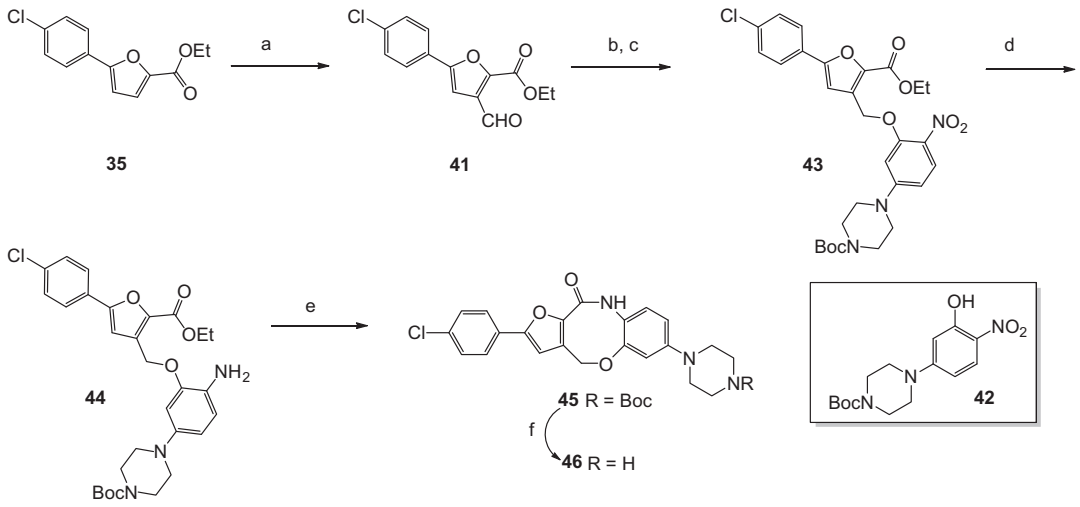


Scheme 4. Synthetic route for the preparation of **40**. Reagents and conditions: (a) 1,2-ethylenediamine, NBS, CH_2Cl_2, 12 h, 55%; (b) $PhI(OAc)_2$, K_2CO_3, DMSO, 12 h, 77%; (c) DIBAL-H, THF, −78 °C, 84%; (d) 3-fluoro-4-iodonitro benzene, Cs_2CO_3, DMF, 65 °C, 12 h, 76%; (e) *t*-BuOK, THF, 6 h, 25%; (f) Zn, AcOH, 2 h, 54%; (g) bis(2-bromoethylamine) hydrogen bromide, Al_2O_3, 120 °C, 12 h, 53%.

45, which could be a useful building block that can be functionalized to give desired tetracyclic products such as imidazo- and triazolo-derivatives as discussed in Scheme 3. Removal of the Boc-group with TFA afforded the tricyclic oxazocine derivative **46** in 75% yield.

Regarding MK2 inhibition (IC_{50})[14] and/or cellular activity (EC_{50}),[15] the conformationally restricted tetracycles proved to be clearly superior to the non-cyclized compound **1** and tricyclic analogs **25**, **26** and **46** (Table 1). Thus, a comparison of **1** and azepine **28** revealed that the former was more potent in cell-based activity, while the cyclized analog **28** was 16-fold more potent against MK2. We attributed the lack of cellular activity due to their differences in solubility (20 μM vs >200 μM). A similar level of potency improvement was observed while comparing **28** with the tricyclic analogs **25** and **26**. The corresponding cyano derivative **29** restored cellular activity at high nanomolar concentration (EC_{50} = 0.26 μM). Compounds **30** and **33** with chloro substitution showed high MK2 affinity but with reduced cellular activity. On the other hand cyano derivative **31** showed similar MK2 and cellular inhibition profiles as **29**. Compound **32** had no major effect on MK2 inhibition however the fluoro substitution had a favorable effect on solubility. Interestingly, the less soluble derivatives **29** and **31** showed improved cellular activity indicating that cellular potency depend upon various additional parameters. The IC_{50} for MK2 inhibition for the oxazocine derivative **40** was determined to be 18 nM with improved solubility and was 6-fold more potent than the corresponding tricyclic analog **46**. The mode of inhibition of MK2 by azepine **31** and oxazocine **40** with respect to peptide substrate, TAMRA peptide was determined by analysis of MK2 activity in the presence of saturating ATP and varying concentrations of peptide substrate and the inhibitor (Fig 3). The results identified a non-ATP competitive binding mode throughout the series.[16]

In conclusion, we have demonstrated practical synthetic routes for generating tetracyclic azepine and oxazocine pharmacophores



Scheme 5. Synthetic route for the preparation of oxazocinone core **45**. Reagents and conditions: (a) TMPMgCl·LiCl, DMF, THF, 2 h, 15%; (b) $NaBH_4$, MeOH, 2 h, 70%; (c) DIAD, PPh_3, **42**, THF, 12 h, 65%; (d) Zn, AcOH, 2 h, 95%; (e) LiHMDS, THF, 0 °C, 3 h, 65%; (f) TFA, CH_2Cl_2, 75%.

Table 1
Conformationally restricted MK2 inhibitors

Compd	MK2[a] IC_{50} (nM)	p-HSP27[b] EC_{50} (μM)	Solubility[d] (μM)
1	110	0.35	>200
25	96 ± 20	ND[c]	ND[c]
26	110 ± 10.0	ND[c]	ND[c]
28	7.0 ± 0.23	1.5 ± 0.14	20
29	2.9 ± 0.31	0.26 ± 0.04	<16
30	9.3 ± 0.92	2.1 ± 0.38	10
31	3.8 ± 0.55	0.26 ± 0.06	16
32	15 ± 0.57	ND[c]	33
33	9.2 ± 0.95	2.5 ± 0.49	20
34	880 ± 22	ND[c]	10
40	18 ± 3.2	ND[c]	38
46	110 ± 11	ND[c]	ND[c]

Data represent the average values of duplicates or triplicates ± standard deviation.
[a] MK2 IMAP assay.
[b] Inhibition of HSP27 phosphorylation in LPS-stimulated THP-1 cells.
[c] ND, not determined.
[d] Kinetic solubility measured at 7.4 pH.

that show potent inhibition against MK2 with a non-ATP competitive binding mode as well as inhibiting HSP27 phosphorylation in THP-1 cells. Highly constrained tetracyclic synthons **21**, **22**, **24** and **45** can be easily generated through the use of the chemistry described herein and are flexible building blocks that can be functionalized to give products such as **28**–**34** and **40**. Compounds of this series may offer an excellent tool for specifically exploring and validating MK2 biology.

Acknowledgments

We thank Dr. Chad Bennett for comments on the preparation of the manuscript and Drs. Malcolm MacCoss and Ismail Kola for their support and encouragement.

References and notes

1. (a) Gaestel, M. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 120; (b) Gaestel, M.; Kotlyarov, A.; Kracht, M. *Nat. Rev. Drug Disc.* **2009**, *8*, 480; (c) Mbalaviele, G.; Monahan, J. B. *Expert Opin. Drug Discov.* **2008**, *3*, 163; (d) Duraisamy, S.; Bajpai, M.; Bughani, U.; Dastidar, S. G.; Ray, A.; Chopra, P. *Expert Opin. Ther. Targets* **2008**, *12*, 921.
2. Kotlyarov, A.; Yannoni, Y.; Fritz, S.; Laass, K.; Telliez, J. B.; Pitman, D.; Lin, L. L.; Gaestel, M. *Mol. Cell Biol.* **2002**, *22*, 4827.
3. Hegen, M.; Gaestel, M.; Nickerson-Nutter, C. L.; Lin, L. L.; Telliez, J. B. *J. Immunol.* **2006**, *177*, 1913.
4. (a) Anderson, D. R.; Hegde, S.; Reinhard, E.; Gomez, L.; Vernier, W. F.; Lee, L.; Liu, S.; Sambandam, A.; Snider, P. A.; Masih, L. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1587; (b) Wu, J.-P.; Wang, J.; Abeywardane, A.; Andersen, D.; Emmanuel, M.; Gautschi, E.; Goldberg, D. R.; Kashem, M. A.; Lukas, S.; Mao, W.; Martin, L.; Morwick, T.; Moss, N.; Pargellis, C.; Patel, U. R.; Patnaude, L.; Peet, G. W.; Skow, D.; Snow, R. J.; Ward, Y.; Werneburg, B.; White, A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4664; (c) Trujillo, J. I.; Meyers, M. J.; Anderson, D. R.; Hegde, S.; Mahoney, M. W.; Vernier, W. F.; Buchler, I. P.; Wu, K. K.; Yang, S.; Hartmann, S. J.; Reitz, D. B. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4657; (d) Goldberg, D. R.; Choi, Y.; Cogan, D.; Corson, M.; DeLeon, R.; Gao, A.; Gruenbaum, L.; Hao, M. H.; Joseph, D.; Kashem, M. A.; Miller, C.; Moss, N.; Netherton, M. R.; Pargellis, C. P.; Pelletier, J.; Sellati, R.; Skow, D.; Torcellini, C.; Tseng, Y.-C.; Wang, J.; Wasti, R.; Werneburg, B.; Wu, J. P.; Xiong, Z. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 938; (e) Xiong, Z.; Gao, D. A.; Cogan, D. A.; Goldberg, D. R.; Hao, M.-H.; Moss, N.; Pack, E.; Pargellis, C.; Skow, D.; Trieselmann, T.; Werneburg, B.; White, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 1994; (f) Anderson, D. R.; Meyers, M. J.; Vernier, W. F.; Mahoney, M. W.; Kurumbail, R. G.; Caspers, N.; Poda, G. I.; Schindler, J. F.; Reitz, D. B.; Mourey, R. J. *J. Med. Chem.* **2007**, *17*, 2647; (g) Lin, S.; Lombardo, M.; Malkani, S.; Hale, J. J.;

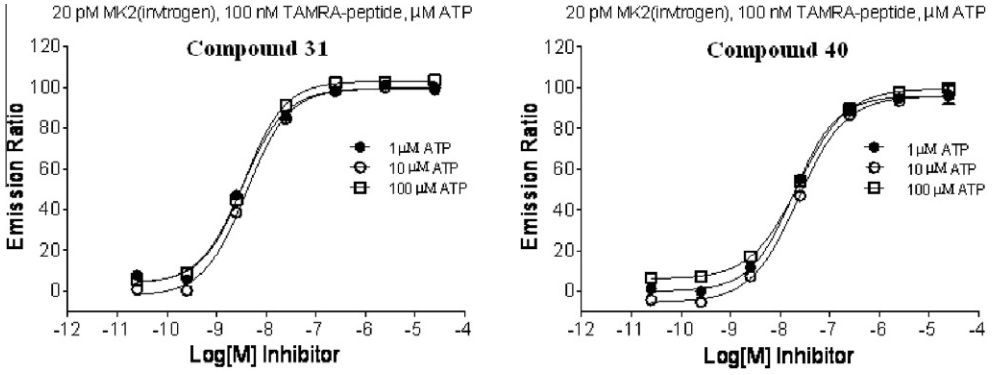


Figure 3. MK2 non-competitive inhibition analysis of compounds **31** and **40**.

Mills, S. G.; Chapman, K.; Thompson, J. E.; Zhang, W. X.; Wang, R.; Cubbon, R. M.; O'Neill, E. A.; Luell, S.; Carballo-Jane, E.; Yang, L. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3238; (h) Anderson, D. R.; Meyers, M. J.; Kurumbail, R. G.; Caspers, N.; Poda, G. I.; Long, S. A.; Pierce, B. S.; Mahoney, M. W.; Mourey, R. J. *Bioorg. Med.Chem. Lett.* **2009**, *19*, 4878; (i) Anderson, D. R.; Meyers, M. J.; Kurumbail, R. G.; Caspers, N.; Poda, G. I.; Long, S. A.; Pierce, B. S.; Mahoney, M. W.; Mourey, R. J.; Parikh, M. D. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 4882; (j) Harris, C. M.; Ericsson, A. M.; Argiriadi, M. A.; Barberis, C.; Borhani, D. W.; Burchat, A.; Calderwood, D. J.; Cunha, G. A.; Dixon, R. W.; Frank, K. E.; Johnson, E. F.; Kamens, J.; Kwak, S.; Li, B.; Mullen, K. D.; Perron, D. C.; Wang, L.; Wishart, N.; Wu, X.; Zhang, X.; Zmetra, T. R.; Talanian, R. V. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 334; (k) Schlapbach, A.; Feifel, R.; Hawtin, S.; Heng, R.; Koch, G.; Moebitz, H.; Revesz, L.; Scheufler, C.; Velcicky, J.; Waelchli, R.; Huppertz, C. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 6142; (l) Velcicky, J.; Feifel, R.; Hawtin, S.; Heng, R.; Huppertz, C.; Koch, G.; Kroemer, M.; Moebitz, H.; Revesz, L.; Scheufler, C.; Schlapbach, A. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1293; (m) Revesz, L.; Schlapbach, A.; Aichholz, R.; Dawson, J.; Feifel, R.; Hawtin, S.; Littlewood-Evans, A.; Koch, G.; Kroemer, M.; Moebitz, H.; Scheufler, C.; Velcicky, J.; Huppertz, C. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4719; (n) Barf, T.; Kaptein, A.; de Wilde, S.; van der Heijden, R.; van Someren, R.; Demont, D.; Schultz-Fademrecht, C.; Versteegh, J.; van Zeeland, M.; Seegers, N.; Kazemier, B.; van de Kar, B.; van Hoek, M.; de Roos, J.; Klop, H.; Smeets, R.; Hofstra, C.; Hornberg, J.; Oubrie, A. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3818; (o) Kaptein, A.; Oubrie, A.; de Zwart, E.; Hoogenboom, N.; de Wit, J.; van de Kar, B.; van Hoek, M.; Vogel, G.; de Kimpe, V.; Schultz-Fademrecht, C.; Borsboom, J.; van Zeeland, M.; Versteegh, J.; Kazemier, B.; de Roos, J.; Wijnands, F.; Dulos, J.; Jaeger, M.; Leandro-Garcia, P.; Barf, T. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 3823.

5. Annis, A.; Chuang, C.-C.; Nazef, N. ALIS: An Affinity Selection Mass Spectrometry System for the Discovery and Characterization of Protein-Ligand Interactions. In Methods and Principles in Medicinal Chemistry; Wanner, K.; Höfner, G., Eds.; In Mass Spectrometry in Medicinal Chemistry: Applications in Drug Discovery; Mannhold, R.; Kubinyi, H.; Folkers, G., Series Eds.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, 2007; Vol. 36, Chapter 3.
6. Huang, X.; Shipps, G. W., Jr.; Cheng, C. C.; Spacciapoli, P.; Zhang, X.; McCoy, M. A.; Wyss, D. F.; Yang, X.; Achab, A.; Soucy, K.; Montavon, D. K.; Murphy, D. M.; Whitehurst, C. E. *ACS Med. Chem. Lett.* **2011**, *2*, 632.
7. (a) Xiao, D.; Palani, A.; Aslanian, R. G.; Degrado, S.; Huang, X.; Zhou, W.; Sofolarides, M.; Chen, X. Patent WO 2011075375 A1, 2011.; (b) Qin, J.; Dhondi, P.; Huang, X.; Aslanian, R. G.; Fossetta, J.; Tian, F.; Lundell, D.; Palani, A. *ACS Med. Chem. Lett.*, Submitted for publication.
8. Krasovskiy, A.; Krasovskaya, V.; Knochel, P. *Angew. Chem., Int. Ed.* **2006**, *45*, 2958.
9. Miyaura, N.; Suzuki, A. *Chem. Rev.* **1995**, *95*, 2457.
10. Di Braccio, M.; Grossi, G.; Roma, G.; Vargiu, L.; Mura, M.; Marongiu, M. E. *Eur. J. Med. Chem.* **2001**, *36*, 935.
11. Fujioka, H.; Murai, K.; Ohba, Y.; Hiramatsu, A.; Kita, Y. *Tetrahedron Lett.* **2005**, *46*, 2197.
12. Ishihara, M.; Togo, H. *Synlett* **2006**, 227.
13. Mitsunobu, O. *Synthesis* **1981**, 1.
14. *MK2 IMAP assay*: All the components of MK2 phosphorylation reaction made are 4× concentrated in 1× reaction buffer containing10 mM Tris, pH 7.2, 10 mM $MgCl_2$, 1 mM DTT, 0.05% azide and 0.01% Tween 20 and the reaction was carried in 384 well black reaction plate at room temperature in dark. Mix 5 μL of 4× inhibitor in 4% DMSO, 5 μL of 400 μM ATP and 5 μL of 200 pM MK2 kinase and incubate for 30 min. Reaction started by adding 5 mL of 400 nM TAMRA labeled peptide and incubating 30 min in dark. The final concentrations are: 1x inhibitor, 1% DMSO, 100 μM ATP, 50 pM MK2 and 100 nM substrate. Reaction was stopped by adding 60 μL of 1:400 diluted Progressive Binding Reagent in 1× Progressive Binding Buffer A and incubating 30 min in dark. Read plate at Analyst HT 96-384 Plate Reader (LJL BioSystem) equipped with Fluorescence Polarization module (Excitation wavelength 530 nm and Emission wavelength 580 nm).
15. *LPS Induced Phospho-HSP27 Serine78 assay*: Bring the THP-1 cells to Log-phase by passing the cells at proper density a day previous to the assay ($\sim 1 \times 10^5$ cells/mL). Spin to collect cells and suspend in fresh complete-RPMI-1640 Medium at cell density of 2.5×10^6 cells/mL. Add equal volume of RPMI-1640 without FBS containing 40 nM Okadaic Acid(final:100,000 cells/80 μL/well). Plate 100,000 cells/80 μL into flat-bottom cell culture plate and incubate at 37 °C for 60 min. Add 10 μL of 10× diluted-compound in 1% DMSO in 5% FBS-RPMI medium and incubate at 37 °C for 60 min. Add 10 μL of 10× LPS in 5% FBS-RPMI medium and incubate at 37 °C for 60 min. Add 25 μL 5× Cell Lysis Buffer containing 5× Halt Inhibitors and incubate on ice for 30 min. Transfer cell lysate to glass fiber filter plate and stack the filter plate on top of a 96-well storage plate. Spin stacked filter-storage plate at 3500 rpm for 5 min at 4 °C. MesoScale pHSP27 S78 Assay 10–20 μL cell lysate was used in this assay.
16. Zhang, R.; Monsma, F. *Curr. Opin. Drug Discov. Dev.* **2010**, *13*, 389.