MedChemComm

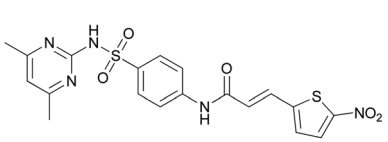
c3md00278k

CONCISE ARTICLE

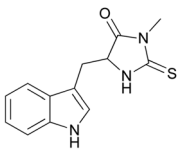
Necrosulfonamide inhibits necroptosis by selectively targeting the mixed lineage kinase domain-like protein

Daohong Liao, Liming Sun, Weilong Liu, Sudan He, Xiaodong Wang and Xiaoguang Lei*

Through high-throughput screening of 200 000 compounds and subsequent structure–activity relationship (SAR) studies we identified necrosulfonamide (NSA) as a potent small molecule inhibitor for necroptosis, induced by a combination of TNF-a, Smac mimetic, and z-VAD-fmk (T/S/Z).



Hit #14



Nec-1

Please check this proof carefully. **Our staff will not read it in detail after you have returned it.**

Translation errors between word-processor files and typesetting systems can occur so the whole proof needs to be read. Please pay particular attention to: tabulated material; equations; numerical data; figures and graphics; and references. If you have not already indicated the corresponding author(s) please mark their name(s) with an asterisk. Please e-mail a list of corrections or the PDF with electronic notes attached - do not change the text within the PDF file or send a revised manuscript. Corrections at this stage should be minor and not involve extensive changes. All corrections must be sent at the same time.

Please bear in mind that minor layout improvements, e.g. in line breaking, table widths and graphic placement, are routinely applied to the final version.

We will publish articles on the web as soon as possible after receiving your corrections; **no late corrections will be made.**

Please return your **final** corrections, where possible within **48 hours** of receipt by e-mail to: medchemcomm@rsc.org

Queries for the attention of the authors

Journal: MedChemComm

Paper: c3md00278k

Title: Necrosulfonamide inhibits necroptosis by selectively targeting the mixed lineage kinase domain-like protein

Editor's queries are marked like this... 1, and for your convenience line numbers are inserted like this... 5

Please ensure that all queries are answered when returning your proof corrections so that publication of your article is not delayed.

Query Reference	Query	Remarks
1	For your information: You can cite this article before you receive notification of the page numbers by using the following format: (authors), J. Mater. Chem. A, (year), DOI: 10.1039/c3ta14532h.	
2	Please carefully check the spelling of all author names. This is important for the correct indexing and future citation of your article. No late corrections can be made.	
3	Please check that the inserted GA text is suitable.	
4	Please check that changes to the English in this sentence have not affected the meaning.	
5	Please check that changes to the English in this sentence have not affected the meaning.	

MedChemComm

CONCISE ARTICLE

Necrosulfonamide inhibits necroptosis by selectively targeting the mixed lineage kinase domain-like protein†

Cite this: DOI: 10.1039/c3md00278k

Daohong Liao,[ab] Liming Sun,[b] Weilong Liu,[b] Sudan He,[b] Xiaodong Wang[b] and Xiaoguang Lei*[ab]

Received 5th November 2013
Accepted 19th January 2014

DOI: 10.1039/c3md00278k

www.rsc.org/medchemcomm

Through high-throughput screening of 200 000 compounds and subsequent structure–activity relationship (SAR) studies we identified necrosulfonamide (NSA) as a potent small molecule inhibitor for necroptosis, induced by a combination of TNF-a, Smac mimetic, and z-VAD-fmk (T/S/Z). Applying a forward chemical genetic approach, we utilized an NSA based chemical probe to further reveal that NSA selectively targeted the Mixed Lineage Kinase Domain-like Protein (MLKL) to block the necrosome formation.

Introduction

Programmed necrosis, also known as necroptosis, is morphologically marked by the swelling of cellular organelles, rapid loss of cell membrane integrity and release of cellular contents. Tumor necrosis factor (TNF) induced necrotic cell death is the most-studied necrotic pathway. Ligation of TNF receptors initiates necrosis under conditions when apoptotic execution is prevented. Initial studies revealed that the necrosis death complex of RIP1/RIP3 assembled at the downstream of TNFα signaling.[1] The formation of the RIP1/RIP3 complex depends on the kinase activity of RIP1. The RIP homotypic interaction motifs (RHIMs) of RIP1 and RIP3 mediate amyloid signaling complex formation,[2] which propagates the kinase signals to downstream substrates. When RIP1 binds with RIP3, the kinase activity of RIP3 is triggered. However, extensive RNAi screenings unfortunately did not provide further information about the downstream substrates of RIP3.

Chemical genetics has been proven to be a powerful means to dissecting complex biological pathways. It could provide us with novel chemical tools to expand our study on the necrosis pathway. Necrostatin-1 (Nec-1), a small molecule that has been discovered as the first chemical inhibitor for necrosis, suppresses necrosis by blocking RIP1 kinase activity.[3] It has been widely used in disease models. In addition, structural analysis revealed necrostatin-1 stabilizes RIP1 into an inactive state through binding with its kinase activation loop.[4] As an upstream inhibitor, Nec-1 blocks necrosis by disrupting necrosome formation. Combined treatment of the necrosis inducer with Nec-1 would interfere with the RIP1/RIP3 interaction and the phosphorylation modifications on RIP1 and RIP3. Moreover, the protective effect conferred by Nec-1 was found to attribute to other targets besides RIP1.[5] Those studies highlighted the need for a potent downstream inhibitor, which does not affect the death complex formation. That will also serve as a powerful chemical tool to help us find new players in this pathway. Herein, we report our recent chemical genetic efforts which lead to the identification of necrosulfonamide (NSA) as the first downstream small molecule inhibitor for necroptosis, as well as the discovery of the Mixed Lineage Kinase Domain-like Protein (MLKL) as a direct cellular target for NSA.[6]

Results and discussion

Synthesis of the analogs of hit #14

Through initial high-throughput screening of ~200 000 compounds we identified a hit compound #**14** (Fig. 1), which specifically blocked necroptosis induced by a combination of TNF-a, Smac mimetic, and z-VAD-fmk (T/S/Z) in both human

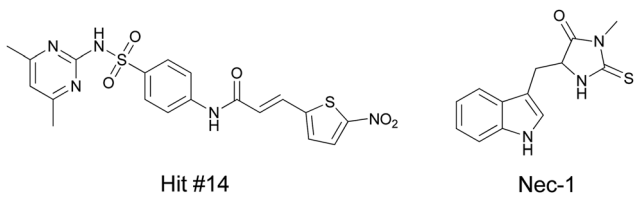


Fig. 1 Structures of chemical inhibitors for necroptosis.

[a]College of Pharmaceutical Science and Technology, Tianjin University, Tianjin 300072, China

[b]National Institute of Biological Sciences (NIBS), Beijing 102206, China. E-mail: leixiaoguang@nibs.ac.cn

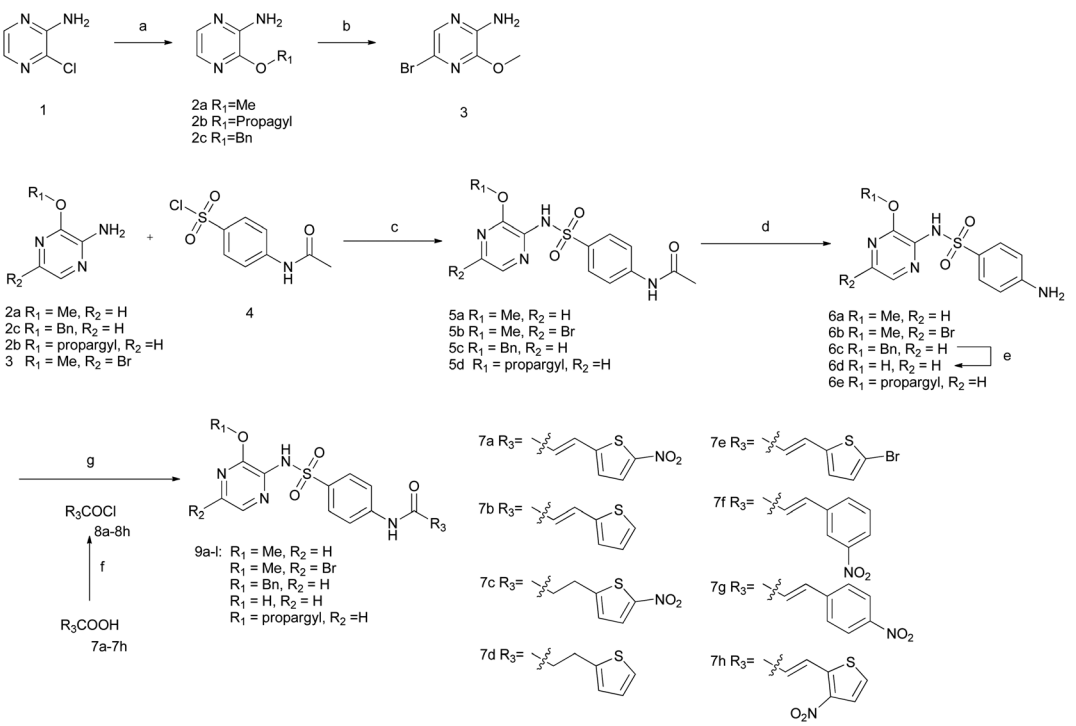
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3md00278k

colon cancer HT-29 cells and FADD null human T cell leukemia Jurkat cells.

Based on the structure of hit #**14**, we designed a synthetic route to access its derivatives and analogs in order to explore the structure–activity relationship (SAR). The synthesis began with the readily available starting material 3-chloropyrazin-2-amine (**1**)[7] (Scheme 1). Substitution of **1** with different alkoxy functionalities to introduce R1 group and furnish the corresponding 2-aminopyrazine derivatives **2a**,[8] **2b**, **2c**.[9] Bromination of **2a** with NBS afforded bromide **3** in moderate to high yield depending on the reaction scale. Pyrazines **2a**, **2b**, **2c** and **3** were further treated with 4-acetamidobenzene-1-sulfonyl chloride (**4**) in pyridine to furnish compounds **5a–5d** in 60–70% yield, respectively. The acetyl protecting group in compounds **5a–5d** was removed by refluxing in 3 M aqueous sodium hydroxide solution. Debenzylation of **6c** was smoothly achieved *via* palladium-catalyzed (5% Pd/C) hydrogenolysis under atmospheric hydrogen gas. Finally, the corresponding anilines **6a–6e** were coupled with a series of acyl chlorides **8a–8h**, *in situ* derived from carboxylic acids **7a–7h**, by refluxing with thionyl chloride in toluene to afford a number of sulfonamides **9a–9l** in 75–93% yield (Scheme 1).

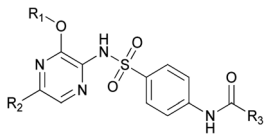
Structure–activity relationship

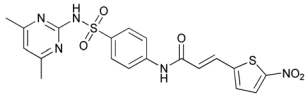
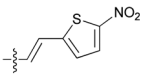
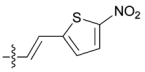
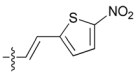
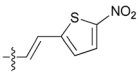
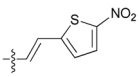
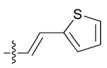
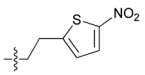
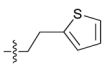
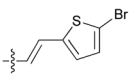
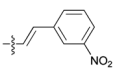
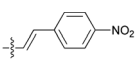
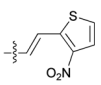
With a number of sulfonamide analogs in hand, we next sought to systematically evaluate the structure–activity relationship (SAR) by cell death assay in HT-29 cells. Necrosis was induced by adding the final concentrations of 20 ng ml^{-1} TNF-α (T), 100 nM Smac mimetic (S), and 20 mM z-VAD (Z) to the cell culture wells. Then the cells were treated with each compound at a concentration of 4 μM for 24 h. The number of surviving cells was normalized to control cells that were treated with DMSO. The biological activities of different analogs (compound **9a–9k**) were compared to that of the hit compound #**14** and the results were summarized in Table 1. Through these biological evaluations we observed that while a pyrazine ring was introduced to replace the original pyrimidine scaffold, the resulting compound **9a** showed similar activity compared with the initial hit compound #**14**. When a bromo substituent was introduced at the C-5 position, to our delight, the resulting compound **9b** was more potent than the initial hit compound #**14**. Replacing the methyl group in **9a** with a bigger benzyl group gave rise to compound **9c**, which showed similar biological activity. The removal of the C-3 methyl group gave **9d** with retained activity compared to **9c**, while the same result was observed for **9e** containing a propargyl substituent. It appeared that the substituent on the pyrazine ring might not adversely affect the potency. The effort was next focused on the evaluation of the thiophene moiety (Table 1). Removal of the nitro group gave **9f**, which showed significantly decreased activity. Similar effects were observed for compounds with other modifications on the thiophene system (**9g**, **9h**, **9i**, **9j**, **9k**), from the reduced double bond in **9g** and **9h**, to replacement of the nitro group with a bromo group in **9i**. In addition, other nitro substituted aromatic rings in **9j**, **9k** and **9l** all reduced the activities. These results highlighted the significance of the α,β-unsaturated enone system, which could be a potential Michael acceptor, as well as the thiophene system



Scheme 1 *Reagent and conditions*: (a) NaH, ROH, rt, 1.5 h, 80 °C, 2 h, 51–95%; (b) NBS, DCM, 0 °C, 45 min, rt, 2 h, 54–91% (different reaction scales); (c) pyridine, 0–60 °C, overnight, 60–77%; (d) 3 M NaOH/H_2O, 105 °C, 4 h, 80–87%; (e) H_2, 5% Pd/C, MeOH, rt, 2 h, 45%; (f) $SOCl_2$, toluene, reflux, 5 h; (g) R_3COCl, toluene, pyridine, 0 °C to rt, 4–10 h, 75–93%.

Table 1 Biological data for sulfonamides **9a–9l**[a]



Compd.	R1	R2	R3	Mp (°C)	Cell survival[a]/(%)
#14				288–290	79.2
9a	Me	H		288–289	80.3
9b	Me	Br		282–283	90.8
9c	Bn	H		238–239	92.2
9d	H	H		264–270	90.4
9e	Propargyl	H		205–207	90.8
9f	Me	H		224–226	29.5
9g	Me	H		164–167	29.9
9h	Me	H		234–236	30.4
9i	Me	H		219–221	31.8
9j	Me	H		280–282	32.0
9k	Me	H		279–281	32.2
9l	Propargyl	H		235–237	19.8

[a] The biological activities of the analogs against TNF-α-induced necrosis was evaluated by a cell death assay in HT-29 cells at a concentration of 4 μM. The values of cell survival rate were evaluated by the means of cellular ATP levels.

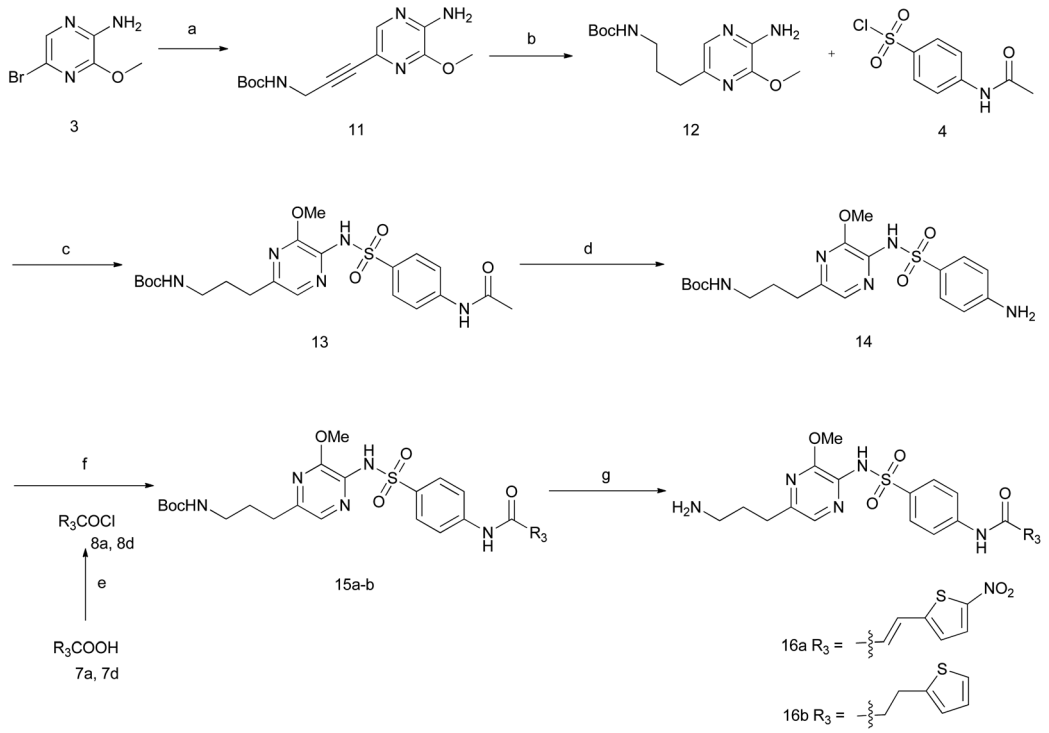
with appropriate substitutions. Meanwhile the presence of different substituents on the pyrazine (**9b**, **9c**, **9d**, and **9e**) did not affect the activities for blocking necrosis. Collectively, the data from SAR studies provided us with a sound direction to design and prepare both the positive and negative chemical probes. Considering both potency and synthetic feasibility of the active analogs we selected compound **9a**, necrosulfonamide (NSA), as our lead compound for subsequent biological studies.

Design and synthesis of the chemical probes

Based on the SAR studies, we envisioned that functionalization of the pyrazine ring in NSA should be tolerated. Accordingly, we planned to add an amino group in NSA, which could be further used to couple with a biotin tag for pull-down experiments with streptavidin-conjugated beads. The synthesis began with bromide **3** (Scheme 2). A Sonogashira cross-coupling reaction[10] between **3** and *tert*-butyl prop-2-yn-1-ylcarbamate (**10**) in the presence of a palladium catalyst afforded alkyne **11**, which was further reduced by palladium-catalyzed (5% Pd/C) hydrogenation under atmospheric hydrogen gas to afford intermediate **12**. Adopting the previously developed route for the synthesis of sulfonamide analogues, compound **12** was treated with 4-acetamidobenzene-1-sulfonyl chloride (**4**) in pyridine to furnish sulfonamide **13**. Deprotection of the acetyl group smoothly generated amine **14**. Amidation of **14** with two different acyl chlorides which were generated *in situ* from the corresponding carboxylic acids **7a** and **7d**, respectively, afforded compounds **15a** and **15b**. Removal of the Boc group under acidic conditions (TFA in DCM) generated the desired amines **16a** and **16b**, respectively, in high yields.

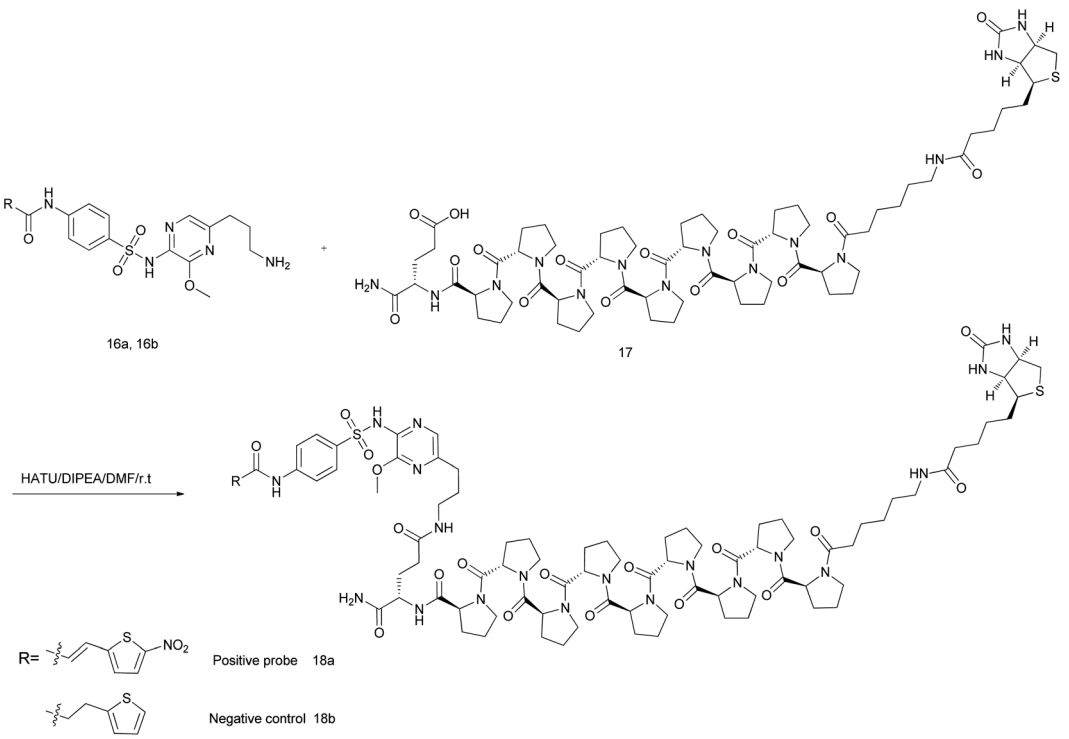
With these probe precursors **16a** and **16b** in hand we began the synthesis of biotin-labelled chemical probes. Biotinylation of small molecules often significantly decreases their aqueous solubility and bioactivity. This problem could be effectively solved by inserting a suitable chemical linker between biotin and the target molecule. Initially we utilized the commonly used triethylene glycol linkers[11] for our chemical probes, however, the result for the pull-down experiment was not satisfying, which did not show a clean background on the silver staining gel, presumably due to non-specific binding. To enhance the capacity of the affinity purification process and to allow for the isolation of low-abundance or low-affinity proteins, a longer linker with optimized chemical and physical properties would be desirable. In 2007, Uesugi and co-workers reported a remarkable polyproline-rod linker,[12] which could be effectively incorporated between the biotin tag and the target compound. This long and rigid polyproline helix spacer was proven to be very effective in facilitating the identification of low-abundance or low-affinity proteins. Therefore, we decided to adopt this strategy in the preparation of NSA-based probes (Scheme 3). Necrosulfonamide (NSA) analogues **16a** and **16b** were coupled with the readily available Biotin-LC-proline acid **17** under effective peptide coupling conditions (HATU/DIPEA/DMF) to afford the desired chemical probes **18a** (positive probe) and **18b** (negative probe), respectively, which were further purified by preparative reverse-phase HPLC. To our delight, the positive chemical probe **18a** showed potent activity to blocking necrosis at 4 μM, while the negative probe **18b** lost most of its activity at the same concentration (Fig. 2). These results encouraged us to use these small molecule chemical probes for the following target identification.

With both positive and negative NSA-based chemical probes **18a** and **18b** in hand, we were able to perform the pull-down experiment to identify the cellular target(s) for NSA. As shown in a previous report,[6] we successfully discovered and elucidated



Scheme 2 *Reagent and conditions*: (a) *tert*-Butyl prop-2-yn-1-ylcarbamate (**10**), $PdCl_2(PPh_3)_2$, CuI, TEA, 80 °C, 6 h, 78–86%; (b) 5% Pd/C, MeOH, H_2, rt, overnight, 99%; (c) pyridine, 0–60 °C, overnight, 60–77%; (d) 3 M NaOH/H_2O, 105 °C, 4 h, 80–87%; (e) $SOCl_2$, toluene, reflux, 5 h; (f) R_3COCl, toluene, pyridine, 0 °C to rt, 4–10 h, 75–93%; (g) TFA, DCM, 0 °C to rt, 99%.

that the Mixed Lineage Kinase Domain-like Protein (MLKL) was a direct target for NSA. Here, we also showed that when we used the positive probe **18a** to pull down MLKL *in vitro*, the non-biotin labelled necrosulfonamide **9a** could efficiently compete



Scheme 3 Synthesis of positive and negative chemical probes.

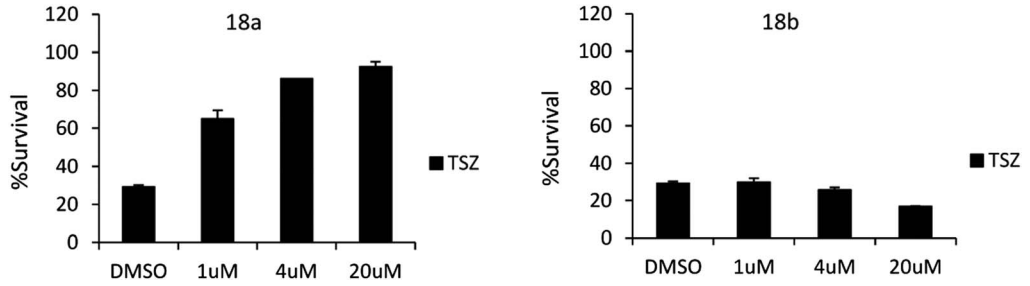


Fig. 2 Biological evaluations of the positive and negative chemical probes against TNF-α-induced necrosis.

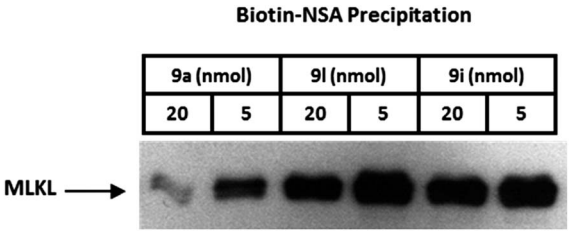


Fig. 3 Identification of MLKL as cellular target for NSA. Precipitation analysis of necrosulfonamide *versus* the derivatives with MLKL. At 24 h post-transfection of Myc-tagged cDNA of MLKL in RIP-HeLa cells, 1 mg aliquots of whole-cell lysates from HeLa cells were used for one condition of precipitation analysis with biotinylated necrosulfonamide. As indicated, the non-biotinylated necrosulfonamide or the derivatives was included in the lysates as competitive baits. The pellets of necrosulfonamide conjugates were analyzed by western blotting using anti-Myc antibody.

with MLKL binding to the beads at the concentration of 20 nM, but the inactive compounds **9i** and **9l** could not (Fig. 3).

Conclusions

In summary, we have described the detailed synthesis and biological evaluation of a series of NSA analogues based on the hit compound #**14** identified from high-throughput screening of ~200 000 compounds. Based on the subsequent SAR study we designed and synthesized the corresponding bioactive chemical probes, which allowed us to identify the Mixed Lineage Kinase Domain-like Protein (MLKL) is the direct cellular target for NSA. In light of these findings on NSA, implementation of chemical genetics will gain us improved understanding on necroptosis signal transduction beyond MLKL in the future.

Acknowledgements

We thank Ms Mingyan Zhao (NIBS) for NMR and HPLC-MS analysis, and Dr Jiang Zhou (Peking University) for HRMS analysis. This work was supported by the National Basic Research Program of China (973 Program 2012CB837400) and the National Natural Science Foundation of China (NNSFC 21072150, 21222209).

References

1 (*a*) Y. S. Cho, S. Challa, D. Moquin, R. Genga, T. D. Ray, M. Guildford and F. K. Chan, *Cell*, 2009, **137**, 1112; (*b*) S. He, L. Wang, L. Miao, T. Wang, F. Du, L. Zhao and X. Wang, *Cell*, 2009, **137**, 1100; (*c*) N. Holler, R. Zaru, O. Micheau, M. Thome, A. Attinger, S. Valitutti, J. L. Bodmer, P. Schneider, B. Seed and J. Tschopp, *Nat. Immunol.*, 2000, **1**, 489; (*d*) D. W. Zhang, J. Shao, J. Lin, N. Zhang, B. J. Lu, S. C. Lin, M. Q. Dong and J. Han, *Science*, 2009, **325**, 332–336.

2 J. Li, T. McQuade, A. B. Siemer, J. Napetschnig, K. Moriwaki, Y. S. Hsiao, E. Damko, D. Moquin, T. Walz, A. McDermott, *et al.*, *Cell*, 2012, **150**, 339.

3 (*a*) A. Degterev, J. Hitomi, M. Germscheid, I. L. Ch'en, O. Korkina, X. Teng, D. Abbott, G. D. Cuny, C. Yuan, G. Wagner, *et al.*, *Nat. Chem. Biol.*, 2008, **4**, 31; (*b*) A. Degterev, Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G. D. Cuny, T. J. Mitchison, M. A. Moskowitz and J. Yuan, *Nat. Chem. Biol.*, 2005, **1**, 112–119.

4 T. Xie, W. Peng, Y. Liu, C. Yan, J. Maki, A. Degterev, J. Yuan and Y. Shi, *Structure*, 2013, **21**, 493.

5 (*a*) Y. Cho, T. McQuade, H. Zhang, J. Zhang and F. K. Chan, *PLoS One*, 2011, **6**, e23209; (*b*) Y. Tamura, Y. Chiba, T. Tanioka, N. Shimizu, S. Shinozaki, M. Yamada, K. Kaneki, S. Mori, A. Araki, H. Ito, *et al.*, *FEBS Lett.*, 2011, **585**, 3058.

6 L. Sun, H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei, *et al.*, *Cell*, 2012, **148**, 213.

7 A. P. Komin and M. Carmack, *J. Heterocycl. Chem.*, 1976, **13**(1), 13.

8 T. Wang, Z. X. Zhang, N. A. Meanwell, J. F. Kadow, Z. W. Yin, Q. M. Xue, US Pat., 20030207910, 2003.

9 J. J. Kaminski, J. M. Hilbert, B. N. Pramanik, D. M. Solomon, D. J. Conn, R. K. Rizvi, A. J. Elliott, H. Guzik, R. G. Lovey, M. S. Domalski, S. C. Wong, C. Puchalski, E. H. Gold, J. F. Long, P. J. S. Chiu and A. T. McPhailt, *J. Med. Chem.*, 1987, **30**, 2031.

10 K. Sonogashira, Y. Tohoda and N. Hagihara, *Tetrahedron Lett.*, 1975, 4467.

11 K. D. McReynolds, M. J. Hadd and J. G. Hague, *Bioconjugate Chem.*, 1999, **10**, 1021.

12 S. Sato, Y. Kwon, S. Kamisuki, N. Srivastava, Q. Mao, Y. Kawazoe and M. Uesugi, *J. Am. Chem. Soc.*, 2007, **129**, 873.